The effects of methyl donor supply and methyl group status on growth performance, health, and methionine metabolism in growing cattle

by

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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
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Abstract

Three experiments were conducted to evaluate the effects of supplemental methyl group donors in growing cattle. In a 3-year experiment, 1440 crossbred beef heifers (480 per year; 224 kg initial body weight) were utilized to study the effects of methionine (Met) or choline supplementation on high-risk receiving cattle. Heifers were limit-fed at 2.2% of body weight daily a corn- and corn-coproduct-based diet for 60 d and supplemented with 1 of 5 treatments: control (nothing), 5 g/d metabolizable Met (8.33 g/d Smartamine M), 15 g/d metabolizable Met (25 g/d Smartamine M), 1.17 g/d available choline (26 g/d Reashure), or 3.5 g/d available choline (78 g/d ReaShure). Treatments were top-dressed at feeding daily. Jugular blood samples were collected on d 0, 14, and 60. Plasma haptoglobin was measured to assess inflammation, and plasma antioxidant potential was measured to assess oxidative balance. Dry matter intake was not affected by treatment ($P \leq 0.25$). Some minor differences among treatments were observed for body weight, average daily gain, and gain:feed throughout the experiment; however, none of the treatments differed from control. Overall prevalence of respiratory morbidity and mortality were 29% and 0.69%, respectively. No treatment effects were detected for first, second, or third respiratory morbidity or mortality ($P \geq 0.30$). Plasma haptoglobin was lesser on d 14 for 1.17 g/d choline than for control, 5 g/d Met, and 3.5 g/d choline ($P \leq 0.01$) and tended to be lesser than 15 g/d Met ($P = 0.06$); plasma haptoglobin did not differ among treatments on d 0 or d 60 ($P > 0.51$). Plasma antioxidant potential was not affected by dietary treatment ($P = 0.53$). Overall, supplemental Met and choline did not affect growth performance or health; however, choline supplementation may decrease inflammation in high-risk, newly received heifers. Two experiments were conducted using ruminally cannulated Holstein steers (Exp. 1, *n*=7, 189 kg; Exp. 2, *n*=6, 161 kg) in 6×6 Latin square designs with 10-d periods. Factorial treatments were

continuously infused abomasally and included 3 methyl group modulators (MGM; Exp. 1&2: control; 15 g/d guanidinoacetic acid [GAA]; or 16.8 g/d creatine) and 2 levels of betaine (Exp.1; 0 or 5.7 g/d betaine) or 2 levels of Met (Exp. 2; 0 or 7.2 g/d Met). Supplemental GAA or creatine increases creatine supply, but GAA consumes methyl groups during conversion to creatine, whereas creatine spares methyl groups by reducing GAA synthesis. Steers in both experiments were fed 3.5 kg/d (DM basis) of a corn-based diet. On d 10 of each period, whole-body Met flux was measured by continuous jugular infusion of 1-^{13}C-L-Met and methyl-$^{2}H_3$-L-Met. Retained N was not affected by MGM in either experiment ($P \geq 0.63$). In both experiments, supplemental GAA increased plasma concentrations and urinary excretion of GAA and creatine ($P < 0.01$), whereas supplemental creatine only increased plasma concentrations and urinary excretion of creatine ($P < 0.01$). Supplemental GAA numerically increased use of Met for methylation reactions and remethylation of homocysteine to regenerate Met; as a result, GAA did not affect Met transsulfuration. In Exp. 1, plasma haptoglobin decreased numerically with GAA but increased numerically with creatine; plasma haptoglobin was not affected by treatment in Exp. 2. Supplemental betaine, but not Met, increased retained N ($P = 0.03$). Supplemental Met, but not betaine, increased urinary creatine excretion ($P = 0.02$). Supplemental Met, GAA, and creatine tended to decrease lipopolysaccharide (LPS)-challenged neutrophil phagocytosis; betaine did not affect phagocytosis in LPS-challenged neutrophils ($P = 0.89$). Overall, our data suggest that our cattle were not deficient in available methyl groups, even when GAA was provided as a methyl group consumer, and effects of Met, creatine, and GAA on inflammation and immune function may not be a result of changes in methyl group status.

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periods. Factorial treatments were continuously infused abomasally and included 3 methyl group modulators (MGM; Exp. 1&2: control; 15 g/d guanidinoacetic acid [GAA]; or 16.8 g/d creatine) and 2 levels of betaine (Exp.1; 0 or 5.7 g/d betaine) or 2 levels of Met (Exp. 2; 0 or 7.2 g/d Met). Supplemental GAA or creatine increases creatine supply, but GAA consumes methyl groups during conversion to creatine, whereas creatine spares methyl groups by reducing GAA synthesis. Steers in both experiments were fed 3.5 kg/d (DM basis) of a corn-based diet. On d 10 of each period, whole-body Met flux was measured by continuous jugular infusion of 1-13C-L-Met and methyl-2H3-L-Met. Retained N was not affected by MGM in either experiment ($P \geqslant 0.63$). In both experiments, supplemental GAA increased plasma concentrations and urinary excretion of GAA and creatine ($P < 0.01$), whereas supplemental creatine only increased plasma concentrations and urinary excretion of creatine ($P < 0.01$). Supplemental GAA numerically increased use of Met for methylation reactions and remethylation of homocysteine to regenerate Met; as a result, GAA did not affect irreversible loss of Met via transsulfuration. In Exp. 1, Plasma haptoglobin decreased numerically with GAA but increased numerically with creatine; plasma haptoglobin was not affected by treatment in Exp. 2. Supplemental betaine, but not Met, increased retained N ($P = 0.03$). Supplemental Met, but not betaine, increased urinary creatine excretion ($P = 0.02$). Supplemental Met, GAA, and creatine tended to decrease lipopolysaccharide (LPS)-challenged neutrophil phagocytosis; betaine did not affect phagocytosis in LPS-challenged neutrophils ($P = 0.89$). Overall, our data suggest that our cattle were not deficient in available methyl groups, even when GAA was provided as a methyl group consumer, and effects of Met, creatine, and GAA on inflammation and immune function were likely not a result of changes in methyl group status.

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Dedication

To my grandma, Dorothy Grant, who always made time to call and check on me.

Thank you for:

Teaching me the "right way" to do math in kindergarten

Keeping me up to date on any and all gossip around home

Listening to me ramble about research that you often didn't understand

Loving and supporting me always and through everything

I love you so much and wouldn't have made it this far without you.

Chapter 1 - Review of Literature

Introduction

Methionine is an essential amino acid and is involved with numerous bodily processes. In addition to its function in protein synthesis, methionine is the most widely utilized methyl donor in the body and participates in over one hundred transmethylation reactions in the body (Lobley, 1992). Methionine and methyl group metabolism is comprised of a series of complex and tightly regulated biochemical reactions that work to maintain methyl group balance in the body. As a methyl donor, methionine is essential for synthesis of phosphatidylcholine and creatine, and methylation of DNA and histones. In addition to methionine alone, other methyl group donors (e.g., choline, betaine, folate, and vitamin B_{12}) have roles in maintenance of methyl group homeostasis.

Availability of dietary methionine and other methyl group sources differs among species. In nonruminants, preformed methyl group sources (i.e., methionine, choline, and betaine) and vitamins essential to methyl group metabolism (e.g., folates and vitamin B_{12}) are primarily obtained from the diet. In contrast, in ruminants, methionine and other methyl group sources are subject to degradation by ruminal microorganisms and their content in feedstuffs rarely reflects their supply available for absorption in the small intestine. In addition, ruminal microorganisms synthesize B vitamins (i.e., including folates and vitamin B_{12}), which are typically available for intestinal absorption in adequate quantities to support normal functions and do not often require supplementation. These key differences in methyl group availability between ruminants and nonruminants can affect how methionine and methyl group sources are metabolized in each respective species.

The objectives of this review are to: 1) outline the current understanding of metabolism of methionine and other methyl group donors, 2) highlight interrelationships between methyl donor supply and methyl group demand and their effects on methionine metabolism across species, and 3) elucidate potential differences in methionine and methyl group metabolism in ruminant and nonruminant species.

Methionine metabolism

Methionine is an essential sulfur-containing amino acid (AA) that participates in numerous essential bodily processes. As a result, determination of methionine requirements and efficiency of use is particularly challenging. In addition to its quantitatively most important role as a proteinogenic AA, methionine is the initiating AA for the translation step of protein synthesis, acts as a substrate for polyamine synthesis, and is the most widely utilized methyl group source in the body.

Methionine serves as a methyl group source when first activated to form *S*-adenosylmethionine (SAM). As SAM, methionine participates in over one hundred transmethylation reactions in the body (Lobley et al., 1992), including phosphatidylcholine and creatine synthesis, and methylation of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and histones (Walker, 1979). After donating its methyl group, SAM becomes *S*-adenosylhomocysteine (SAH), which is hydrolyzed to form homocysteine. Free homocysteine is either remethylated to regenerate methionine or undergoes transsulfuration to produce cysteine. Together, transmethylation, remethylation, and transsulfuration are the primary reactions required for effective use of methionine in the body.

Methionine activation to SAM occurs when methionine adenosyl transferase (MAT) transfers an adenosyl group from ATP to methionine. Three isoforms of MAT (MAT I, MAT II, and MAT III) exist and vary in tissue distribution in and activity. Both MAT I and MAT III are expressed in hepatic tissues, whereas MAT II predominates in all other tissues (Finkelstein, 2006). Together, the three MAT variants work to tightly regulate methionine metabolism, and conserve methionine when supply is limited and dispose methionine when in surplus. Increases in methionine supply decrease activity of MAT I and II, but increase activity of MAT III (Finkelstein, 2006). Similarly, MAT I and II are inhibited by SAM, whereas SAM activates MAT III. When methionine is in excess, MAT III allows hepatic SAM production to continue due to its low affinity and high capacity for methionine, which ultimately allows the liver to rapidly adapt to surplus methionine (Finkelstein 1990). Following activation by MAT, SAM undergoes either decarboxylation to produce polyamines (minor reaction, less than 10% of total SAM) or, more quantitatively, serves as a methyl donor for hundreds of transmethylation reactions (Finkelstein, 1998). These reactions are essential for synthesis of phosphatidylcholine, creatine, and neurotransmitters and methylation of DNA, RNA, and histones (Walker, 1979). Although these reactions are carried out by various SAM-dependent methyltransferases, all are inhibited by SAH, the product of SAM demethylation (Finkelstein, 1990).

Because SAH inhibits both use of SAM for transmethylation reactions and its own production, removal of SAH is essential for SAM-dependent reactions to proceed (Finkelstein, 1998). This balance is maintained by *S*-adenosylhomocysteine hydrolase (SAHH), which catalyzes the reversible hydrolysis of SAH to form homocysteine and adenosine (Finkelstein, 1990). The equilibrium of this reaction thermodynamically favors production of SAH from homocysteine and adenosine (de la Haba and Cantoni, 1959), and only proceeds if homocysteine

and adenosine are metabolized and removed from the system (Finkelstein, 1990). Homocysteine has two primary fates, which include remethylation to produce methionine or transsulfuration to form cysteine.

Regeneration of methionine via homocysteine remethylation occurs through one of two reactions. In one reaction, methionine synthase (i.e., 5-methyltetrahydrofolate-homocysteine methyltransferase; MS) transfers a methyl group from 5-methyltetrahydrofolate (5-MTHF) to homocysteine and depends on vitamin B_{12} (methylcobalamin) as a cofactor (Finkelstein, 1990). In addition to its function in producing methionine, homocysteine remethylation is essential for replenishment of tetrahydrofolate (THF), which allows for the closely related folate cycle to proceed. In a reaction catalyzed by serine hydroxymethyltransferase and vitamin B_6, free THF reacts with serine, which yields 5,10-methylenetetrahydrofolate (5,10-MTHF) and glycine (Brosnan and Brosnan, 2006). Next, the methylene bond of 5,10-MTHF is reduced by methylenetetrahydrofolate reductase (MTHFR) and flavin adenine dinucleotide (FAD; a vitamin B_2 derivative) to form 5-MTHF, which is available to further methylate homocysteine (Finkelstein, 1998). In the second remethylation reaction, betaine-homocysteine methyltransferase (BHMT) transfers a methyl group from betaine (i.e., trimethylglycine) to homocysteine, which yields methionine and dimethylglycine (Mudd et al., 2007). Betaine used to remethylate homocysteine is either obtained through the diet directly or is produced as a product of choline oxidation. Dimethylglycine and later methylglycine (i.e., sarcosine) donate methyl groups to produce 5,10-MTHF, which ultimately provide additional 5-MTHF for homocysteine remethylation via MS (Ducker and Rabinowitz, 2017). Low intracellular concentrations of SAM (methionine limiting conditions) activate MS and BHMT, whereas high intracellular concentrations of SAM (methionine excess) activate transsulfuration and inhibit MS and BHMT

(Finkelstein, 1998). In addition, both remethylation enzymes have a high affinity for homocysteine, which further supports that methionine is conserved when supply is low (Finkelstein, 1998). If homocysteine is not remethylated, it is diverted to the transsulfuration pathway and maintains the equilibrium of SAH hydrolysis in favor of homocysteine production.

The transsulfuration pathway is a two-step process that ultimately produces cysteine. First, in a vitamin B_6 dependent reaction, cystathionine-β-synthase (CBS) irreversibly combines homocysteine with serine that yields cystathionine (Finkelstein, 1998). In a second vitamin B_6 dependent reaction, cystathionase (cystathionine-γ-lyase) hydrolyzes cystathionine to produce cysteine, α-ketobutyrate, and an ammonium ion (Brosnan and Brosnan, 2006). Once synthesized, cysteine can be used for protein synthesis or further metabolized to produce key intracellular antioxidants glutathione and taurine, or sulfate (Finkelstein, 2006). Cystathionine-β-synthase is activated by both SAM and SAH accumulation, which leads to greater transsulfuration of homocysteine when dietary methionine is in excess (Finkelstein, 1998). Because transsulfuration of homocysteine is irreversible, it represents a permanent loss of methionine via oxidation rather than methionine conservation (Brosnan and Brosnan, 2006).

Surplus methionine in excess of protein synthesis and methyl group requirements can be disposed of by glycine *N*-methyltransferase (GNMT). As discussed previously, MAT III is activated by high dietary methionine and continues to produce SAM in the liver beyond the body's need for available methyl groups. Accumulation of SAM activates GNMT, which transfers a SAM methyl group to glycine to produce sarcosine (i.e., methylglycine; Finkelstein, 1998). Once formed, sarcosine can donate its methyl group to form 5,10-MTHF, which is reduced to 5-MTHF and can be used to regenerate methionine by MS (Finkelstein, 1998). Accumulation of 5-MTHF inhibits GNMT, which serves as a regulatory step to prevent

excessive use of SAM methyl groups for sarcosine synthesis (Wagner et al., 1985). The GNMT pathway is particularly useful because it disposes of excess SAM methyl groups, produces a relatively inert product, and is tightly regulated to prevent excessive inefficient use of methionine.

Choline and betaine metabolism

Choline is a nutrient present in some foods and feedstuffs, and it can be synthesized endogenously in the liver. In humans and other monogastric species, most choline is obtained from dietary sources (e.g., eggs, liver, fish, dairy, legumes, cruciferous vegetables); however, most humans do not consume enough choline to meet recommendations for adequate choline intake (Zeisel and Da Costa, 2009). As a result, most humans rely on de novo choline synthesis to varying degrees to support choline needs in the body. In contrast, ruminants rely almost exclusively on de novo choline synthesis because choline is extensively degraded by ruminal microorganisms and limited dietary choline is available for absorption in the small intestine (Neill et al., 1979; Sharma and Erdman, 1989). As a result, choline metabolism differs somewhat between ruminant and monogastric animals.

Choline is synthesized endogenously during phospholipid modification when phosphatidylethanolamine *N*-methyltransferase (PEMT) sequentially transfers three methyl groups from SAM to phosphatidylethanolamine, which yields phosphatidylcholine (Vance, 2014). Once formed, phosphatidylcholine can be utilized in cell membranes or can be incorporated into very low density lipoproteins (VLDL) for hepatic triglyceride export. When phosphatidylcholine is in excess of biological needs, phospholipase D can cleave the choline ion from phosphatidylcholine to yield free choline (Zeisel, 2006). In addition, dietary choline can be

phosphorylated and incorporated into phosphatidylcholine (Vance, 2014). Free choline can undergo acetylation by choline acetyltransferase, which produces the neurotransmitter acetylcholine. Because this reaction is reversible, at least half of choline used for acetylcholine synthesis is likely recycled from hydrolyzed acetylcholine (Taylor and Brown, 1999). Choline of either endogenous or dietary origin can also serve as a methyl group source if first oxidized to betaine by choline oxidase.

In addition to betaine produced during choline oxidation, betaine also can be obtained through the diet in monogastrics. Similar to choline, betaine is rapidly degraded by ruminal microorganisms (Mitchell et al., 1979). Regardless of origin, betaine serves as a methyl group donor when BHMT catalyzes donation of one of its three methyl groups to homocysteine to resynthesize methionine (Mudd et al., 2007). The coproduct of this reaction, dimethylglycine, is then demethylated to form sarcosine, which is further demethylated to glycine. Methyl groups provided by dimethylglycine and sarcosine are transferred to 5,10-MTHF, which is further reduced to 5-MTHF to remethylate homocysteine to methionine.

Enzymatic control of choline and betaine metabolism differ among species. Because bioavailability of dietary choline and betaine is low in ruminants, choline synthesis by PEMT is a major consumer of SAM methyl groups in ruminants (Lobley et al., 1996; Stead et al., 2006). In rats maintained on a choline-deficient diet, hepatic PEMT activity nearly doubled relative to rats supplemented with adequate choline (Cui and Vance, 1996). Both ruminants and choline-deficient rats absorb negligible amounts of choline in the small intestine. As a result, ruminant choline availability is directly impacted by availability of methionine methyl groups for its synthesis and is regulated as such for ruminants under normal dietary conditions. Oxidation of choline to betaine is an active pathway in rat liver, but it is strikingly less active in sheep liver

(Snoswell and Xue, 1987). Because choline oxidation occurs primarily when choline supply exceeds bodily requirements for choline and phosphatidylcholine needs are met, it is not surprising that choline oxidation is low in ruminants, which rely almost exclusively on de novo choline synthesis. Lambert et al. (2002) reported low hepatic BHMT activity in growing steers, which is likely reflective of low choline oxidation and limited betaine available for methyl group donation. Together, low activities of choline oxidase and BHMT suggest that endogenously produced choline is not quantitatively used to support homocysteine remethylation in ruminants.

Folate metabolism and related methyl group sources

Folate is a generic term used to describe folic acid (pteroylmonoglutamic acid; vitamin B_9) and folic acid derivatives that exhibit similar biological activity (McDowell, 2000; Combs and McClung, 2017). Folates have metabolic functions in amino acid metabolism (e.g., methionine, serine, glycine, and histidine), methyl group metabolism, and nucleotide synthesis. For humans and other monogastric species, folate is a required nutrient and must be obtained from the diet. In contrast, ruminants are not typically deficient in folate because folates and other B vitamins are synthesized by ruminal microorganisms (NASEM, 2016; NASEM, 2021). As a result, research investigating metabolism of folates and related methyl group sources in ruminants has been largely ignored in recent decades.

Dietary folic acid is reduced by NADPH-dependent dihydrofolate reductase to produce dihydrofolate, which is further reduced to tetrahydrofolate (THF) by the same enzyme. Fully reduced THF can accept single-carbon units at the 5 and/or 10 positions. The primary reaction, catalyzed by vitamin B_6-dependent enzyme serine hydroxymethyltransferase, transfers a single-carbon unit from serine to THF to produce 5,10-methylenetetrahydrofolate (5,10-MTHF) and

glycine. Glycine catabolism through the glycine cleavage pathway (i.e., glycine synthase) also provides single-carbon units to convert THF to 5,10-MTHF (Kikuchi, 1973). Next, 5,10-MTHF is reduced to 5-MTHF by methylenetetrahydrofolate reductase (MTHFR) in a reaction dependent on flavin adenine dinucleotide (FAD), a riboflavin derivative. Alternatively, 5,10-MTHF can be converted to 10-methyl-THF, and further 10-formyl-THF, which is used for purine synthesis (Combs and McClung, 2017). As mentioned previously, MS catalyzes the transfer of a methyl group from 5-MTHF to homocysteine to yield methionine and free THF. Homocysteine remethylation via MS requires vitamin B_{12} (i.e., cobalamin) as a cofactor.

Vitamin B_{12} is a cobalt-containing, water-soluble vitamin and serves as an intermediate methyl group carrier during homocysteine remethylation to form methionine. In this reaction, a methyl group is transferred from 5-MTHF to cobalamin to form methylcobalamin, which donates its methyl group to homocysteine to produce methionine. Without adequate vitamin B_{12}, MS cannot remethylate homocysteine, which causes accumulation of 5-MTHF and homocysteine and prevents regeneration of methionine and free THF (Combs and McClung, 2017). As a result, vitamin B_{12} deficiency creates a "folate trap" because the folate cycle cannot proceed past 5-MTHF, which ultimately creates a secondary folate deficiency despite adequate folate supply. As a result, clinical presentation of vitamin B_{12} deficiency is generally very similar to folate deficiency (e.g., pernicious anemia, neurological symptoms; McDowell, 2000). Vitamin B_{12} is synthesized almost exclusively via bacterial fermentation and, as a result, is only found in fermented foods or meat from animals that obtained vitamin B_{12} through ruminal or intestinal microbial fermentation or from dietary sources (Combs and McClung, 2017). Because microbial fermentation in the digestive tract of monogastrics occurs in the hindgut (i.e., subsequent to absorption), vitamin B_{12} must be obtained primarily through the diet. For ruminants, ruminal

microorganisms typically produce vitamin B_{12} in quantities large enough to support animal requirements if adequate dietary cobalt is available to support its synthesis (McDowell, 2000).

Metabolism of methionine and folate overlap at the point of methionine resynthesis from homocysteine. As a result, tight regulation of both the methionine and folate cycles is required to maintain both methyl group and folate balance in the body. Transfer of single-carbon units from serine to THF by serine hydroxymethyltransferase is inhibited by 5-MTHF (Stover and Schirch, 1991). This prevents excessive consumption of serine one-carbon units and prevents synthesis of 5,10-MTHF and later 5-MTHF beyond the capacity of MS to remethylate homocysteine. In addition, accumulation of SAM inhibits reduction of 5,10-MTHF to 5-MTHF by MTHF, which limits use of THF to produce 5-MTHF. Together, inhibition of serine hydroxymethyltransferase and MTHFR when methyl group supply is high (i.e., elevated SAM and 5-MTHF) prevents excessive de novo synthesis of additional methyl groups in the body (Bailey and Gregory, 1999). In contrast, when methionine (i.e., SAM) supply is low, the folate cycle can proceed as normal and allows for optimized methionine conservation via homocysteine remethylation.

Folate and vitamin B_{12} supply directly impact methionine metabolism. Marginal or deficient folate status decreases THF available for synthesis of 5-MTHF, which reduces homocysteine remethylation even when methionine supply is low. As a result, the equilibrium between SAH and homocysteine favors SAH, which inhibits use of SAM for transmethylation reactions (Selhub and Miller, 1992). Vitamin B_{12} deficiency leads to similar disruptions of normal methionine metabolism as a result of the co-dependence of MS on 5-MTHF and vitamin B_{12} for homocysteine remethylation. The close interrelationship between methionine and folate metabolism allows for conservation of folate or methionine when their respective supplies are deficient and limits unproductive use of excess methionine or folate when supplies are adequate.

Effects of decreased methyl group availability on methionine metabolism

Balance of methionine and methyl groups is maintained between available methyl group donors and consumers. Methyl group balance can be disrupted via dietary restriction of methionine and other key compounds essential for methionine metabolism (e.g., choline, folates, vitamin B_{12}) or supplementation of compounds that consume methyl groups or decrease methyl group availability in the body. Among these, some are naturally produced in the body or are involved in normal metabolic processes, whereas others do not function in normal metabolic activities. For example, guanidinoacetic acid (GAA), the body's sole creatine precursor, is synthesized endogenously and consumes a methyl group from SAM to produce creatine and SAH (Brosnan and Brosnan, 2007). Creatine synthesis is a major consumer of methyl groups in humans (Stead et al., 2006) and is not regulated at the point of GAA methylation, but rather, accumulation of creatine inhibits GAA synthesis (Walker, 1979). Thus, providing exogenous GAA will consume methionine methyl groups to produce creatine, potentially to the point of methyl group deficiency (Stead et al., 2001; Setoue et al., 2008).

Depletion of methyl group supply through provision of large amounts of supplemental GAA has been observed in rats (Stead et al., 2001; Setoue et al., 2008), humans (Ostojic et al., 2013), swine (McBreairty et al., 2015) and cattle (Ardalan et al., 2020; 2021) as evidenced by increases in circulating homocysteine concentrations. As mentioned previously, homocysteine is metabolized through either remethylation to replenish methionine or via transsulfuration to produce cysteine and when Met and other methyl groups are adequate, homocysteine does not accumulate. Thus, circulating homocysteine is an effective biomarker for methyl group status (Setoue et al., 2008). In addition, co-supplementation of GAA with methionine (Ardalan et al., 2020; 2021) and other methyl donors (e.g., choline, betaine, folic acid, vitamin B_{12}; Stead et al.,

2001; Setoue et al., 2008; Ostojic et al., 2013) can prevent accumulation of homocysteine in blood. This is primarily due to either increased remethylation of homocysteine to produce Met or by increased transsulfuration to cysteine.

In Yucutan miniature pigs, McBreairty et al. (2013, 2015) demonstrated that supplemental GAA drastically increased use of methionine methyl groups for creatine synthesis and, as a result, decreased use of methionine methyl groups for phosphatidylcholine synthesis. The diversion of methionine methyl groups away from phosphatidylcholine synthesis could decrease phosphatidylcholine available for incorporation into VLDL for hepatic lipid transport. In addition, availability of free choline for betaine synthesis would likely be depleted, which would decrease methyl groups available for remethylation of homocysteine by BHMT (Setoue et al., 2008). In growing cattle, Ardalan et al. (2021) observed greater plasma concentrations and urinary excretion of creatine in response to supplemental GAA in growing steers; however, overall transmethylation reactions were not affected by supplemental GAA. In agreement with work of McBreairty et al. (2013, 2015), they suggested that obligatory use of methionine methyl groups for creatine synthesis likely decreased use of methionine methyl groups for phosphatidylcholine synthesis (Ardalan et al., 2021). In marginally methionine deficient growing steers, Speer et al. (2022) observed a similar trend when supplemental GAA was provided alone; however, when supplemental methionine was provided, use of methionine for transmethylation reactions increased. This suggests that in the presence of methionine adequacy, other transmethylation reactions (i.e., primarily phosphatidylcholine synthesis) were not likely decreased by supplemental GAA.

Some vitamins can deplete methyl group supply if consumed in excess. For example, excess niacin (vitamin B_3) in its active forms nicotinamide or nicotinic acid consumes methyl groups

during degradation for urinary excretion (Finkelstein et al., 1988). Excess nicotinic acid is either converted to nicotinuric acid prior to excretion or can be converted to nicotinamide. Prior to excretion, nicotinamide undergoes SAM-dependent methylation to produce N^1-methylnicotinamide, which is further converted to pyridinones for excretion (Shibata and Matsuo, 1989). As a result, large quantities of excess dietary niacin could deplete methyl group supply. Work in rats has demonstrated increases in circulating and liver homocysteine in response to supraphysiological doses of supplemental niacin (Finkelstein, 1988; Basu et al., 2002). In addition, Basu et al. (2002) observed decreases in plasma B_{12} concentrations but not folate in response to supplemental niacin, which could suggest that use of folate for to remethylate excess homocysteine produced by niacin supplementation was limited by vitamin B_{12} availability. Because this has not been widely investigated, it is challenging to extrapolate these results to humans or other species.

Effects of supplemental methyl group donors on methionine metabolism

Methionine and Cysteine

In addition to its function as a methyl donor, methionine's role as the precursor to cysteine synthesis via homocysteine transsulfuration is important in some species. When methionine supply is adequate to support cysteine synthesis, cysteine is not typically considered an essential AA for mammals (Brosnan and Brosnan, 2006). Adequate cysteine supply can spare use of methionine for cysteine synthesis in rats (Finkelstein et al., 1988), and work in monogastrics has demonstrated that up to 50% of total sulfur AA requirements (i.e., methionine + cysteine) can be met by cysteine alone (Chung and Baker, 1992; Graber and Baker, 1971). In ruminants, methionine sparing through supplemental cysteine has not been demonstrated alone

(Campbell et al., 1997) or in combination with betaine and choline as methyl donors (Loest et al., 2002). In part, this could be attributed to the generally low methyl group availability in ruminants compared to monogastrics (Cambell et al., 1997). In addition, Lambert et al. (2002) reported low CBS relative to MS in growing steers compared to rats, which suggests that methionine conservation is likely more important in ruminants than is supporting cysteine synthesis.

In transition cows, Osorio et al. (2014) reported decreases in milk choline and a tendency for decreased hepatic concentrations of phosphatidylcholine in cows supplemented with methionine, which could suggest increased incorporation of phosphatidylcholine into VLDL for hepatic lipid transport and improved lipid metabolism. In addition, supplemental methionine improved hepatic glutathione concentrations, which is a downstream product of cysteine metabolism, suggesting greater use of methionine for transsulfuration, likely due to supplemental methionine meeting and exceeding needs for protein synthesis or as a methyl donor (Osorio et al., 2014). Similarly, in response to methionine supplementation, Zhou et al. (2017) observed increases in circulating cystathionine, homocysteine, taurine, and sulfur containing compounds, all of which indicate diversion of homocysteine towards transsulfuration (Guttormsen et al., 2004).

Choline and betaine

Choline serves as both a consumer and source of methyl groups, which gives it a potential for both “pull” and “push” effects on overall methyl group metabolism. In addition, although betaine synthesis from choline does not directly consume methyl groups, its synthesis from choline in the body makes it an indirect consumer of methyl groups. As previously discussed, betaine serves as a methyl group source either directly through donation of a methyl

group to homocysteine via BHMT activity or indirectly through donation of its additional two methyl groups, which are available to replenish methionine via 5-MTHF and MS.

Work in non-ruminants has consistently demonstrated increased remethylation of homocysteine to methionine in response to supplemental betaine (Finkelstein et al., 1983; Emmert et al., 1996, 1998; Robinson et al., 2018). This effect has not been demonstrated in ruminants (Loest et al., 2002), likely due to low activity of choline oxidase and BHMT observed in ruminants (Xue and Snoswell, 1986; Lambert et al., 2002). Similarly, Zhou et al. (2017) observed no change in circulating cystathionine, homocysteine, or homocysteine in response to supplemental choline in transition cows. This may further support the low choline oxidase activity that has been observed in ruminants (Xue and Snoswell, 1986). In contrast, Coleman et al. (2019a,b) observed increases in BHMT and MS activity and decreases in CBS activity in response to supplemental choline in nutrient restricted dairy cows. This may suggest improvements in homocysteine use towards remethylation rather than transsulfuration, which would support greater methionine regeneration. Although it is unclear why cattle of Loest et al. (2002) did not also divert use of homocysteine towards remethylation, Lambert et al. (2004) suggested that a deficiency in one or more vitamin cofactors (i.e., folate, vitamin B_{12}, or pyridoxine) may have limited methionine remethylation.

Vitamin B_{12} and folate

The effects of supplemental vitamin cofactors used in methyl group metabolism (i.e., vitamin B_{12} and folate) on methionine metabolism have been investigated in both ruminants and nonruminants. As mentioned previously, B vitamins are considered essential nutrients for nonruminants and must be obtained from the diet. In contrast, because ruminal microorganisms produce folate and vitamin B_{12} their supplementation is not typically required in ruminants.

Under conditions of methyl group deficiency, supplemental folates can improve methionine status through increased remethylation of homocysteine to conserve methionine in neonatal piglets (McBreairty et al., 2016; Robinson et al., 2018). Because ruminal synthesis is the primary source of folates and vitamin B_{12} in ruminants, the ability of supplemental folate or vitamin B_{12} to improve methionine use in ruminants is dependent on adequate ruminal synthesis to meet ruminant folate and vitamin B_{12} needs. In growing steers maintained under a Met deficiency model, Lambert demonstrated increases in protein deposition when folic acid, vitamin B_{12}, and pyridoxine were provided. Although specific effects of these vitamins on methionine metabolism were not measured, increases in methionine availability for protein synthesis via increases in homocysteine remethylation by 5-MTHF and MS and decreases in transsulfuration are a reasonable explanation for this observation (Lambert et al., 2004). In lactating cows, Girard et al. (2005) observed decreases in circulating homocysteine concentrations in response to supplemental folate, and decreases in circulating cysteine when folic acid was supplemented in combination with methionine but not folate alone. This could suggest that supplemental folic acid diverted homocysteine use towards methionine resynthesis, particularly when combined with supplemental methionine. In a companion study, Girard and Matte (2005) reported improvements in lactational performance in methionine and folate supplemented cows in response to vitamin B_{12} injections. Because vitamin B_{12} is an integral cofactor for remethylation of homocysteine via 5-MTHF and MS, these data could suggest that B_{12} supply was not optimal to promote homocysteine remethylation with supplemental folate and methionine alone. Preynat et al. (2009) observed a tendency for intramuscular folic acid and vitamin B_{12} injections to decrease transsulfuration of homocysteine in cows receiving supplemental methionine. Independent of methionine supplementation, vitamin injections decreased plasma homocysteine

concentrations in cows. Their observations most likely resulted from a diversion of methionine use away from transsulfuration and towards protein synthesis, as evidence by increased milk protein yield in cows receiving vitamin injections (Preynat et al., 2009). In sheep, Xue and Snoswell (1985) demonstrated that MS activity was more important for homocysteine remethylation than BHMT, which could suggest an even more important role of adequate folate and B_{12} availability in ruminants than in monogastrics. Although supplementation of folates and B_{12} are not likely limiting under all conditions for ruminants, it appears that their supplementation may have some benefit in improving methionine use when methionine supply is very low (Lambert et al., 2004) or under high methionine demand (Girard et al., 2005; Girard and Matte 2005; Preynat et al., 2009).

Conclusions

Methionine and methyl group metabolism is complex and heavily regulated in both ruminant and nonruminant species. Ruminants typically absorb much smaller quantities of preformed methyl group sources than do nonruminants. As a result, regulation of methyl group metabolism in ruminants has evolved to adapt to low methyl group intake. Ruminants rely heavily on de novo synthesis of methyl groups via folate metabolism to support methyl group needs. In addition, ruminant methyl group metabolism appears to support conservation of methyl groups as evidenced by low relative enzymatic activity associated with choline oxidation and transsulfuration. As a result, ruminants require much smaller intakes of preformed methyl groups than nonruminants. Further investigation of effects of alternative methyl group sources (e.g., choline, betaine, folate, vitamin B_{12}) on methionine metabolism in ruminants, particularly those with altered methyl group availability, could provide additional insight to this issue.

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Chapter 2 - Effects of supplemental methionine or choline on growth performance, health, acute protein response, and antioxidant status in high-risk newly received beef heifers [1]

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Abstract

In a 3-year experiment, 1,440 crossbred beef heifers (480 per year; 224 ± 0.9 kg initial body weight; southeastern United States origin) were received in 15 truckloads (blocks; 5 per year) in October 2020, 2021, and 2022. Individual body weights were measured at arrival (d -1). The following day (d 0) cattle were vaccinated for viral and clostridial diseases, received 2.5 mg tulathromycin/kg body weight, and were stratified within block by arrival body weight to 1 of 8 pens containing 12 heifers each. Cattle (40 pens per year; 120 pens total) were limit-fed at 2.2% of body weight daily (dry matter basis) a ration containing 39.5% dry-rolled corn, 40% branded wet corn gluten feed, 13% chopped prairie hay, and 7.5% supplement for 60 d. Pens were assigned to 1 of 5 treatments: Control (nothing), 5 g/d metabolizable methionine (Met; 8.33 g/day Smartamine M; Adisseo USA Inc., Alpharetta, GA), 15 g/day metabolizable Met (25 g/day Smartamine M), 1.17 g/day available choline (26 g/day ReaShure; Balchem Corp., Montvale, NJ), or 3.5 g/day available choline (78 g/day ReaShure). Treatments were top-dressed at feeding daily. Heifers were revaccinated and weighed on day 14, and pen weights were measured weekly thereafter to adjust feed offered the following week. Heifers were observed twice daily for clinical signs of respiratory illness and treated as follows: first morbidity, florfenicol; second morbidity, enrofloxacin; third morbidity, oxytetracycline. Upon third treatment, heifers were declared chronic and removed from the experiment. Performance data from chronic and dead heifers were removed before analysis. In each year, there were 8 pens per treatment, for a total of 24 pens per treatment across 3 years. No overall effects of treatment on dry matter intake were detected ($P \geq 0.25$). There were some minor differences among treatments for body weight, average daily gain, and gain:feed throughout the experiment, but none of the treatments were significantly different from control for these responses. Overall

prevalence of respiratory morbidity and mortality were 29% and 0.69%, respectively. No treatment effects were detected for first, second, or third respiratory morbidity or mortality ($P \geq 0.30$). Plasma haptoglobin was lesser on d 14 for 1.17 g/d choline than for control, 5 g/d Met, and 3.5 g/d choline and tended to be lesser than 15 g/d Met; plasma haptoglobin did not differ among treatments on d 0 or d 60 ($P > 0.51$). Plasma antioxidant potential was not affected by dietary treatment ($P = 0.53$). Overall, supplemental methionine and choline did not affect growth performance or health; however, choline supplementation may decrease inflammation in high-risk, newly received heifers.

Introduction

Newly received beef cattle face numerous physiological stressors during transfer from the cow-calf to feedyard sectors of the beef industry (e.g., weaning, marketing, commingling, low feed and water intake, and pathogen exposure; Van Engen and Coetzee, 2018). As a result, many cattle become ill shortly after feedyard arrival, primarily with respiratory illnesses (i.e., bovine respiratory disease). Respiratory disease is the most frequent and expensive illness faced by the U.S. cattle feeding sector (NAHMS, 2013). In addition to the large cost of treating sick cattle, pressures from consumer and regulatory groups to decrease antimicrobial use in livestock production are increasing. Thus, nutritional strategies that improve health and immune function of newly received cattle may have value in the beef industry.

Methionine (Met) is an often-limiting amino acid for growth in cattle, particularly when microbial protein is the main source of metabolizable protein (Richardson and Hatfield, 1978). This is a result of low Met content of microbial protein produced by ruminal microorganisms (Orskov, 1982). In addition to its primary function in protein synthesis, Met can be converted to *S*-adenosylmethionine, which is the most widely utilized methyl group donor in the body. Methionine, as *S*-adenosylmethionine, participates in over 100 transmethylation reactions (Lobley, 1992), including synthesis of phosphatidylcholine and creatine as well as regulation of gene expression through methylation of deoxyribonucleic acid, ribonucleic acid, and histones (Walker, 1979). Synthesis of phosphatidylcholine is one of the quantitatively most important transmethylation reactions (Stead et al., 2006). Phosphatidylcholine is used in cell membrane structures, aids hepatic lipid transport through its incorporation into very low density lipoproteins, and can be cleaved to yield free choline (Zeisel, 2006). Although choline synthesis consumes methyl groups, choline also has potential to serve as a methyl group source if first

oxidized to betaine, which donates methyl groups to synthesize Met from homocysteine. The role of Met in choline synthesis is particularly important in ruminants because choline is rapidly degraded by ruminal microorganisms (Sharma and Erdman, 1989); thus, ruminants rely almost exclusively on de novo choline synthesis. Both methionine and choline have important roles in mitigation of inflammation and oxidative stress. Methionine is a precursor to intracellular antioxidants glutathione and taurine (Brosnan and Brosnan, 2006), which work to maintain oxidative balance in tissues. In addition, choline's role in hepatic triglyceride export prevents accumulation of lipids in liver tissue (Cole et al., 2012). Disruptions in normal oxidative balance and lipid transport can contribute to inflammation (Drackley, 1999; Sordillo et al., 2009), and supplemental methionine and choline have potential to mitigate oxidative stress and inflammation.

In the transition period between late gestation and early lactation, dairy cattle enter a period of negative energy and nitrogen balance due to decreased dry matter intake near calving coupled with increased partitioning of nutrients towards late-stage fetal growth and lactation initiation (Drackley, 1999). In this period, cows are at risk of developing metabolic disorders (e.g., fatty liver, ketosis, milk fever) and also face inflammation and oxidative stress. Supplementation of ruminally protected methyl donors (e.g., methionine, choline) to dairy cows in the transition period can improve lactational performance, health, and immune function (Ardalan et al., 2010, Osorio et al., 2014; Zhou et al., 2016a,b; Batistel et al., 2017, 2018; Arshad et al., 2020). Improvements in performance and health likely result from corrections to disrupted antioxidant balance, decreased inflammation, or both (McFadden et al., 2020). Because stressors faced by newly received beef cattle may be physiologically similar to those faced by periparturient dairy cattle, stressed beef calves may respond positively to methionine or choline

supplementation. Ruminally protected methionine supplementation may decrease inflammation in high-risk, newly received beef heifers (Grant et al., 2022). In addition, post-ruminal choline infusion may reduce inflammation in growing Holstein steers (Grant, 2020). To our knowledge, no previous work has evaluated the effects of ruminally protected choline supplementation on health or immune function in receiving cattle. In addition, no direct comparisons between ruminally protected methionine and choline are available in receiving cattle. Our objective was to determine the effects of supplemental methionine or choline on growth performance, health, inflammation, and antioxidant status in high-risk newly received beef heifers.

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

In a 3-year experiment, 1440 crossbred beef heifers (480 per year; 224 ± 0.9 kg initial body weight) of southeastern U.S. origin were purchased at auction markets in Tennessee, commingled at an order buyer's facility in Dickson, TN, and then transported 1,086 km to the Kansas State University Beef Stocker between October 2020 and October 2022. Heifers were used in a replicated balanced incomplete block design to evaluate the effects of methionine and choline supplementation on health and performance in three 60-d receiving studies. Calves were blocked by truckload (15 blocks; 5 per year) and stratified by individual arrival weight within block to 1 of 8 pens containing 12 heifers each. Within each block of 8 pens, 5 pens were used to house each of the 5 treatments and the additional 3 pens were allocated to treatments, in sequence as blocks were filled, such that no treatment was over-represented. Across the 5 blocks in each replicate year, this created a total of 8 pens/treatment, with a total of 40 pens for each year. Pens (9.1 × 15.2 m) were soil surfaced with a concrete fenceline bunk (9.1 m) and a 3.6-m bunk apron. A common diet was offered at 2.2% of body weight daily (DM basis; Table 2.1) and was calculated to contain 1.31 Mcal/kg NE_g based on tabular values (NASEM, 2016). Experimental treatments (per heifer) were as follows: control (nothing), 5 g/d supplemental metabolizable Met provided as 8.33 g/d Smartamine M, 15 g/d supplemental metabolizable Met provided as 25 g/d Smartamine M, 1.17 g/d available choline provided as 26 g/d ReaShure, or 3.5 g/d available choline provided as 78 g/d ReaShure. Amounts of Smartamine M and ReaShure

provided to meet targeted available methionine or choline were based on bioavailability data of Rulquin and Kowalczyk (2003) and de Veth et al. (2016), respectively. The 5 g/d Met and 3.5 g/d choline treatments provided equimolar amounts of methionine and choline. The 5 g/d Met and 1.17 g/d choline treatments as well as the 15 g/d Met and 3.5 g/d choline treatments provided equal amounts of methyl groups (methionine contains one methyl group, whereas choline contains three methyl groups). Treatments were top-dressed daily immediately after feed was delivered to each pen.

On arrival (d -1), heifers were weighed individually, assigned an individual identification ear tag, and grossly assessed for disease or lameness. An ear notch was collected from each heifer and immediately placed on ice; following processing, samples were transported to the Kansas State University Veterinary Diagnostic Laboratory to identify animals persistently infected with Bovine Viral Diarrhea Virus; heifers identified as positive were excluded from the experiment. Heifers not displaying illness or lameness were randomly assigned to pens containing 12 animals and offered 0.5% body weight (DM basis) prairie hay and ad libitum access to water overnight.

The next morning (d 0) heifers were weighed individually, assigned an ear tag with pen number, and were vaccinated for respiratory and clostridial disease. For respiratory pathogens, Express 5 (Boehringer Ingelheim, Duluth, GA), 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 used. For protection against clostridial pathogens, Vision 7 Somnus with Spur (Merck Animal Health, Rahway, NJ) was administered. All heifers were treated at initial processing (d 0) for subclinical respiratory disease with 2.5 mg/kg tulathromycin (Draxxin; Zoetis Inc., Parsippany, NJ).

Additionally, heifers were treated on d 0 for internal parasites with albendazole (Valbazen; Zoetis Inc., Parsippany, NJ) and external parasites with doramectin (Dectomax; Zoetis Inc., Parsippany, NJ). On d 14, heifers were revaccinated for respiratory pathogens with Express 5 (Boehringer Ingelheim).

Individual body weights were measured at initial processing (d 0), at revaccination (d 14), and at the conclusion of the receiving period (d 60). A pen scale (Rice Lake Weighing Systems, Rice Lake, WI) was used to measure pen weights weekly (d 0, 14, 21, 28, 35, 42, 49, and 60). Pen weights were used to calculate feed offered for the following week. Pens were fed once daily at 0700 h using a Roto-Mix feed wagon (Model #414-14B; Roto-Mix, Dodge City, KS). On d 0 (following initial processing), cattle were offered the experimental diet at 1% of body weight (DM basis). After d 0, feed offered was increased by 0.2% of body weight each day if the previous day's feed was completely consumed until the pen reached a dry matter intake of 2.2% of body weight. During step-up, feed bunks were evaluated each morning prior to feeding, and all refusals were left in the feed bunk. If more than 5% of offered feed was present in the bunk, feed offered was decreased accordingly to prevent excessive refusals from accumulating in the bunk the following day. By d 14, all pens were fully stepped up to feed delivered at 2.2% of body weight (DM basis), and cattle consistently consumed all feed prior to the following day's feeding. Each day, the amount of feed delivered to each pen was recorded at feeding.

Animals were observed twice daily for clinical signs of illness including depression, nasal or ocular discharge, anorexia, and gauntness. Heifers displaying signs of morbidity were walked to treatment facilities where rectal temperature was measured and clinical illness score (CIS) was determined. The CIS 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 rectal temperature ≥ 40 °C and CIS ≥ 2 or with rectal temperature ≤ 40 °C and CIS ≥ 3 were treated for bovine respiratory disease. At first morbidity, heifers were treated subcutaneously with florfenicol (40 mg/kg body weight; Norfenicol, Norbrook Laboratories, Newry, United Kingdom). After first treatment, if symptoms did not resolve or improve within 72 h, second morbidity was declared and heifers were treated subcutaneously with enrofloxacin (10 mg/kg body weight; Enroflox 100, Norbrook Laboratories). Upon third morbidity, heifers were treated subcutaneously with oxytetracycline (30 mg/kg body weight; 300 Pro LA, Norbrook Laboratories). At third treatment, heifers were considered chronically infected with bovine respiratory disease and removed from the study.

Sample Collection and Analysis

Feed ingredient samples were collected weekly and frozen at −20 °C until analysis. Frozen ingredient samples were composited, dried in a forced-air oven at 55 °C for 48 h, and ground to pass through a 1-mm screen using a Thomas Wiley Mill (Thomas Scientific, Swedesboro, NJ). Additionally, a subsample (100 g) of each weekly ingredient sample was dried in a forced-air oven at 105 °C for 24 h to determine weekly dry matter content. All samples were analyzed in duplicate. Ground samples were dried at 105 °C in a forced-air oven to determine total dry matter content and then ashed at 450 °C in a muffle furnace for 8 h to determine organic matter content. Samples were analyzed for neutral detergent fiber content with alpha amylase and sequentially analyzed for acid detergent fiber content using an ANKOM Fiber Analyzer (Model 200, ANKOM Technology, Macedon, NY). Feed ingredients were analyzed for N content using a LECO TruMac N Analyzer (LECO Corporation, Saint Joseph, MI). Measured

nitrogen content was multiplied by 6.25 to determine crude protein content. Dietary dry matter, organic matter, neutral detergent fiber, acid detergent fiber, and crude protein content were calculated from measured ingredient sample values on a dry matter basis.

Prior to feeding on d 0, 14, and 60, a jugular vein blood sample was collected from each heifer into a 10-mL evacuated blood tube containing sodium heparin (BD Vacutainer, Beckton, Dickinson, and Company, Franklin Lakes, NJ). Immediately after collection, blood tubes were gently inverted several times and stored on ice. Samples were centrifuged at 1000 × *g* at 4 °C for 20 min. Plasma was harvested and stored in 2-mL microcentrifuge tubes at −20 °C until analysis. Plasma samples were analyzed individually for haptoglobin concentration to assess inflammation and acute phase protein response. For each blood collection time point (d 0, 14, or 60), plasma samples were pooled within pen and analyzed for plasma antioxidant potential and concentrations of amino acids, glucose, and urea.

Plasma haptoglobin concentrations were measured by colorimetric assay based on peroxidase activity using methods modified from Cooke and Arthington (2013) and Smith et al. (1998). Briefly, 5 µL of plasma or serially diluted plasma standard was added to wells of a 48-well plate. Next, 25 µL hemoglobin solution (0.3 g/L bovine hemoglobin dissolved in deionized water) and 1 mL *o*-dianisidine solution (0.6 g/L *o*-dianisidine, 13.8 g/L EDTA, and 13.8 g/L sodium phosphate monobasic in deionized water) were added to each well. Then, plates were covered with a thermoplastic film and incubated at 37 °C for 45 min in a gravity convection incubator. Immediately following incubation, 10 µL of a freshly prepared 312 mM hydrogen peroxide solution was added to each well, followed by incubation at room temperature for 1 h. After incubation, absorbance at 450 nm was measured using a microplate reader (BioTek PowerWave XS, BioTek Instruments, Inc., Winooski, VT). Samples were analyzed in triplicate

and the median value was utilized. A pooled standard plasma sample was created from individual bovine plasma samples that were previously identified with high haptoglobin concentration, and the concentration of the pooled sample was measured by the Kansas State University Veterinary Diagnostic Lab (Manhattan, KS) as described by Smith (1998).The pooled standard was serially diluted with water, analyzed identically to samples in each plate, and used to calculate haptoglobin concentrations for all samples for the experiment.

Plasma AOP, an estimate of the total antioxidant activity, was measured by Trolox equivalent antioxidant capacity as described by Re et al. (1999). Plasma glucose concentrations were measured using a commercially available 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). Concentrations of plasma urea were measured by the method of Marsh et al. (1965).

Plasma amino acid concentrations were measured using ultra-high pressure liquid chromatography (Waters Acquity UPLC; Waters Corporation, Milford, MA). Deproteinized samples underwent precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Tag Ultra Derivatization Kit; Waters Corporation, Milford, MA), were separated on a C18 column, and detected with a tunable UV spectrophotometer at 260 nm wavelength (Waters, 2007). Plasma was prepared for amino acid analysis by adding 10% (wt/vol) sulfosalicylic acid containing 600 μM norvaline as an internal standard 1:1 to deproteinize samples, then vortexed and frozen. Samples were then thawed and 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 a microcentrifuge tube. Samples were then derivatized using a commercially available kit (AccQ-

Tag Ultra Derivatization Kit; Waters Corporation, Milford, MA) and stored at 4 °C until analysis.

Calculations

Average pen body weights and calculations of average daily gain and gain:feed were based on pen scale weights. All intake and performance data calculations were made with data from chronic and dead heifers removed. To achieve this, weekly pen weights were adjusted to remove the proportion the dead or chronic individual contributed to their respective pen at the most recent individual body weight measurement (i.e., d 0 or 14). For example, for a d 14 individual weight of 250 kg and pen weight of 3000 kg, (3000 – 250) / 3000 = 0.917, all subsequent pen weights prior to the heifer's removal or death would be multiplied by a factor of 0.917.

Statistical Analyses

Performance data and were analyzed as a replicated balanced incomplete block design using the MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC) with the final model including fixed effects of treatment and year and random effect of block nested within year. Health data were analyzed as binomial proportions of first, second, and third respiratory treatment and mortality using the GLIMMIX procedure of SAS (version 9.4; SAS Institute., Cary, NC). The model included fixed effects of treatment and year, and random effect of block nested within year. The treatment × year interaction was tested for all analyses but was never significant ($P \geq 0.18$); thus, it was removed from the final model.

A large proportion of plasma samples contained some degree of hemolysis. Because hemolysis interferes with the haptoglobin assay, samples containing any visible hemolysis were not analyzed for haptoglobin concentration. Thus, only 2202 individual animal observations (51% of total samples) of plasma haptoglobin concentration were available across the 3 blood sampling days. Pen means were generated for plasma haptoglobin concentration for each of the 3 sampling days using the MIXED procedure of SAS with a model including fixed effects of pen, day, and pen × day and random effect of heifer (pen). The 360 total pen means were then analyzed using the MIXED procedure of SAS in a model including fixed effects of treatment, day, year, treatment × day, and day × year and random effect of block nested within year. Day was considered a repeated measure with spatial power as the covariance structure. Pen means for plasma AOP, glucose, and urea (samples pooled within pen and day; 360 total pen means per analyte) were analyzed identically to plasma haptoglobin pen means. The SLICE option of SAS was used to separate treatment effects within day for plasma measures. Interactions for treatment × year and treatment × day × year were evaluated for haptoglobin, AOP, glucose, and urea but were never significant ($P \geq 0.22$) and were excluded from the final models. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 \leq P \leq 0.10$.

Results

Growth Performance

Effects of year

No effects of year were observed for body weight or average daily gain during any period of the experiment ($P \geq 0.23$; Table 2.2). An effect of year on dry matter intake was observed for all time periods throughout the experiment ($P \leq 0.01$). From d 0 to 14, dry matter intake was greater in year 1 than 3 ($P < 0.01$) and tended to be greater in year 2 than year 3 ($P = 0.07$). From d 14 to 60, dry matter intake was greater in year 1 and 3 compared with year 2 ($P \leq 0.01$) and tended to be greater in year 1 than year 3 ($P = 0.06$). Overall, average dry matter intake was greater between d 0 and 60 in year 1 than in years 2 and 3 ($P \leq 0.02$) but did not differ between years 2 and 3 ($P = 0.17$). Feed efficiency tended to differ among years d 0 to 14 and d 14 to 60 ($P = 0.09$ and $P = 0.06$, respectively). Between d 0 and 14, gain:feed was greater in year 3 than in year 2 ($P = 0.04$) but was not different among other years ($P \geq 0.13$). From d 14 to 60, gain:feed tended to be greatest in year 2 ($P \leq 0.10$) but did not differ between years 1 and 3. Overall gain:feed (d 0 to 60) averaged 0.21 kg gain/kg dry matter intake across the 3-year experiment and did not differ among years ($P = 0.22$).

Effects of treatment

Dry matter intake was unaffected by treatment for all time periods ($P \geq 0.25$; Table 2.3) and averaged 5.5 kg/d over the 60-d trial. Body weights did not differ among treatments on d 0 ($P = 0.28$) and were not different among treatments through d 35 ($P \geq 0.26$). A main effect of treatment on body weight was observed on d 42 ($P = 0.01$) and d 49 ($P = 0.05$), and a tendency was observed for d-60 body weight to differ among treatments ($P = 0.09$). On d 42 and d 49, body weights were greater for 15 g/d Met than for 5 g/d Met, 1.17 g/d choline or 3.5 g/d choline

($P \leq 0.03$) and tended to be greater than control ($P \leq 0.10$). Final (d 60) body weights were greater for 15 g/d Met than for 5 g/d Met, 1.17 g/d choline, and 3.5 g/d choline ($P \leq 0.03$) but none of the treatments differed from control ($P \geq 0.17$). Average daily gains were similar among treatments from d 0 to 14 and d 14 to 60 ($P = 0.69$ and $P = 0.21$, respectively). Overall average daily gain (d 0 to 60) tended to be affected by treatment ($P = 0.09$). Overall, calves supplemented with 15 g/d Met gained faster than calves supplemented with 1.17 g/d choline or 3.5 g/d choline ($P \leq 0.02$) and tended to gain faster than calves supplemented with 5 g/d Met ($P = 0.06$); however, none of the treatments differed from control ($P \geq 0.12$). No differences among treatments were observed for gain:feed between d 0 to 14 or d 14 to 60 ($P = 0.55$ and $P = 0.32$, respectively); however, a tendency for a main effect of treatment on overall gain:feed (d 0 to 60) was detected ($P = 0.10$); however, none of the treatments differed from control ($P > 0.15$). In agreement with observations for body weight, gain:feed was numerically greatest for the 15 g/d Met treatment. Heifers supplemented with 15 g/d Met gained more efficiently than calves supplemented with 1.17 g/d choline or 3.5 g/d choline ($P \leq 0.03$) and tended to gain faster than heifers supplemented with 5 g/d Met ($P = 0.09$).

Animal Health

Effects of year

Incidence of first, second, and third respiratory treatment was variable by year (effect of year, $P < 0.001$; Table 2.2). First respiratory treatment was greatest in year 3 (53.5%, $P \leq 0.01$), and tended to be greater in year 2 than in year 1 (21.5% vs. 9.4%, $P = 0.06$). Incidence of second respiratory treatment was greatest in year 3 (19.8%), intermediate in year 2 (6.2%), and least in year 2 (1.2%; $P \leq 0.04$). Third respiratory treatment was greatest in year 3 (4.8%; $P \leq 0.01$) but

was similar in years 1 and 2 (1.0% and 0.2%, respectively). The incidence of mortality did not differ among the three replicate years ($P = 0.24$) and averaged 0.81%. The distribution of days to first and second respiratory treatment differed among replicate years ($P < 0.01$; Figure 2.1). Days to first treatment did not differ between years 1 and 2 ($P = 0.25$) and averaged 22 d; however, first respiratory treatment occurred earlier in year 3 (13 d on average, $P < 0.01$) because a large proportion of cattle became ill shortly after arrival. For second respiratory treatment, days to treatment did not differ between years 1 and 3 and averaged 20 d ($P = 0.43$) but average days to second treatment was later in year 2 (40 d, $P < 0.001$).

Effects of treatment

Incidence of first, second, and third respiratory treatment and mortality were not affected by dietary treatment ($P \geq 0.30$; Table 2.3). Overall, first respiratory treatment averaged 28.1% and mortality averaged 0.72%. The distribution of days to first and second respiratory morbidity did not differ among treatments and averaged 19 d and 26 d, respectively ($P \geq 0.12$). Days to third respiratory morbidity tended ($P = 0.06$) to differ among treatments and was greater for 1.17 g/d choline than control and 3.5 g/d choline; the measurements were based on a small number of observations. Days to mortality did not differ among treatments ($P = 0.67$) and averaged 22 d.

Plasma Acute Phase Protein and Oxidative Stress Response

Effects of year

An interaction between year and sampling day was detected for plasma haptoglobin (year × day interaction, $P < 0.01$; Figure 2). On day 0, plasma haptoglobin concentrations were similar in years 1 and 2 but were much greater at arrival in year 3. Between d 0 and 14, haptoglobin

increased in year 1, remained low in year 2, and remained high in year 3. By day 60, plasma haptoglobin decreased for all years and was similar among the 3 experimental years.

An interaction between year and sampling day was detected for plasma AOP (year × day interaction, $P < 0.01$; Figure 3). On day 0, plasma AOP was substantially lower in year 1 than in years 2 and 3. By day 14, plasma AOP increased in years 1 and 3 relative to day 0 with a larger increase during year 1 than year 3. Plasma AOP was stable between d 0 and 14 in year 2. Plasma AOP was stable between days 14 and 60 for all years but lower in year 1 than years 2 and 3.

Effects of treatment

No treatment × day interactions were detected for plasma haptoglobin concentrations or plasma AOP (Figure 6 and Figure 7, respectively; $P \geq 0.18$). A tendency for a main effect of treatment was detected for plasma haptoglobin ($P = 0.06$; Table 2.4). Plasma haptoglobin did not differ among treatments on d 0 ($P = 0.95$) but was affected by treatment on d 14 ($P < 0.01$). On d 14, haptoglobin was lesser for heifers receiving 1.17 g/d choline than for heifers receiving control, 5 g/d Met, or 3.5 g/d choline ($P < 0.01$) and tended to be lesser than heifers receiving 15 g/d Met ($P = 0.06$). By d 60, plasma haptoglobin was not different among treatments ($P = 0.51$).

No main effect of treatment was detected for plasma AOP ($P = 0.33$; Table 2.4). Plasma AOP was not different among treatments on d 0 ($P = 0.79$), d 14 ($P = 0.38$), or d 60 ($P = 0.13$).

Plasma Metabolites

Effects of year

An interaction between year and day was detected for plasma glucose concentrations (year × day interaction, $P < 0.01$; Figure 4). In years 1 and 3, plasma glucose was relatively stable throughout the 60-d experiment, whereas plasma glucose was much lower on day 0 in year

2 but increased drastically by d 14 and was relatively stable between d 14 and 60. An interaction between year and day was detected for plasma urea-N (year × day interaction, $P < 0.01$; Figure 5). Plasma urea-N increased between d 0 and 14 in year 1 but decreased over the same period in years 2 and 3. Plasma urea-N increased between d 14 and 60 in all years but was greatest on d 60 in year 1, intermediate in year 3, and least in year 3.

Effects of treatment

A main effect of treatment was detected for plasma glucose ($P = 0.02$; Table 2.4). Plasma glucose did not differ among treatments on d 0 or d 60 ($P \geq 0.74$) but was affected by treatment on d 14 ($P < 0.01$; Figure 8). On d 14, plasma glucose was greater for 15 g/d Met and 1.17 g/d choline than for control ($P \leq 0.03$) and tended to be greater for 5 g/d Met relative to control ($P = 0.06$). Plasma urea-N was not affected by treatment ($P = 0.83$; Figure 9) and plasma urea-N did not differ among treatments on d 0, d 14, d 60 ($P \geq 0.41$).

Discussion

Growth Performance

In our experiment, heifers were limit-fed at a target of 2.2% of body weight daily (dry matter basis). Over the 60-d feeding period, dry matter intake averaged 5.46 kg/d and average bodyweight across all treatments was 257 kg, which equates to cattle consuming 2.12% of body weight daily. The discrepancy is due to feed consumption during the initial 14 d averaging 1.81% of body weight daily as heifers were stepped up to the targeted intakes.

Work investigating performance responses to ruminally protected Met in growing and finishing cattle has yielded variable results. In steers fed a diet based on corn and corn silage, Deetz et al. (1985) observed similar average daily gain but lower dry matter intake when ruminally protected Met was supplemented, which led to greater feed efficiency for Met-supplemented steers. Wright and Loerch (1988) observed no differences in intake or finishing performance when ruminally protected Met was supplemented at four levels of metabolizable Met between 0.04% to 0.16% of dietary dry matter (approximately 3.5 to 15 g metabolizable Met/d) to finishing steers fed diets based on high-moisture corn. More recent studies have shown a similar lack of response to supplemental ruminally protected Met in finishing cattle fed diets based on high-moisture corn (Oney et al., 2016) or steam-flaked corn (Baggerman et al., 2021). The lack of response among trials conducted in growing and finishing cattle is most likely explained by either a lack of a Met deficiency or possibly another amino acid being co-limiting for growth. Cattle fed corn-based diets are most likely to be first-limited in lysine rather than methionine for growth due to the low lysine content of corn (Titgemeyer et al., 1988). Thus, because growth performance was only modestly affected by supplemental Met, and health was not different among treatments, it is unlikely that Met was clearly first-limiting in our cattle.

Work investigating ruminally protected Met supplementation to stressed, newly received beef cattle is limited. In a recent study, supplementing approximately 5.4 g/d metabolizable Met did not improve growth performance of 222-kg high-risk beef heifers fed a diet based on corn and wet corn gluten feed in a 45-d receiving trial (Grant et al., 2022). Like heifers in the present study, cattle of Grant et al. (2022) were of southeastern U.S. origin and were managed under nearly identical conditions.

Work in transition dairy cows suggests that peripartal supplementation of Met can improve lactation performance. Osorio et al. (2013) observed greater milk fat and protein yield and a tendency for increased milk yield in cows supplemented with approximately 10 g/d metabolizable Met throughout the transition period (21 d prepartum to 30 d postpartum). Similar responses were observed by Zhou et al. (2016a) in cows supplemented with approximately 13 g/d metabolizable Met and Batistel et al. (2017) in cows receiving approximately 15.6 g/d metabolizable Met. One explanation for improved performance of cows receiving supplemental Met is a consistently observed increase in dry matter intake for Met-supplemented cows during early lactation (Osorio et al. 2013; Zhou et al., 2016a; Batistel et al., 2017). In early lactation, cows are unable to consume adequate dry matter intake to support lactational demands for energy and protein and enter negative energy and nitrogen balance (NASEM, 2021). Thus, cows that maintain greater dry matter intake in the transition period may be able to better support greater milk production in early lactation. Because our cattle were limit-fed, improvements in gain or health resulting from increased voluntary dry matter intake during the receiving period could not be evaluated.

Few studies have investigated ruminally protected choline supplementation in growing and finishing beef cattle. Bryant et al. (1999) observed maximal average daily gain and feed

efficiency when 5 g/d choline ion was supplemented for the entire finishing period to beef steers fed a 90% concentrate diet for 112 or 140 d. Bindel et al. (2000) demonstrated greatest average daily gain and feed efficiency when 10 g/d available choline ion was supplemented to finishing beef heifers fed corn-based diets for 120 d. In both studies, authors observed no added benefit to choline doses greater than 10 g/d (i.e., 10 or 20 g/d choline, Bryant et al., 1999; 10 or 15 g/d choline Bindel et al., 2000). In contrast, supplementing 5 g/d available choline ion in the last 29 days of the finishing period failed to improve finishing performance of steers consuming a diet based on steam-flaked corn (Sexson et al., 2010). Cattle of Bryant et al. (1999) and Bindel et al. (2000) were supplemented with choline for the entire finishing period (112 to 140 d and 120 d, respectively), whereas cattle of Sexson et al. (2010) were only fed choline for the final 29 d of the finishing period. Taken together, it appears that choline supplementation can improve feedlot performance; however, short-term choline supplementation (i.e., < 30 d) may not be adequate to generate a statistically significant change in performance. To our knowledge, no work prior to the present study has investigated ruminally protected choline supplementation in newly received beef cattle. In our heifers, providing either 1.17 or 3.5 g/d available choline for 60 d (26 or 78 g ReaShure/d) did not improve growth performance. Bryant et al. (1999) suggested that choline may have improved finishing performance through altered lipid metabolism (e.g., phosphatidylcholine synthesis), so it is possible our heifers were depositing too little adipose tissue to benefit from changes in lipid metabolism.

Choline supplementation has been thoroughly investigated in transition dairy cows. In a recent meta-analysis summarizing 21 experiments, Arshad et al. (2020) determined that providing 12.9 g/d choline ion throughout the transition period increased pre- and post-partum dry matter intake and yields of milk, energy-corrected milk, fat, and protein. It is currently

unclear the exact mechanism by which choline improves performance in transition cows; however, it is likely that multiple functions of choline in the body (e.g., methyl group metabolism, absorption and transport of lipids, phospholipid synthesis, and reduced inflammation) contribute to its efficacy in transition cows (Baldi and Pinotti, 2006; Chandler and White, 2017, Zenobi et al., 2018a; Bollatti et al., 2018). Because our cattle were limit-fed, our cattle could not benefit from stimulation of feed intake.

Although none of the methionine or choline treatments altered growth performance relative to control, final body weight, overall average daily gain, and feed efficiency tended to be greater for heifers receiving 15 g/d Met compared with 5 g/d Met or 1.17 or 3.5 g/d choline. To our knowledge, no other experiments offering a direct comparison of ruminally protected Met and choline supplementation are available in beef cattle. Löest et al. (2002) supplemented Met-deficient growing Holstein steers with Met or choline by abomasal infusion and observed an expected increase in protein deposition when Met was supplemented but N retention did not respond to choline infusion. Limited work has been conducted offering a direct comparison of ruminally protected Met and choline in transition dairy cows. Ardalan et al. (2010) supplemented transition cows with Met or choline alone or in combination and observed greater milk yield and fat-corrected milk yield for cows supplemented with choline alone or in combination with Met relative to control; Met supplementation alone did not improve lactational performance. In contrast, Zhou et al. (2016a) supplemented Met or choline alone or in combination to transition cows and observed greater dry matter intake and yield of milk and milk components when cows were supplemented with Met relative to control; choline did not improve intake or lactational performance. In a similar study, Potts et al. (2020) reported greater milk yield in primiparous cows supplemented with choline alone or in combination with Met; however, Met alone did not

affect lactational performance. In contrast, in multiparous cows, increased milk yield was observed when cows received choline alone but not in combination with Met; Met alone did not affect milk yield (Potts et al., 2020). Arshad et al. (2020) reported that lactational responses to choline decreased as metabolizable Met supply increased, so it is possible that some differences could be attributed to differences in Met supplied by diets of Ardalan et al. (2010), Zhou et al. (2016a), and Potts et al. (2020). The lack of a large response to supplemental Met in our cattle suggests that our cattle were not first-limiting in Met, which could similarly explain why our cattle also did not respond to supplemental choline.

Animal Health

Effects of year

Overall incidence of morbidity and mortality can vary greatly among groups of newly received cattle. In our study, we observed large variation in health status among the three replicate years (Figure 1) and further among individual loads (blocks) of cattle (data not shown). In year 1, 21.5% of cattle were treated at least once for respiratory illness, which ranged from 9.4 to 32.3% of the five loads of cattle received. Heifers tended to have lower first treatment morbidity in year 2, where 9.3% of heifers were treated at least once, which ranged from 0 to 18.8% of the five loads received. Year 3 morbidity was greatest with 53.5% of heifers treated at least once, and ranged from 41.7 to 67.7% among the five blocks. Compared with years 1 and 2, incidence of first respiratory morbidity was 2.5- and 6-fold greater in year 3, respectively; however, it isn't clear why heifers in year 3 faced a substantially greater respiratory challenge.

In years 1 and 2, most of the first respiratory treatments occurred between d 14 and d 30 of the feeding period. Conversely, in year 3, cattle became ill shortly after arrival and the greatest

proportion of first respiratory treatments occurred between d 7 and 14 of the feeding period. On d 7 (i.e., first day that heifers were eligible for treatment following the 7-d metaphylaxis moratorium), 23.3% of heifers were treated for respiratory illness, some of which displayed signs of clinical illness prior to d 7. Thus, our heifers were either already infected or were exposed to infected cattle before arrival at our facility. Although a large proportion of cattle were treated very early in year 3 (likely before dietary treatments could affect health), heifers treated for respiratory disease on d 7 were distributed evenly among dietary treatments, so this was unlikely to have biased our data.

Effects of treatment

Because our heifers were both stressed and exposed to pathogens, it is not surprising that a portion of our cattle became sick shortly after arrival. We did not detect any differences among treatments for first, second, or third respiratory morbidity or mortality. On average across the five treatments, 76.8% of heifers treated for respiratory disease once responded to treatment, and 81.5% of heifers treated twice responded to their second treatment. Because heifers treated thrice were removed from the experiment, response to third treatment was not measured. Among heifers treated once, 0.72% died prior to third treatment; chronic heifers that died after removal from the experiment were not included in final death loss. Overall, 2.9% of heifers treated at least once died, and 7.2% of heifers treated at least once either died or were declared chronic.

Limited work has been conducted investigating effects of Met or choline on health in newly received beef cattle. Grant et al. (2022) supplemented newly received beef heifers of southeastern U.S. origin with 0 or 5 g/d metabolizable Met under management conditions similar to ours and observed very low incidence of morbidity and mortality (2.1% and 0.5%, respectively); due to low incidence, effects of Met on health could not be evaluated. In a similar

experiment conducted at this research facility, Spore et al. (2019) observed overall morbidity and mortality of 13% and 4%, respectively, in newly received beef heifers of similar origin and treated with metaphylaxis at arrival. Administering metaphylaxis at arrival provides therapy to cattle that may be subclinically ill and temporarily delays onset of a respiratory disease outbreak following feedlot arrival (Vander Ley, 2019). By administering metaphylaxis, differences in respiratory disease incidence among dietary treatments could be fairly evaluated in our experiment and were not biased by cattle that were ill at arrival. In addition, metaphylaxis typically decreases incidence of respiratory disease and mortality by approximately 50% (Wileman et al., 2009). Thus, without metaphylaxis, respiratory disease incidence may have approached 60% in our heifers. Although our cattle faced an illness challenge large enough to evaluate potential health responses to treatment, supplemental Met or choline did not reduce clinical illness in our cattle.

Plasma Acute Phase Protein and Oxidative Stress Responses

Effects of year

Haptoglobin is a positive acute phase protein and is produced in the liver during an inflammatory response and released into circulation (Ackerman, 2017). Thus, circulating haptoglobin concentrations are a useful indicator of inflammation in the body. Our observation of elevated plasma haptoglobin at trial initiation in year 3 matches well with the early occurrence of respiratory illness in year 3 compared with years 1 and 2. In year 3, 40% of heifers received at least one respiratory treatment on or before d 14, whereas only 7.5% of heifers in year 1 and 0.6% of heifers in year 2 were treated at least once in the same period (Figure 2.1). Although cattle in year 3 were not visibly ill upon arrival at our facility, they clearly were in an elevated

inflammatory state at arrival. In addition, the observed greater haptoglobin on d 14 in years 1 and 3 compared with year 2 matches well with overall respiratory morbidity observed among years (Figures 2.1 and 2.2). Our observation of elevated day 14 plasma haptoglobin in year 1 compared with year 2 suggests an elevated inflammatory status in year 1 cattle at d 14, which correlates well with numerically larger respiratory morbidity in year 1. Very few respiratory treatments occurred in the last 30 d of our 60-d experiment, which matches well with plasma haptoglobin concentrations being lowest on d 60 for all 3 years.

The balance between oxidative compounds (e.g., superoxide, hydroxyl radicals, hydrogen peroxide) and existing antioxidant mechanisms (e.g., antioxidant enzymes and compounds) is integral to maintenance of cellular integrity (Freitas et al., 2016). Oxidative stress occurs when oxidative compounds overwhelm the body's antioxidant capacity, which exacerbates inflammation and is associated with greater disease susceptibility in dairy cattle (Bernabucci et al., 2005; Castillo et al., 2005). Plasma AOP reflects the cumulative circulating antioxidant activity standardized to the reducing power of Trolox, rather than measurement of individual antioxidants. In addition, greater plasma AOP indicates less oxidative stress, which could prevent inflammation and decrease disease susceptibility. In contrast, decreases in plasma AOP could indicate greater oxidative stress and susceptibility to inflammation and disease. It is unclear why AOP was depressed in year 1 compared with years 2 and 3, particularly because incidence of respiratory illness and overall inflammatory status was greater in year 3 compared with years 1 and 2. These data are in contrast with our expectation that decreases in plasma AOP would be related to increases in inflammatory status and disease incidence; plasma AOP was not related to inflammation or disease incidence in our cattle.

Effects of treatment

Plasma haptoglobin concentrations were similar among treatments on d 0 (i.e., prior to treatment application), which indicates that baseline inflammatory status was evenly distributed across treatments at trial initiation. Between d 0 and 14, plasma haptoglobin tended to increase for control cattle and heifers supplemented with equimolar amounts of Met or choline (i.e., 5 g/d Met and 3.5 g/d choline). In contrast, haptoglobin concentrations were stable between d 0 and 14 for heifers supplemented with 15 g/d Met and tended to decrease for 1.17 g/d choline. Our data indicate that the trend for haptoglobin (i.e., inflammation) to increase between d 0 and 14 for control cattle was mitigated by supplementing 1.17 g/d choline and was numerically mitigated by 15 g/d supplemental Met. It is unclear why changes in plasma haptoglobin were so dose dependent for both Met and choline; however, dose-dependent responses to anti-inflammatory agents are not surprising. It is possible that 5 g/d Met was not a large enough dose to reduce inflammation compared with 15 g/d Met, although Grant et al. (2022) reported that supplementing 6 g/d Met mitigated an increase in plasma haptoglobin that was observed in heifers managed similarly to those in the current study. Health status of the cattle may play a role, because cattle of Grant et al. (2022) only experienced 2.1% morbidity compared with 24% in the current experiment. Thus, it is possible that providing 6 g/d Met successfully mitigated inflammation in healthy cattle, whereas 5 g/d Met may have been inadequate to manage inflammation in disease-susceptible cattle. In transition dairy cows, plasma haptoglobin responses to ruminally protected Met supplementation has been variable; in some experiments, supplemental Met decreases haptoglobin (Zhou et al., 2016; Batistel et al., 2018), whereas other experiments have not observed changes in plasma haptoglobin in response to Met (Osorio et al., 2014; Vailati-Riboni et al., 2017). Because Met supplementation did not strongly impact growth

performance or health in our cattle, the decrease in inflammation in response to 15 g/d Met most likely did not result from remediation of a Met deficiency. For heifers supplemented with choline, it is unclear why providing 1.17 g/d choline effectively decreased inflammation on d 14, whereas 3.5 g/d choline did not. In growing steers, Grant (2020) reported a trend for post-ruminal infusion of 5 g/d choline to decrease plasma haptoglobin, which was attributed to a likely decrease in systemic inflammation. Because we provided choline in a ruminally protected form, it is difficult to directly compare choline doses between our experiment and that of Grant (2020). Work in dairy cattle has demonstrated decreases in plasma haptoglobin in response to ruminally protected choline supplementation in feed restriction models designed to induce fatty liver disease (Zenobi et al., 2018; Arshad et al., 2023). Zenobi et al. (2018) observed a linear decrease in haptoglobin with increasing choline dose after feed restriction and pulse dosing of supplemental fat. In contrast, Arshad et al. (2023) observed an approximate 40% decrease in plasma haptoglobin in choline-supplemented cows regardless of choline dose (12.9 or 25.8 g/d choline ion) compared to control cattle in response to feed restriction. Supplemental choline has not decreased circulating haptoglobin in transition cow models (Zhou et al., 2016; Bollatti et al., 2020; Zenobi et al., 2020). In humans, individuals consuming diets rich in choline and betaine have consistently lower circulating inflammatory biomarkers (Detopoulou et al., 2008; Cho et al., 2008; Fargnoli et al., 2008). Similarly, individuals with greater choline intake have decreased risk for development of non-alcoholic fatty liver disease, which can lead to hepatic inflammation and fibrosis (Chai, 2023). In pregnant rats subjected to an in vivo lipopolysaccharide (LPS) induced inflammatory challenge, supplemental choline decreased circulating proinflammatory cytokines and improved fetal survivability relative to rats receiving LPS alone (Zhang et al.,

2018). Although choline's attenuation of inflammation is well documented in other species, these responses are less consistent in cattle.

In transition dairy cows, ruminally protected Met supplementation consistently increases plasma and hepatic concentrations of antioxidants glutathione and taurine (Osorio et al., 2014; Zhou et al., 2018; Batistel et al., 2018); however, plasma AOP was improved in some experiments (Osorio et al., 2014; Batistel et al., 2018) but not others (Zhou et al., 2018). Because we did not measure individual antioxidants, we cannot conclude if glutathione or taurine were affected by Met. In transition dairy cows supplemented with ruminally protected choline, Swartz et al. (2023) reported increased AOP for choline-supplemented cows prepartum; however, this increase was not observed postpartum. In contrast, other work in transition cows has not demonstrated improvements in AOP in response to supplemental choline (Zhou et al., 2016; Coleman et al., 2019). Because choline improves AOP in transition cows in some cases but not others, our observed numeric increase in AOP for choline supplemented heifers on d 60 is not surprising.

Plasma Metabolites

Effects of year

Blood glucose levels are typically elevated immediately following long-distance road transport in cattle but return to baseline levels within 24 h (Galyean et al., 1981; Earley and O'Riordan, 2006; Earley et al., 2006). Although our cattle were rested for approximately 24 h prior to trial initiation on d 0, stressors other than transport (e.g., commingling, novel environment, initial processing, etc.) could have contributed to elevated plasma glucose on d 0 in years 1 and 3. Elevated circulating glucose on arrival has been associated with greater respiratory

morbidity and mortality in newly received cattle in some experiments (Donley et al., 2009), which agrees with our observation of greater morbidity in years 1 and 3 than year 2. In contrast, other work has reported greater respiratory morbidity for cattle with low circulating glucose on arrival (Montgomery et al., 2009; Oosthuysen et al., 2016). Plasma glucose concentrations were generally sustained between d 14 and 60 at levels above baseline, which likely resulted from stable feed intake from d 14 to 60 in our cattle.

Circulating urea is also often increased during stress (e.g., marketing, transport) and immune system activation due to muscle protein degradation and amino acid catabolism to provide glucose precursors and support synthesis of acute phase proteins during inflammation (Elsasser et al., 1995). The decrease in plasma urea between d 0 and 14 could suggest that by d 14 cattle were no longer mobilizing muscle tissue to support energetic needs; however, because plasma haptoglobin increased between d 0 and 14, it is clear that our cattle were still in an inflammatory state on d 14. Plasma urea did not change between d 0 and 14 in year 1; dry matter intake tended to be greater between d 0 and 14 in year 1 compared with years 2 and 3, which would be expected to improve protein status. The increase in plasma urea between d 14 and 60 was likely due to greater feed and N intake on d 60 than on d 14.

Effects of treatment

Circulating glucose levels can be indicative of general energy status in cattle. Greater plasma glucose for heifers receiving 5 or 15 g/d Met or 1.17 g/d choline could result from decreased inflammation on d 14 relative to control and 3.5 g/d choline, which would require less glucose to support the inflammatory response. In transition dairy cattle, Sun et al. (2016) observed greater plasma glucose at calving for cows supplemented with Met, or choline, or both. In contrast, other work in transition cows has not observed effects of Met on glucose during the

transition period (Osorio et al., 2013; Zhou et al., 2016a; Batistel et al., 2017). In a recent meta-analysis of 21 experiments, Arshad et al. (2020) reported greater pre- and post-partum glucose concentrations for cows supplemented with ruminally protected choline. These data support our observation of greater plasma glucose for heifers receiving 1.17 g/d choline. The lack of a similar response for plasma glucose for 3.5 g/d choline may be related to greater inflammation (i.e., haptoglobin) on d 14 for heifers receiving 3.5 g/d choline compared with 1.17 g/d choline.

Circulating urea-N concentrations can be an indicator of overall protein and N balance in cattle. In transition cows, Zhou et al. (2016) observed no differences in milk urea-N in cows supplemented with Met or choline. The lack of differences among treatments for plasma urea-N across sampling days suggests that overall N utilization in our cattle was not largely affected by Met or choline.

Conclusions

Our data suggest that Met was not clearly the first-limiting amino acid for growth in our heifers. In addition, choline supplementation did not improve performance in our heifers. Clinical illness was not affected by methionine or choline in our high-risk receiving cattle. Despite this, choline supplementation increased plasma antioxidant potential and decreased plasma haptoglobin concentrations, which suggests that choline may ameliorate oxidative stress and inflammation at sub-clinical levels. Moreover, the trend for a linear decrease in haptoglobin with increasing supplemental Met supports previous work suggesting that Met may mitigate inflammation in receiving cattle. The lack of response to supplementation with ruminally protected Met or choline with regard to growth performance and clinical illness does not support their use as economically feasible immunomodulators for newly received calves fed diets based on corn and corn gluten feed.

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Table 2.1. Composition of diet fed to receiving heifers

Item	Year 1	Year 2	Year 3
Ingredient		% of dietary dry matter	
Corn, dry-rolled	39.5	39.5	39.5
Wet corn gluten feed[1]	40.0	40.0	40.0
Prairie hay, chopped	13.0	13.0	13.0
Supplement pellet[2]	7.5	7.5	7.5
Chemical composition			
Dry matter, % as is	76.2	75.1	75.2
Organic matter	92.9	93.6	94.0
Neutral detergent fiber	27.9	27.1	26.9
Acid detergent fiber	10.1	9.9	9.9
Crude protein	14.8	14.5	14.9

[1] Sweet Bran (Cargill Corn Milling, Blair, NE)

[2] Supplement pellet formulated to contain (dry matter basis) 8.5% calcium, 0.64% phosphorus, 0.76% potassium, 5.0% salt, and 307 g/ton monensin (Rumensin; Elanco, Greenfield, IN)

Table 2.2. Summary of growth performance and health of beef heifers by replicate year

	Year				
Item	1	2	3	SEM[1]	*P*-value
BW, kg					
Day 0	225	225	221	1.6	0.28
Day 14	243	240	241	2.0	0.64
Day 21	247	250	250	1.7	0.29
Day 28	255	254	255	2.0	0.90
Day 35	260	262	263	1.8	0.68
Day 42	269	270	271	2.0	0.78
Day 49	277	279	278	1.8	0.91
Day 60	289	293	290	3.0	0.69
ADG, kg/d					
Days 0 to 14	1.31	1.11	1.43	0.133	0.27
Days 14 to 60	1.01	1.15	1.05	0.053	0.23
Days 0 to 60	1.08	1.14	1.14	0.050	0.66
DMI, kg/d					
Days 0 to 14	4.43^{a}	4.23^{ab}	3.99^{b}	0.087	0.01
Days 14 to 60	5.99^{a}	5.68^{b}	5.87^{a}	0.042	0.007
Days 0 to 60	5.63^{a}	5.33^{b}	5.43^{b}	0.050	0.004
G:F, kg/kg					
Days 0 to 14	0.295^{xy}	0.265^{y}	0.360^{x}	0.0283	0.09
Days 14 to 60	0.169^{x}	0.202^{y}	0.179^{x}	0.0090	0.06
Days 0 to 60	0.192	0.214	0.210	0.0087	0.22
Morbidity, %					
Treated once	21.5^{b}	9.4^{b}	53.5^{a}	7.75	0.0007
Treated twice	6.2^{b}	1.2^{c}	19.8^{a}	4.20	0.0004
Treated thrice	1.0^{b}	0.2^{b}	4.8^{a}	1.35	0.007
Mortality, %	0.6	0.4	1.4	0.54	0.24
Days to					
First treatment	21^{a}	24^{a}	13^{b}	1.9	0.003
Second treatment	21^{b}	40^{a}	19^{b}	3.2	0.0004
Third treatment	29	29	29	9.4	1.00
Mortality	18	25	23	24.2	0.97

[1] Average SEM across all years

[a-c] Within row, means without a common superscript differ, $P \leq 0.05$

[x-z] Within row, means without a common superscript differ, $P \leq 0.10$

Table 2.3. Effects of methionine and choline on growth performance and health in high risk beef heifers

	Treatment						
		Methionine, g/d		Choline, g/d			
Item	Control	5	15	1.17	3.5	SEM[1]	*P*-value
BW, kg							
Day 0	223	223	224	224	224	0.9	0.26
Day 14	241	241	242	241	241	1.3	0.61
Day 21	249	249	248	249	248	1.4	0.91
Day 28	256	254	256	254	255	1.4	0.27
Day 35	262	261	263	261	261	1.4	0.26
Day 42	270^{aby}	270^{b}	273^{adx}	269^{b}	268^{b}	1.4	0.01
Day 49	278^{abe}	277^{b}	281^{ad}	277^{b}	277^{b}	1.4	0.05
Day 60	291^{xy}	290^{y}	293^{x}	290^{y}	289^{y}	2.1	0.09
ADG, kg/d							
Days 0 to 14	1.29	1.30	1.33	1.26	1.24	0.087	0.69
Days 14 to 60	1.09	1.06	1.11	1.05	1.05	0.037	0.21
Days 0 to 60	1.14^{xy}	1.11^{y}	1.16^{x}	1.10^{y}	1.09^{y}	0.034	0.09
DMI, kg/d							
Days 0 to 14	4.22	4.18	4.18	4.29	4.20	0.062	0.32
Days 14 to 60	5.86	5.82	5.88	5.84	5.84	0.029	0.25
Days 0 to 60	5.48	5.44	5.48	5.47	5.45	0.033	0.48
G:F, kg/kg							
Days 0 to 14	0.306	0.313	0.321	0.296	0.298	0.0195	0.55
Days 14 to 60	0.186	0.182	0.189	0.180	0.180	0.0061	0.32
Days 0 to 60	0.208^{xy}	0.205^{xy}	0.212^{x}	0.201^{y}	0.201^{y}	0.0058	0.10
Morbidity, %							
Treated once	23.9	24.1	22.6	23.1	27.6	4.50	0.71
Treated twice	4.9	6.7	4.4	4.9	7.2	1.78	0.30
Treated thrice	1.2	1.3	1.4	0.5	0.8	0.67	0.52
Mortality, %	0.60	0.60	0.60	0.91	0.91	0.54	0.97
Days to							
First treatment	19.3	19.9	17.6	20.9	18.2	1.39	0.15
Second treatment	25.4	30.3	21.3	28.1	27.1	2.66	0.12
Third treatment	18.1^{z}	32.5^{xy}	28.2^{y}	41.4^{x}	26.1^{yz}	6.9	0.06
Mortality	21.5	28.6	7.9	22.4	29.6	23.5	0.67

[1] Average SEM across all treatments

[a-c] Within row, means without a common superscript differ, $P \leq 0.05$

[x-z] Within row, means without a common superscript differ, $P \leq 0.10$

Table 2.4. Effects of supplemental methionine or choline on plasma glucose, urea, haptoglobin, and Trolox-equivalent AOP in high-risk beef heifers

		Methionine, g/d		Choline, g/d			*P*-value		
Item	Control	5	15	1.17	3.5	SEM[1]	SLICE	TRT	D × T
Number of pens	24	24	24	24	24	–	–	–	–
Glucose, mM									
Day 0	4.61	4.70	4.73	4.63	4.65	0.103	0.78	0.02	0.40
Day 14	5.10[bc]	5.29[ab]	5.41[a]	5.33[a]	5.06[c]	0.103	<0.01		
Day 60	5.10	5.11	5.17	5.05	5.05	0.103	0.74		
Urea, mM									
Day 0	4.80	4.60	4.64	4.68	4.64	0.111	0.41	0.83	0.51
Day 14	4.26	4.20	4.14	4.26	4.18	0.111	0.82		
Day 60	5.40	5.44	5.50	5.40	5.57	0.111	0.52		
Haptoglobin, µg/mL									
Day 0	893	840	872	892	813	116.7	0.95	0.06	0.18
Day 14	1076[a]	1087[a]	900[ab]	670[b]	1004[a]	116.7	<0.01		
Day 60	555	596	441	451	435	116.7	0.51		
AOP, µM[2]									
Day 0	83.2	83.8	85.3	86.6	85.0	2.91	0.79	0.33	0.40
Day 14	92.2	94.9	95.1	91.5	96.5	2.91	0.38		
Day 60	92.9	91.3	91.6	97.4	96.1	2.91	0.13		

[1] Average SEM across all treatments

[a-c] Means without a common superscript differ, $P \leq 0.05$

[2] Trolox-equivalent antioxidant potential

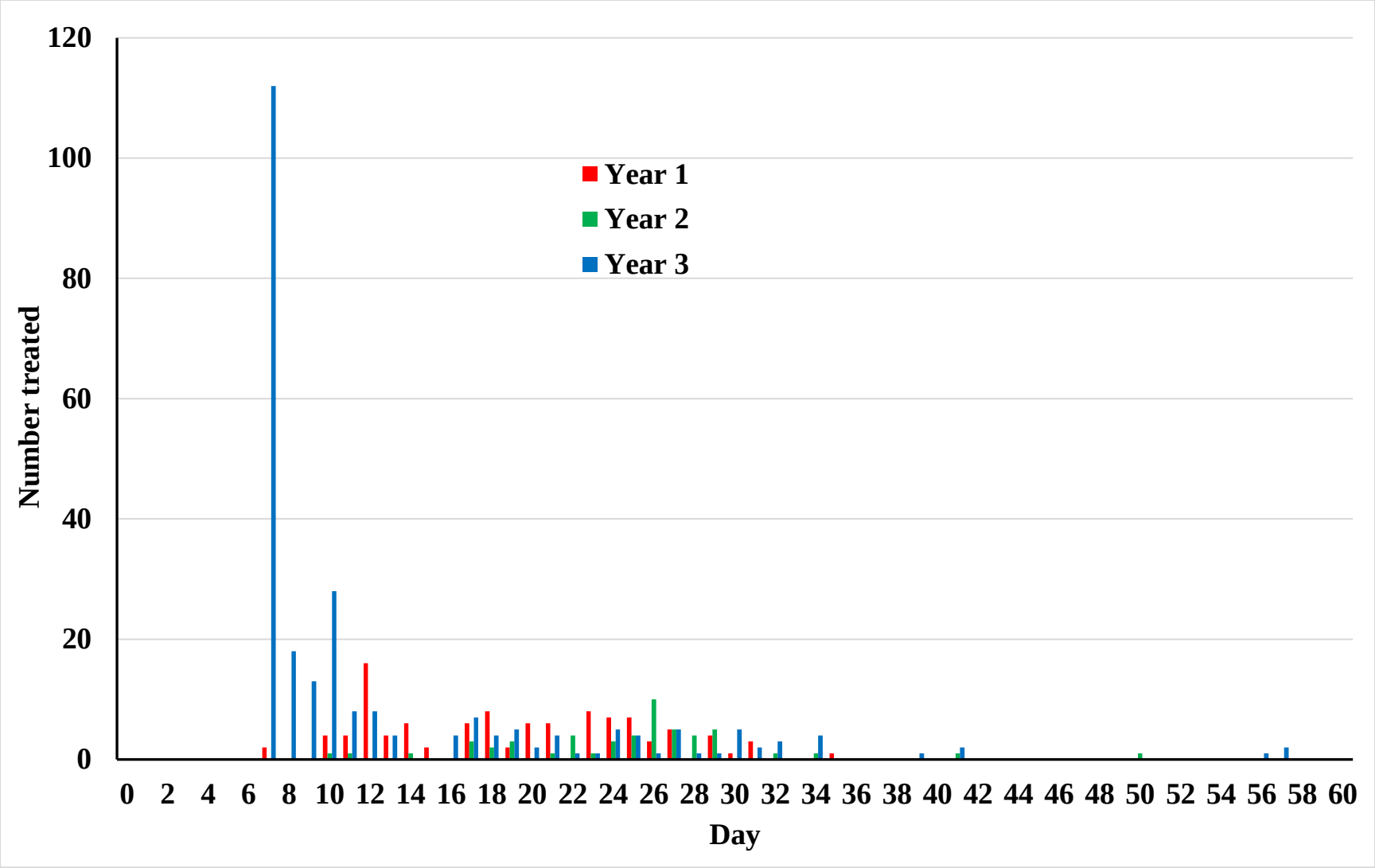

Figure 2.1. Distribution of days to first respiratory morbidity in high-risk beef heifers across replicate years. Effect of year, *P* <0.01), SEM = 1.71.

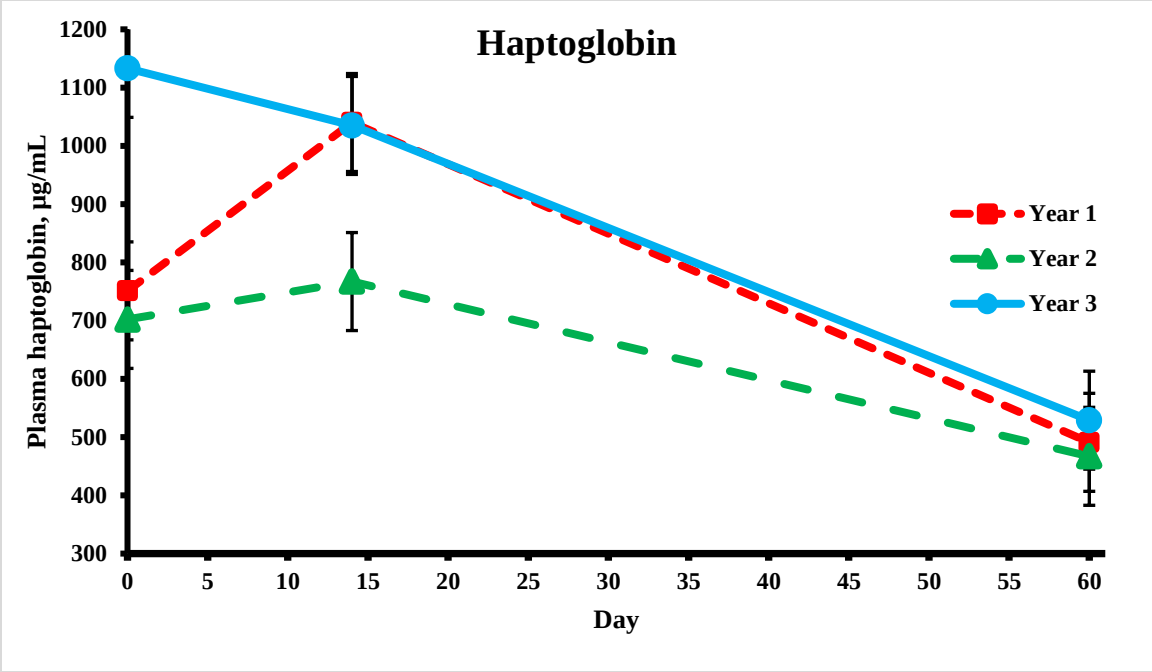

Figure 2.2. Effects of trial year on plasma haptoglobin concentrations in high-risk beef heifers. Effect of year (P = 0.05); SEM = 65.2.

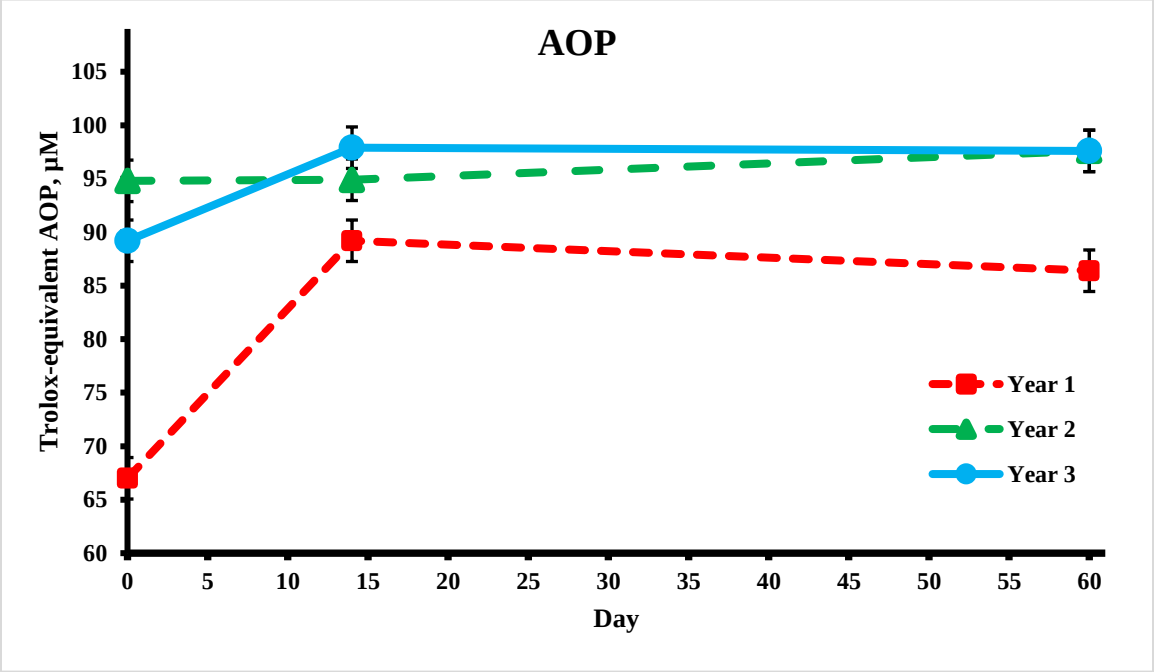

Figure 2.3. Effects of trial year on plasma Trolox-equivalent antioxidant potential (AOP) in high-risk beef heifers. Effect of year ($P < 0.01$); SEM = 1.4.

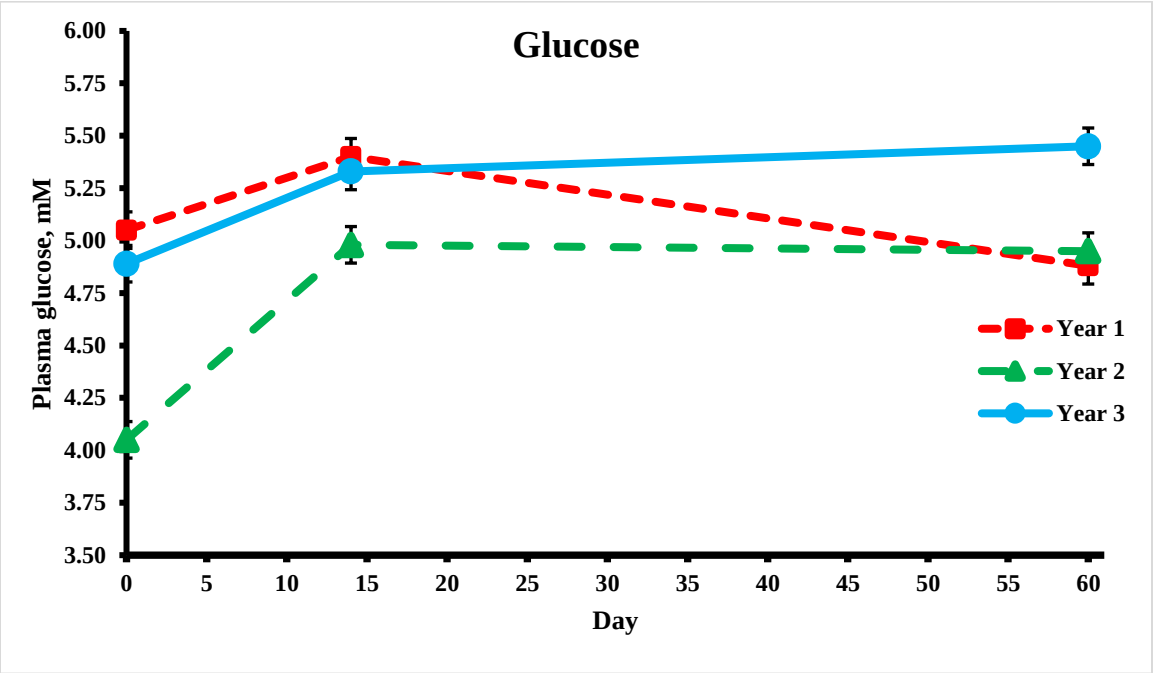

Figure 2.4. Effects of trial year on plasma glucose concentrations in high-risk beef heifers. Effect of year ($P < 0.01$); SEM = 0.0585)

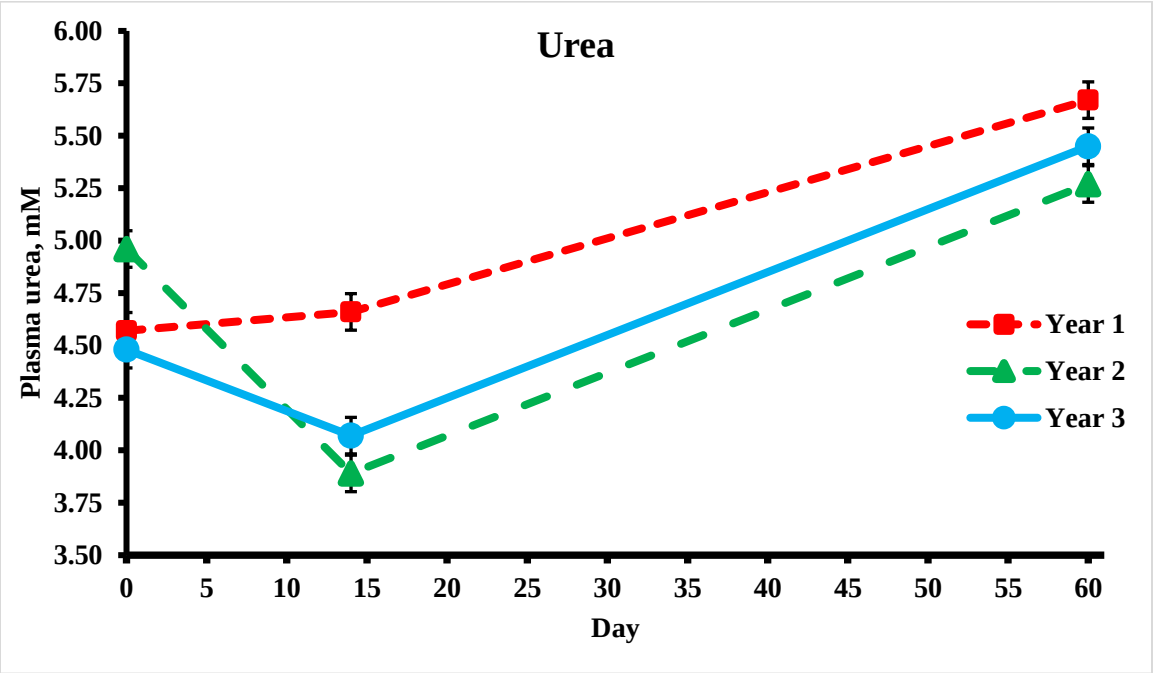

Figure 2.5. Effects of trial year on plasma urea concentrations in high-risk beef heifers. Effect of year (P = 0.02); SEM = 0.0727.

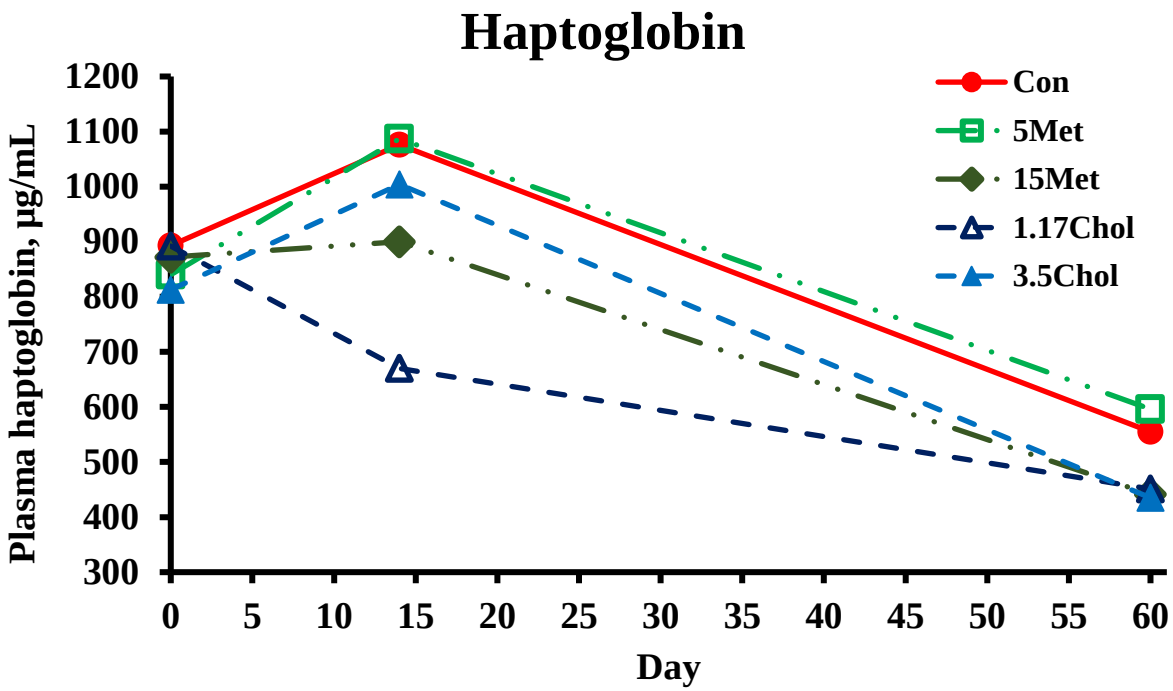

Figure 2.6. Effects of methionine and choline on plasma haptoglobin concentrations in high-risk beef heifers. Effect of treatment ($P = 0.06$), day ($P < 0.05$), treatment × day ($P = 0.18$). Effect of treatment within day, day 0 ($P = 0.95$), day 14 ($P < 0.01$), day 60 ($P = 0.51$). SEM = 116.7.

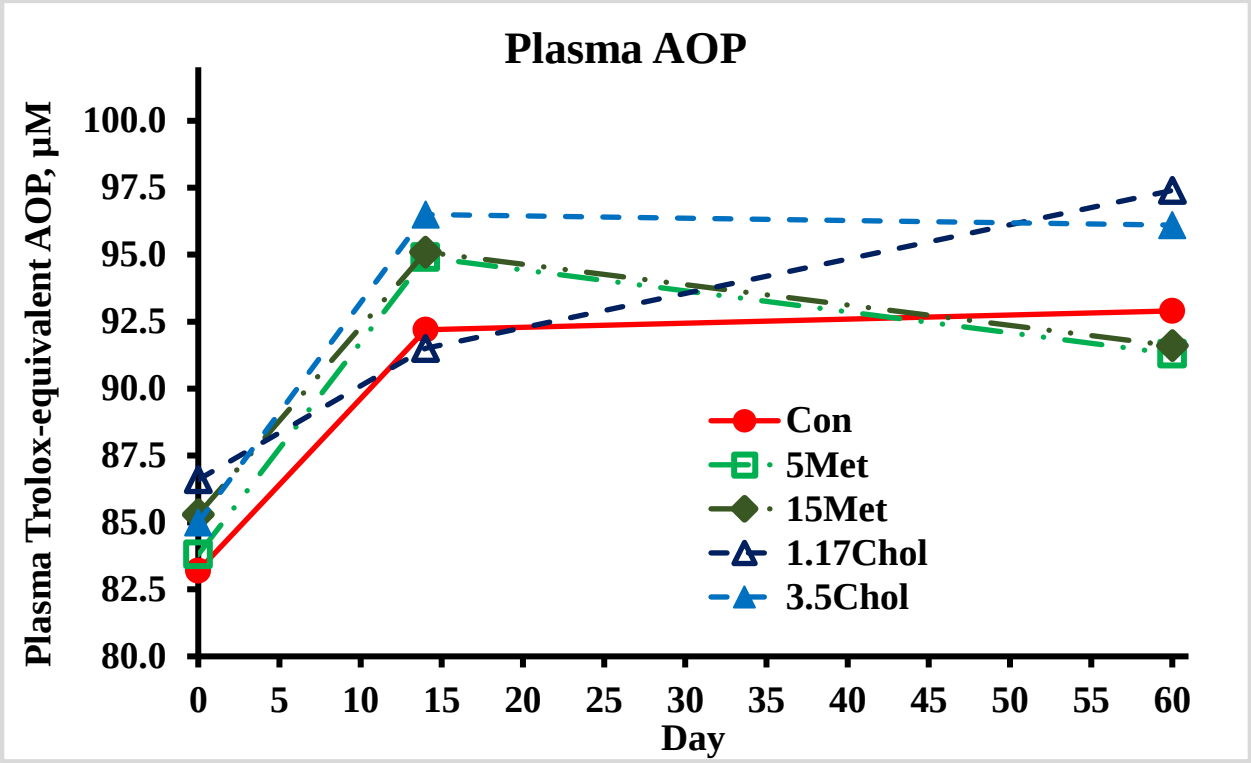

Figure 2.7. Effects of methionine and choline on plasma Trolox-equivalent antioxidant potential (AOP) in high-risk beef heifers. Effect of treatment ($P = 0.33$), day ($P < 0.01$), treatment × day ($P = 0.40$). Effect of treatment within day, day 0 ($P = 0.79$), day 14 ($P = 0.38$), day 60 ($P = 0.13$). SEM = 2.92.

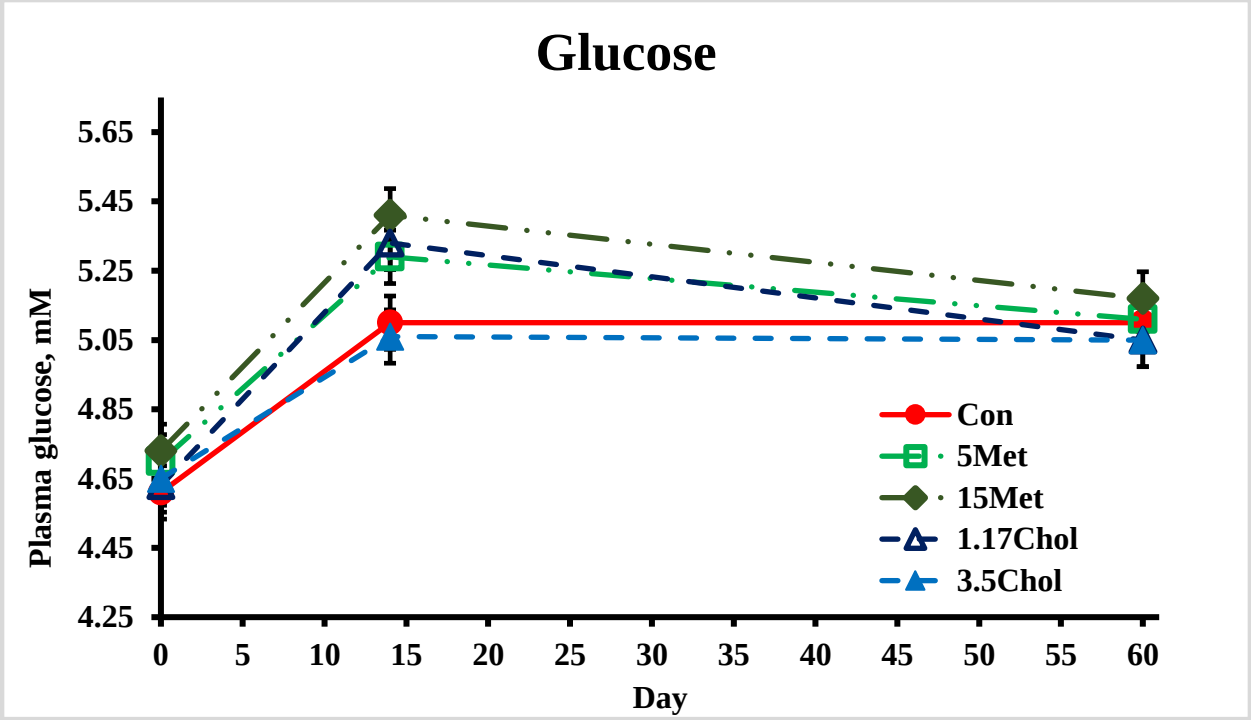

Figure 2.8. Effects of methionine and choline on plasma glucose concentrations in high-risk beef heifers. Effect of treatment ($P = 0.02$), day ($P < 0.01$), treatment × day ($P = 0.40$). Effect of treatment within day: day 0 ($P = 0.78$), day 14 ($P < 0.01$), day 60 ($P = 0.78$). SEM = 0.103.

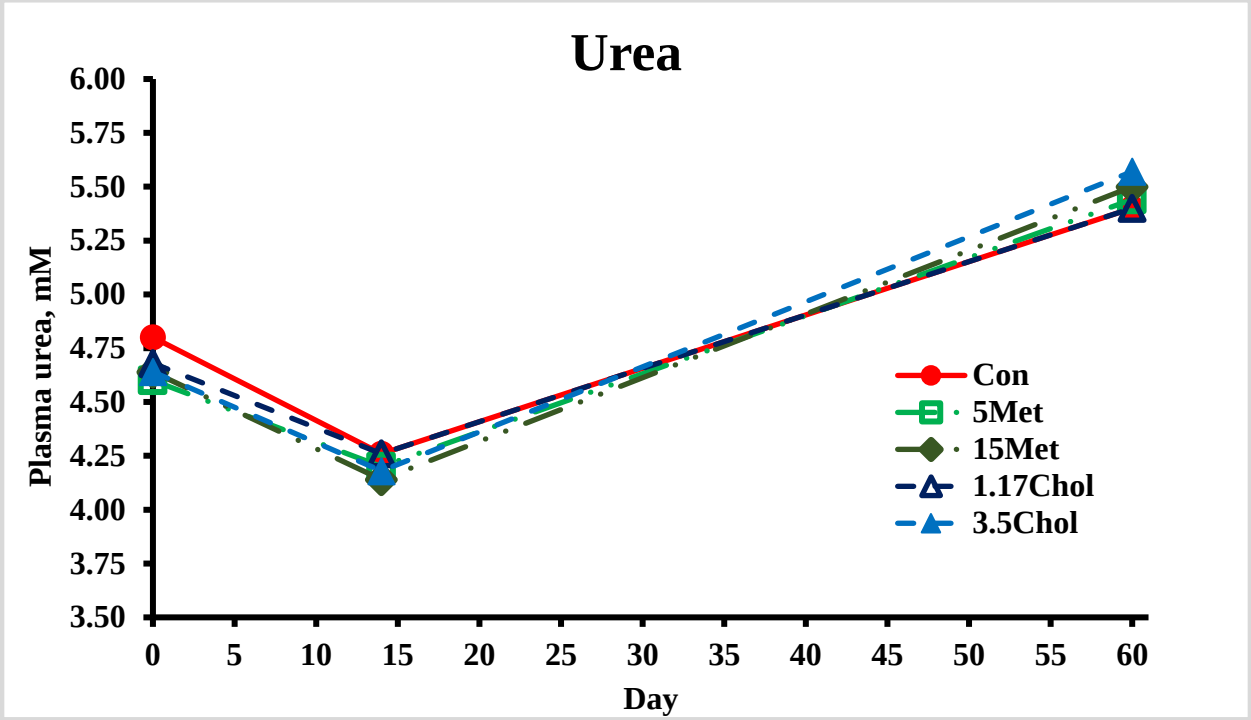

Figure 2.9. Effects of methionine and choline on plasma urea concentrations in high-risk beef heifers. Effect of treatment ($P = 0.82$), day ($P < 0.01$), treatment × day ($P = 0.51$). Effect of treatment within day, day 0 ($P = 0.41$), day 14 ($P = 0.82$), day 60 ($P = 0.52$). SEM = 0.111.

Chapter 3 - Effects of supplemental betaine, guanidinoacetic acid, and creatine on protein deposition, whole-body methionine metabolism, and immune function in growing steers [1]

[1]This work is supported by Agricultural and Food Research Initiative grant no. 2020-67015-30826/project accession no. 1021931 from the USDA National Institute of Food and Agriculture

Abstract

Methyl donors (e.g., methionine, choline) can improve lactational performance and immune function in transition dairy cows. Betaine is a product of choline oxidation and a methyl donor. Our objective was to determine if betaine supplementation would affect protein deposition and immune function in growing cattle with altered methyl group status. Ruminally cannulated Holstein steers (189 kg) were used in a 6 × 6 Latin square design. Factorial treatments, which were infused abomasally, included 3 methyl group modulator treatments (MGM: control; 15 g/d guanidinoacetic acid [GAA]; or 16.8 g/d creatine) and 2 levels of betaine (0 or 5.6 g/d betaine). Providing GAA or creatine increases body creatine supply, but GAA consumes methyl groups to synthesize creatine, whereas supplemental creatine spares methyl groups that would otherwise be used for its synthesis. Steers received 3.5 kg/d (dry matter basis) of a corn-based diet. Whole-body Met flux was measured by continuous jugular infusion of 1-^{13}C-L-Met and methyl-$^{2}H_3$-L-Met. Jugular blood was collected to measure polymorphonuclear (PMN) cell function, haptoglobin, antioxidant capacity, and plasma metabolites. Betaine supplementation improved N retention ($P = 0.03$). Betaine did not affect plasma concentrations or urinary excretion of GAA, creatine, or creatinine ($P \geq 0.14$). Betaine increased plasma concentrations of Met and Cys-Cys ($P \leq 0.04$). Betaine increased Met deposition ($P = 0.03$), which subsequently decreased disposal of Met via transsulfuration ($P = 0.03$); betaine did not affect protein synthesis or degradation, use of Met for methylation reactions, or remethylation of homocysteine ($P \geq 0.57$). Betaine did not affect plasma antioxidant potential (AOP), interleukin-10 (IL-10), haptoglobin, or hematological parameters ($P \geq 0.20$). Retained N was not affected by MGM ($P = 0.63$), although GAA and creatine increased fecal N excretion ($P = 0.01$). Supplemental GAA increased plasma concentrations of GAA, creatine, and creatinine ($P < 0.01$),

whereas supplemental creatine increased plasma creatine and creatinine ($P \leq 0.02$) but did not affect plasma GAA ($P = 0.16$). Supplemental GAA increased urinary excretion of GAA, creatine, and creatinine ($P \leq 0.02$), whereas creatine supplementation only increased creatine and creatinine excretion ($P \leq 0.02$). Concentrations of total plasma amino acids (AA), total branched chain AA, and Cys-Cys decreased in response to GAA. No effects of MGM were observed for measures derived from whole body Met flux ($P \geq 0.34$). Plasma AOP, haptoglobin, and IL-10 were not affected by MGM ($P \geq 0.14$). Oxidative burst and bacterial phagocytosis were not affected by treatments. Overall, betaine improved N balance in our cattle, likely due to improved utilization of Met for protein deposition, but these effects were independent of methyl group status.

Introduction

Betaine, or trimethylglycine, is a nutrient present in some feedstuffs and is synthesized in the body as a product of choline oxidation. Betaine serves as a methyl group donor in the body and to regenerate methionine from homocysteine (Mudd et al., 2007). Both betaine and choline are rapidly degraded in the rumen (Mitchell et al., 1979; Sharma and Erdman, 1989). As a result, ruminants absorb only small amounts of methyl group sources from the diet and rely heavily on *de novo* choline synthesis to support choline and betaine needs. In addition to its function as a methyl donor, betaine is also an osmolyte and chemical chaperone, and works to maintain cell volume and stability during stress (Craig et al., 2004).

Guanidinoacetic acid (GAA) is the sole precursor to creatine, which acts as a source of high-energy phosphate bonds to replenish ATP when energy demand is high (Wyss and Kaddurah-Douk, 2000). Creatine is synthesized in a two-step process and begins predominantly in the kidney when GAA is produced from arginine and glycine (Brosnan and Brosnan, 2007). Next, GAA enters circulation and can be absorbed by the liver, where guanidinoacetate *N*-methyltransferase (GAMT) transfers a methyl group from *S*-adenosylmethionine to GAA, which yields creatine. Creatine supply is regulated at the point of GAA synthesis, and available GAA obligatorily consumes SAM methyl groups to produce creatine if methionine is available. Thus, providing exogenous GAA increases creatine production; creatine synthesis consumes methyl groups from SAM, which may deplete available methyl groups if Met supply is limiting (Stead et al., 2001; Setoue et al., 2008). Providing supplemental methyl donors (e.g., methionine, choline, or betaine) in combination with GAA could prevent development of a methyl group deficiency (Tehlivets et al., 2013). In contrast, providing creatine directly increases creatine supply, which may decrease demand for methyl groups that would otherwise be used for its synthesis.

In cattle, GAA tended to improve nitrogen retention when methionine was supplemented to meet requirements, but not when cattle were deficient in methionine (Ardalan et al., 2021). In steers fed corn-based diets, GAA supplementation did not improve protein deposition (Speer et al., 2022), with or without methionine supplementation. In contrast, supplemental GAA, but not creatine, increased protein deposition (Grant et al., 2021); moreover, cattle did not respond to supplemental choline alone or in combination with GAA or creatine.

Our objective was to evaluate the effects of supplemental betaine alone or in combination with GAA or creatine on protein deposition, methyl group utilization, 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

Seven ruminally cannulated Holstein steers (average initial body weight 189 ± 6.7 kg) were used in a 6 × 6 Latin square design with 10-d periods. Treatments were arranged in a 2 × 3 factorial, and treatment sequence was balanced for carryover effects. Treatments included 3 levels of methyl group status created by providing saline solution (5.3 g/d NaCl; control), 15 g/d GAA (which consumes methyl groups), or 16.8 g/d creatine (which spares methyl groups; equimolar with the GAA treatment) along with either 0 or 5.6 g/d of betaine. All treatments were infused abomasally.

Steers were housed in a temperature-controlled room (20 °C). They had ad libitum access to water and were limit-fed a corn-based diet (3.5 kg/d dry matter; DM) in 2 equal portions at 12-h intervals (0700 and 1900 h). Three weeks before trial initiation, calves were adapted to the diet and housed in tie-stalls. The diet (Table 3.1) was based on that of Speer (2019). 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. Treatments were infused continuously with a peristaltic pump (Ismatec ISM404-0121 MCP Standard; Cole-Parmer Instrument Company, Vernon Hills, IL) at a rate of 2.77 mL/min, with total infusion amounts of 4 kg/d per steer. Each

experimental period was 10 d in length and included 6 d of adaptation to treatment infusions, 3 d for total collection of urine and feces, and 1 d for blood sampling.

The GAA treatment infusion was prepared as described by Speer et al. (2022). Briefly, 91 g GAA was dissolved in 17,450 g water that contained 218.4 g of 9.25% wt/wt HCl. After all GAA was dissolved, approximately 436 g of 5% wt/wt NaOH was added to increase final solution pH between 6 to 7. Then, water was added to reach a final solution weight of 18.2 kg, resulting in a 5 g/kg GAA solution containing 1.76 g/kg NaCl. Creatine treatment infusions were prepared by dissolving 102 g creatine (as 116 g creatine monohydrate) and 32 g NaCl in 17,500 kg water. Once all creatine and NaCl were dissolved, water was added to bring final solution weight to 18.2 kg, for a final concentration of 5.56 g/kg creatine and 1.76 g/kg NaCl. For the control (saline) solution, 32 g NaCl was dissolved in 17,500 g water, and additional water was added to bring final solution weight to 18.2 kg, for a final concentration of 1.76 g/kg NaCl. For the creatine and control treatment solutions, NaCl was added to equalize electrolyte content across treatments; solutions were balanced specifically for Na and Cl content. The betaine treatment solution was prepared by dissolving 57 g betaine anhydrous (98% purity; 56 g betaine) into a 2-L solution with deionized water, creating a 2.8% wt/vol betaine solution. The GAA, creatine, and betaine solutions were stored at 4°C until use; control (saline) solution was stored at room temperature. Infusion bottles were prepared daily by adding 1.5 kg of the respective methyl group modulator treatment (i.e., saline, GAA, or creatine) to each bottle (3 kg/d per steer). For steers receiving 5.6 g/d betaine, 100 mL betaine solution was added to each bottle (200 mL/d per steer). Infusion bottles were filled to a final weight of 2 kg with water (total infusion weight 4 kg/d per steer) and stored at 4°C until use; infusion bottles were changed at 12-h intervals (0700 and 1900 h).

Sample Collection

At diet mixing, samples from individual feed ingredients were collected and stored at −20 °C until analysis. On d 6 to 8 of each period, a representative diet sample was collected and frozen at −20 °C. Within period, diet samples were composited, subsampled, and frozen at −20 °C until analysis. If present, orts were weighed at 1900 h daily from d 6 to 8 of each period and frozen at −20 °C. Within period, orts were composited by steer, subsampled, and frozen at −20 °C until analysis. From d 7 to 9 of each period, total outputs of urine and feces were collected and weighed daily for each steer. Urine was collected in buckets containing 900 mL of 10% (wt/wt) H_2SO_4 to decrease urine pH to ≤ 3 and prevent ammonia volatilization. Urine buckets were mixed thoroughly prior to sampling. Feces were collected into pans lined with plastic bags and mixed thoroughly prior to sampling. After total output was weighed, a representative sample of urine and feces was collected from each steer daily and frozen at −20 °C. Within period, daily urine and fecal samples were composited by steer in proportion to total outputs and frozen at −20 °C until analysis.

On d 10 of each period, jugular blood was collected into vacutainer tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ) containing sodium heparin (50 mL) or potassium EDTA (10 mL) 1 h after feeding (0800 h). Tubes were immediately inverted several times to disperse the anticoagulant and placed on ice. For blood containing sodium heparin, 20 mL was centrifuged at 1,200 × *g* at 4 °C for 20 min, and plasma was harvested and stored at −20 °C until analysis. Frozen plasma was used to measure plasma GAA, creatine, creatinine, amino acids, urea, glucose, haptoglobin, Trolox-equivalent antioxidant capacity, and Interleukin-10. The remaining 30 mL of heparinized whole blood was stored on ice until use for polymorphonuclear cell isolation and analysis of phagocytosis and oxidative burst. Blood containing potassium

EDTA (10 mL) was analyzed for complete blood count (CBC) at the Kansas State University Veterinary Diagnostic Lab.

On d 10 of each period, whole-body Met flux was also measured. Plasma collected at 0800 h was used to measure background enrichment of methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met. At 0900 h on d 10, an indwelling jugular catheter (MILACATH #LA1420; MILA International Inc., Florence, KY) was placed for continuous infusion of methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met. Catheters were placed using sterile technique in the jugular vein contralateral to site of jugular blood collection. Immediately after placement, a 76.2-cm catheter extension (Hospira Inc., Lake Forest, IL) was attached to the catheter and the extension and catheter were flushed with heparinized saline (4 U/mL heparin in 0.9% sterile saline solution). After catheters were placed in all steers, a line of flexible Tygon tubing (1.5 mm i.d.; Fisher Scientific, Pittsburgh, PA) was attached to each catheter extension. Immediately prior to initiation of label infusions at 1100 h, a pulse dose of 0.04 g each of methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met was administered through a 0.22-µm membrane syringe filter (Millex-GV; MilliporeSigma, Burlington, MA). Then, the filter and infusion line were connected to a 60-mL syringe attached to a programmable syringe pump (BS-9000-6 Multi-Phaser; Braintree Scientific, Inc., Braintree, MA). A solution containing methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met was continuously infused at a rate of 10 mL/h (0.04 g/h each of methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met) for 4 h (1100 h to 1500 h). At 1500 h, blood (20 mL) was collected contralateral to the catheter via jugular venipuncture into vacutainer tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ) containing sodium heparin. Samples were immediately placed on ice and centrifuged at 1,200 × *g* at 4 °C for 20 min. Plasma was isolated and frozen at −20 °C to measure plasma enrichment of methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met.

The methyl-$^{2}H_3$-L-Met and 1-^{13}C-L-Met solution was prepared in a laminar flow hood using aseptic technique. Methyl-$^{2}H_3$-L-Met and 1-^{13}C-L-Met (98% and 99% purity; Cambridge Isotope Laboratories, Inc., Andover, MA) were dissolved in sterile saline (0.9% NaCl) to produce a solution containing 4 g/L each of both labeled Met isotopes. Then, the solution was sterilized by filtration through a 0.22-µm syringe filter (Millex-GV; MilliporeSigma, Burlington, MA) into a 500-mL serum bottle. After filtration, the bottle was sealed with a crimped rubber septum and stored at 4 °C until use.

Laboratory Analyses

Diet and orts subsamples were dried at 55 °C in a forced-air oven for 48 h, air equilibrated for 24 h, and weighed to calculate partial dry matter. Samples were then ground to pass through a 1-mm screen using a Thomas Wiley Mill (Thomas Scientific, Swedesboro, NJ). Ground feed and orts subsamples and wet fecal samples were dried at 105 °C in a forced-air oven for 24 h to determine dry matter and heated to 450 °C in a muffle oven for 8 h to determine organic matter content. Feed samples were sequentially analyzed for NDF (with α-amylase and sodium sulfite) and ADF using a ANKOM Fiber Analyzer (ANKOM Technology, Macedon, NY). Feed and orts samples, wet fecal samples, and urine samples were analyzed for N content with a LECO TruMac N Analyzer (LECO Corporation, Saint Joseph, MI). For feed samples, crude protein content was calculated by multiplying N × 6.25.

Plasma and urine samples were analyzed for urea concentration as described by Marsh et al. (1965). Total urinary urea excretion was calculated by multiplying urea concentration by total urine output. Plasma glucose concentration was measured using a commercially available kit 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 haptoglobin concentrations were measured using methods modified from Cooke and Arthington (2013) and Smith et al. (1998) as described in Chapter 2 as a biomarker for inflammation status; samples were analyzed in quintuplet and the median value was utilized. Plasma antioxidant potential (AOP) was measured using Trolox-equivalent antioxidant capacity as described by Re et al. (1999). Briefly, plasma antioxidant potential was determined by comparison to standardized reduction by Trolox, a 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). Plasma AOP estimates total circulating antioxidant activity. Plasma concentrations of interleukin-10 were measured using a commercially available ELISA kit (RayBio Bovine IL-10 ELISA Kit; RayBiotech, Norcross, GA) at the University of Minnesota Cytokine Reference Laboratory (Minneapolis, MN).

Plasma and urinary GAA, creatine, and creatinine concentrations were measured using procedures similar to those described by Ardalan et al. (2020), which were based on the HPLC method developed by Shingfield and Offer (1999). For plasma amino acids, samples were deproteinized by adding equal amounts of 10% (wt/vol) sulfosalicylic acid that contained 600 mM norvaline as an internal standard. Samples were vortexed, placed on ice for 30 min, and centrifuged at 13,000 × *g* at 4°C for 10 min; then, the supernatant was filtered through a 0.22-μm membrane syringe filter into a microcentrifuge tube and frozen at −20°C until analysis. Plasma amino acid concentrations were measured using ultra-high pressure liquid chromatography (Waters Acquity UPLC; Waters Corporation, Milford, MA). Deproteinized samples underwent precolumn derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AccQ-Tag

Ultra Derivatization Kit; Waters Corporation, Milford, MA), separated on a C18 column, and detected with a tunable UV spectrophotometer at 260 nm wavelength (Waters, 2007).

Plasma samples were analyzed for methyl-$^{2}H_3$-L-Met and 1-^{13}C-L-Met using modified methods of Guiraud et al. (2017). Plasma samples were thawed and vortexed for 10 seconds. Next, 20 µL plasma was added to a 1.5-mL microcentrifuge tube. Ten microliters of an internal standard solution was added to each tube and then 170 µL of acetonitrile/water (80:20) (vol/vol) containing 1% formic acid was added to the mixture. The tubes were vortexed for 10 seconds, incubated for 10 min on ice, and centrifuged at 13,000 rpm for 10 min at 4 °C. Then, 100 µL of supernatants were pipetted and filtered through a 0.22 µm filter and placed into vials for UPLC–MS/MS analysis. The analysis was carried out by UPLC–ESI–MS/MS instrument, consisted of a Waters Acquity Ultra Performance LC with a Waters column manager and heater/cooler, binary system manager, sample manager coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) (Waters Acquity Ultra Performance LC, Xevo TQ-S MS/MS, Waters Co., Milford, MA, USA). An Acquity UPLC C18 column Waters HSS T3, 1.8 µ, 2.1 x 50 mm was held at 40 °C with eluents composed of mobile phase A (0.1% formic acid in water) and mobile phase B (acetonitrile). The flow rate was 0.4 mL/min. The following gradient program was used: from 0-1 min phase A (95%), at 2 min phase A (5%), and at 2.01 min phase A (95% for 2.99 min). Total run time was 5 min. The triple quadrupole mass spectrometer was Xevo TQ-S MS/MS equipped with an electrospray ionization (ESI) interface. This ionization mode used was positive ionization (+ESI). The operating parameters for the mass spectrometer were as follows: capillary voltage was 2.7 kV, source and desolvation temperatures were 150 °C and 500 °C, respectively. The cone energy was set at 23 V. Nitrogen was used as the desolvation gas and cone gas. Helium was used as the collision gas. The

collision gas flow was 0.15 mL/min. Desolvation and cone gas flow were 800 and 150 L/h, respectively. The data acquisition and quantification were performed using the Waters MassLynx and TargetLynx software 4.1, respectively.

To measure neutrophil phagocytic and oxidative burst activity, polymorphonuclear cells (PMN; primarily neutrophils) were isolated similarly to methods used by Garcia et al. (2018). Briefly, 25 mL heparinized whole blood was diluted with 25 mL phosphate buffered saline (PBS; approximately 4°C) and then underwent differential centrifugation with 20 mL Ficoll-paque PLUS (GE Healthcare; Pittsburg, PA) at 1,700 × *g* at 4 °C for 30 min. After centrifugation, the upper layers containing plasma, mononuclear cells, and Ficoll were removed with a vacuum pump, leaving red blood cells and PMN in the tube. Red blood cells were lysed with 5 mL ACK lysing buffer (Gibco by Life Technologies; Thermo Fisher Scientific, Waltham, MA; approximately 4 °C), and then cells were washed with Hanks' balanced salt solution (HBSS; Gibco by Life Technologies; Thermo Fisher Scientific, Waltham, MA; approximately 4 °C) until only PMN remained. Cell viability of PMN was measured using trypan blue exclusion and averaged 96%.

Isolated PMN were diluted to 3×10^5 live cells/mL, 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. Next, dihydrorhodamine (DHR; 100 µM final concentration; Invitrogen, Waltham, MA) was added and cells were incubated at 37°C for 10 min. Then, *E. coli* covalently labeled with Texas Red (Molecular Probes Inc., Eugene, OR) were added to cells at a ratio of 20 bacteria per neutrophil and incubated at 37°C for 40 min. Following incubation, PMN were cold shocked to halt activity and washed with PBS and pelleted thrice. Finally, PMN were reconstituted with 200 µL PBS for fluorescence

measurement. A capillary flow cytometer (Guave EasyCyte Mini Clow Cytometry System; Millipore Sigma, Billerica, MA) was used to measure PMN activity. Five thousand cells were counted from each sample and PMN were gated based on side scatter- and forward scatter-area. Cells incubated with DHR but without Texas Red-labeled *E. coli* served as negative controls to correct for nonspecific fluorescence. The proportion of neutrophils containing red fluorescence represented phagocytosis of Texas Red labeled *E. coli*. The proportion of neutrophils with green fluorescence represented 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 contributing intake (i.e., intake via abomasal infusion was not included). Methionine flux was calculated as described by Preynat et al. (2009). Whole-body flux of methyl-$^{2}H_{3}$-L-Met was defined as: Q(methyl-$^{2}H_{3}$-L-Met) = protein synthesis (PS) + transmethylation reactions = protein degradation (PD) + dietary intake + remethylation. The whole-body flux rate of 1-^{13}C-L-Met was defined as: Q(1-^{13}C-L-Met) = PS + transsulfuration = PD + dietary intake. The PD was calculated as 1-^{13}C-L-Met flux – metabolizable Met. Protein synthesis was calculated as the sum of PD and Met deposition. Methionine deposition was predicted as: N retention, g N/h × 6.25 g protein/g N × 0.134 mmol Met/g protein (Ainslie et al., 1993). Metabolizable Met intake was estimated from the diet as described by Speer et al. (2022).

Statistical Analysis

Data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). Our model included period and treatments (choline, MGM, and choline × MGM) as fixed effects and steer as a random effect. The LSMEANS statement was used to calculate treatment means. Individual MGM treatment means 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 one steer was removed from periods 1 and 2 due to excessive feed refusals.

Results and Discussion

Nutrient Intake and Digestibility

Dry matter and organic matter intakes were similar among treatments and averaged 3.5 kg/d and 3.3 kg/d, respectively (Table 3.2, data not analyzed statistically). No differences among betaine or MGM were observed for apparent digestibility of DM or OM ($P \geq 0.61$); moreover, no interactions between betaine and MGM were observed for DM or OM apparent digestibility. Apparent digestibility of DM and OM were 77.1% and 77.8%, respectively. Speer et al. (2022) a fed similar diet to 256-kg growing steers and observed lesser apparent DM and OM digestibilities of 73.9% and 74.5%, respectively. Cattle of Speer et al. (2022) consumed more DM than our cattle (5.3 kg/d vs. 3.5 kg/d, respectively) and consumed slightly more as a percentage of BW (2.1% vs. 1.9%, respectively). Grant (2020) fed a similar diet to 150-kg growing steers and observed numerically greater DM and OM digestibilities of 78.8% and 79.7%, respectively. Cattle of Grant (2020) had DM intakes similar to our cattle (average 3.5 kg/d for both) but consumed more as a percentage of BW (2.4% vs. 1.9%, respectively). It is unclear why our apparent digestibilities and those of Grant (2020) were greater than those of Speer et al. (2022).

Nitrogen Retention

No interactions between betaine and MGM were observed for N intake, N excretion, or N retention measures ($P \geq 0.71$; Table 3.2). Total N intakes were slightly greater for betaine, GAA, and creatine supplemented steers compared to unsupplemented controls (data not analyzed statistically), which was expected due to the treatment structure.

Effects of betaine

Excretion of total urinary N and urinary urea-N were not affected by betaine supplementation ($P \geq 0.50$; Table 3.2). Additionally, fecal N output was not significantly affected by betaine ($P = 0.16$). Retained N increased by 13% (2.9 g/d) for steers supplemented with 5.6 g/d betaine ($P = 0.03$), principally due to 0.67 g/d greater N intakes and 1.9 g/d less fecal N.

Limited work has investigated betaine supplementation in growing cattle. Across two experiments, Löest et al. (2002) supplemented Met-deficient growing steers with 1.6, 8, or 16 g/d betaine, which led to numerical increases in retained N of 1.3, 2.2, and 1.9 g/d, respectively. In both experiments, responses to betaine were approximately 20% as large as responses observed in the experiment when 2 g/d Met was supplemented to Met-deficient steers (Löest et al., 2002). Although our experiment did not compare betaine and Met supplementation, Speer et al. (2022) fed a similar diet growing steers and observed a 5.8 g/d increase in retained N when 5 g/d Met was supplemented. Relative to cattle of Speer et al. (2022), response of our cattle to 5.6 g/d betaine was about half as large as their response to 5 g/d methionine; however, cattle of Loest et al. (2002) were more severely limited in Met and methyl groups than cattle of Speer et al. (2022). In growing heifers limit-fed soyhull-based diets, no improvements in growth performance were observed when either lipid-coated betaine (4.2 g/d betaine) or concentrated separator byproduct (15.5 g/d betaine) was supplemented (Löest et al., 2001). Although cattle consuming soybean hull-based diets are limiting in Met (Campbell et al., 1997), it is possible that betaine sources provided by Löest et al. (2001) were degraded extensively in the rumen or that methyl groups were not limiting.

Responses to betaine supplementation in finishing cattle are inconsistent. In finishing steers and heifers, Löest et al. (2002) observed no improvements in growth performance in response to supplemental betaine provided as ruminally protected betaine or concentrated separator byproduct; however, betaine increased carcass dressing percentage and overall carcass fatness. Moreover, Bock et al. (2004) observed similar increases in dressing percentage and carcass fatness in steers fed betaine, and they reported minor improvements in growth performance when betaine was supplemented on both pasture and in the finishing period. Recent work in finishing Angus bulls suggests that betaine supplementation may improve average daily gain by 13-15% when provided at 0.6 g betaine/kg DM (7 to 8 g/d; Wang et al., 2020; Liu et al., 2021). Responses to betaine reported by Wang et al. (2020) and Liu et al. (2021) are greater than other work in finishing cattle, and it is not clear why their cattle responded to a greater degree than others. Although responses to betaine are conflicting in finishing cattle, it appears that observed differences may be due to changes in lipid deposition and metabolism in this stage of production. Because we utilized growing cattle and did not measure biomarkers associated with lipid metabolism, we cannot speculate if lipid metabolism was altered in our cattle.

Effects of methyl group modulators

We observed a tendency for a main effect of MGM on total urinary N excretion (P = 0.08; Table 3.2). Relative to unsupplemented controls, providing GAA or creatine tended ($P \leq$ 0.08) to increase urinary N excretion by similar amounts. Although urinary urea-N excretion was not significantly affected by MGM treatments (P = 0.19), the treatment pattern was similar to that for total urinary N excretion. Also, MGM affected fecal N excretion ($P < 0.01$) with supplemental GAA or creatine increasing fecal N excretion similarly. Despite differences among treatments for urinary and fecal N excretion, N retention averaged 24.1 g/d and was unaffected

by MGM treatment (P = 0.63). Due to MGM treatment infusions, N intakes were 5.4 g/d greater for both GAA and creatine supplemented steers, which corresponds to 7.0 and 6.2 g/d increases in total N excretion, respectively, and similar retained N among MGM treatments.

Previous work in our lab demonstrated that growing steers fed a diet identical to the diet in our experiment were limited by methionine supply, as evidenced by an increase in retained N when 5 g/d Met was infused abomasally (Speer et al., 2022). Accordingly, we expected steers to be marginally deficient in Met in our experiment, although corn-based diets are most typically limited in lysine (Burris et al., 1976; Hill et al., 1980; Titgemeyer et al., 1988). Because our cattle were likely limited in Met and perhaps methyl group supply, supplemental GAA would be expected to exacerbate this deficiency, whereas creatine was expected to possibly spare Met due to reduced methyl group demand to support creatine synthesis. Speer et al. (2022) did not observe significant improvements in N retention when GAA was provided abomasally (0, 7, or 15 g/d GAA) with or without 5 g/d supplemental methionine; however, a numerical trend for decreasing retained N with increasing GAA dose was observed. In growing steers fed the same diet, Grant et al. (2021) observed a 3.1 g/d increase in N retention when 15 g/d GAA was provided with or without 5 g/d choline. Although cattle in the present experiment were maintained under nearly identical experimental conditions as Speer et al. (2022) and Grant et al. (2021), it is unclear why responses to supplemental GAA are variable in growing steers fed corn-based diets. Grant et al. (2021) postulated that their cattle may have responded to supplemental GAA more readily than cattle of Speer et al. (2022) due to differences in body weight, dry matter intake, or creatine requirements. In comparison, cattle of Grant et al. (2021) weighed less initially (146 kg vs. 189 kg) but had similar dry matter intake (3.5 kg/d), which led to greater intake as a percentage of body weight (2.4% vs. 1.9%) when compared to our cattle. Protein

deposition was slightly greater for control cattle of Grant et al. (2021) compared to our control cattle (27.2 g N/d vs. 24.1 g N/d, respectively); this remains true if protein deposition is scaled to body weight (0.19 g retained N/kg BW vs. 0.13 g retained N/kg BW, respectively). Conversely, cattle of Speer et al. (2022) were heavier at trial initiation (256 kg vs. 189 kg) and consumed more dry matter (5.3 kg/d vs. 3.5 kg/d) daily and slightly more as a percentage of body weight (2.1% vs. 1.9%) compared to our cattle. In addition, their control cattle deposited more protein (38.5 g/d vs. 24.1 g/d) compared to our control cattle, but this difference was much smaller when scaled to body weight (0.15 g retained N/kg BW vs. 0.13 g retained N/kg BW, respectively). Relative to mature animals, growing animals require a greater creatine supply to support protein deposition and tissue development (Walker, 1979). Because our cattle and those of Speer et al. (2022) were larger and likely older than those of Grant et al. (2021), their creatine requirement may have been smaller and less responsive to exogenous GAA provision to support creatine synthesis. Supplementation of 16.8 g/d creatine (equimolar to 15 g/d GAA treatment) did not improve protein deposition in our cattle. Grant et al. (2021) also observed a lack of response to 16.8 g/d creatine provided abomasally and suggested that creatine may not have been absorbed well in the small intestine, which would limit its ability to act in the body. Similar to Grant et al. (2021), fecal N excretion increased when creatine was provided compared to controls, which supports the theory that creatine may not have been efficiently absorbed. Supplemental GAA supplementation also increased fecal N excretion in our cattle and cattle of Grant et al. (2021), but to a lesser extent than for creatine in both cases; this increase in fecal N was not observed by Speer et al. (2022). This could suggest that GAA, like creatine, was not efficiently absorbed in the small intestine; however, it is unclear why this observation is not consistent among experiments.

In growing steers starkly deficient in methionine, Ardalan et al. (2021) observed a tendency for an interaction between GAA and methionine supplementation, where GAA linearly increased N retention when 6 g/d methionine was provided but linearly decreased N retention in absence of methionine. Supplementing 0.6 g GAA/kg DM (approximately 6 to 8 g/d) to Angus bulls has improved average daily gain by 10 to 20% in the finishing period (Li et al., 2020; Liu et al., 2020; Liu et al., 2021). Li et al. (2021) supplemented GAA at increasing doses (0, 0.05, 0.1, 0.2, and 0.4% of diet dry matter) to Angus bulls and reported a 44% increase in average daily gain when GAA was provided at 0.2% of diet dry matter (approximately 17 g/d GAA). Interestingly, cattle of Li et al. (2021) supplemented with GAA at 0.05, 0.1, or 0.4% (approximately 4, 8, or 34 g/d GAA) did not perform better than control cattle. It is unclear why responses to supplemental GAA are variable among groups of cattle, but some key differences among experiments include: 1) weight, breed, and maturity of cattle, 2) route of GAA delivery (i.e., abomasal infusion vs. provided in diet directly), and 3) duration of supplementation. Improvements in diet digestibility and ruminal fermentation characteristics have been consistently reported in Angus bulls supplemented with GAA (Li et al., 2020; Liu et al., 2021a,b). Because we infused GAA abomasally, we could not evaluate potential effects of GAA on ruminal fermentation.

Limited work has investigated direct (i.e., postruminal) creatine supplementation in ruminants beyond the present experiment and that of Grant et al. (2021). Early work reported that creatine is degraded by ruminal microorganisms (Belasco et al., 1954) and may be comparable to urea as a source of non-protein nitrogen for growing lambs, as evidenced by similar diet digestibilities and nitrogen metabolism (Anderson et al., 1959; Campbell et al., 1959; McLaren et al., 1965). Recent work showed beneficial effects of creatine pyruvate

supplementation on heat and transport stress in beef cattle (Liu et al., 2020; Li et al., 2021; Lu et al., 2022; Mao et al., 2022), which authors attributed to improvements in ruminal fermentation; however, it is not possible to separate individual effects of creatine and pyruvate in these experiments. Because creatine was infused post-ruminally in our cattle, we could not evaluate potential benefits of creatine on ruminal fermentation.

Plasma and Urinary Creatine Metabolites

An interaction between betaine and MGM was observed for plasma creatinine concentrations ($P = 0.03$; Table 3.3). Supplemental GAA increased plasma creatinine concentrations regardless of betaine supplementation ($P < 0.01$), whereas supplemental creatine provided with 5.6 g/d betaine increased plasma creatinine ($P < 0.01$), but only a numeric increase was observed for supplemental creatine alone ($P = 0.12$). A tendency for an interaction between betaine and MGM was observed for plasma creatine ($P = 0.08$) and followed a similar treatment pattern to observations for plasma creatinine.

Effects of betaine

No main effects of betaine were detected for plasma concentrations of GAA, creatine or creatinine ($P \geq 0.14$; Table 3.3); however, a few nuanced interactions were detected between betaine and MGM and are discussed in the section below. No effects of betaine supplementation on urinary excretion of GAA, creatine, or creatinine were observed ($P \geq 0.51$).

Effects of methyl group modulators

Plasma GAA concentrations increased with supplemental GAA ($P < 0.01$; Table 3.3), whereas supplemental creatine numerically decreased plasma GAA concentrations ($P = 0.16$). Urinary GAA excretion increased with GAA supplementation ($P < 0.0001$), but not in response

to creatine ($P = 0.39$). Increased plasma GAA concentrations in response to GAA are consistent with observations in growing steers and dairy heifers at similar levels of supplementation (Ardalan et al., 2020, 2021; Grant et al., 2021). Urinary excretion of GAA averaged 0.17 and 0.23 g/d for control and creatine treatments, respectively, and 0.45 g/d for the GAA treatment. Although urinary GAA excretion was approximately two-fold larger for calves supplemented with GAA compared to control or creatine, the 0.28 g/d increase in GAA excretion accounted for only 1.9% of GAA infused daily. Similar increases in urinary GAA excretion in response to GAA supplementation have been observed in other experiments in growing cattle (Ardalan et al., 2020; Speer et al., 2022; Grant et al., 2021). Grant et al. (2021) also observed no change in urinary GAA excretion with creatine supplementation. Our observed numeric decrease in plasma GAA with supplemental creatine is consistent with results of Grant et al. (2021) and could suggest feedback inhibition of L-arginine:glycine amidinotransferase (AGAT; which synthesizes GAA) by creatine (Stead et al., 2021).

Supplemental GAA or creatine increased plasma concentrations of creatine ($P < 0.01$; Table 3.3), and the increase was greater for GAA than for creatine ($P < 0.01$). Providing either GAA or creatine increased urinary creatine excretion ($P < 0.01$), and creatine excretion was greater for calves supplemented with GAA compared with creatine ($P = 0.01$). Increases in plasma creatine concentration in response to GAA supplementation confirms that supplemental GAA was largely methylated to produce creatine. This observation agrees with other work in growing cattle under both methionine deficient conditions (Ardalan et al., 2021) and in cattle fed corn-based diets (Ardalan et al., 2020; Speer et al., 2021; Grant et al., 2021). Urinary creatine excretion averaged 2.2 g/d for control, 4.6 g/d for creatine, and 6.5 g/d for GAA. The increase in creatine excretion when creatine was supplemented represents 14.3% of the 16.8 g/d creatine

infused. In addition, the almost three-fold increase in creatine excretion when GAA was provided represents 25.6% of the 16.8 g/d creatine that would be synthesized from 15 g/d GAA. The greater increase in urinary creatine excretion for steers receiving GAA compared with creatine further supports the theory that intestinal absorption of creatine may have been lower than GAA. Our observed greater increase in urinary creatine excretion for GAA supplementation compared with creatine supplementation agrees with data of Grant et al. (2021), where GAA and creatine supplementation led to increases in creatine excretion of similar to the current study for both GAA and creatine treatments. In humans, Ostojic et al. (2016) reported numerically larger increases in serum creatine with supplemental GAA relative to creatine. Moreover, GAA supplementation more effectively increased muscle and brain creatine concentrations relative to creatine supplementation in humans (Ostojic et al. 2016). In contrast, Stead et al. (2001) observed similar increases in muscle creatine concentrations when GAA or creatine were supplemented, but total muscle creatine (i.e., creatine + phosphocreatine) was greater for creatine-supplemented than GAA-supplemented rats. In Yucatan miniature pigs, McBreairty et al. (2015) observed larger increases in hepatic and muscle creatine concentrations when GAA was supplemented compared to creatine. In contrast to tissue concentrations, supplementation with creatine increased plasma creatine concentrations more than supplementation with GAA (McBreairty et al., 2015). Our data, in conjunction with work in cattle and other species, confirms the effectiveness of GAA to improve creatine status, and possibly to a greater degree than direct creatine supplementation.

Plasma creatinine concentrations increased for supplemental GAA or creatine ($P \leq 0.02$; Table 3.3) and the increase was greater for GAA than creatine ($P < 0.01$). Providing GAA or creatine increased urinary creatinine excretion ($P \leq 0.02$), and excretion of creatinine tended to

be greater for GAA-supplemented calves compared to creatine-supplemented calves ($P = 0.10$). Creatinine excretion averaged 5.5 g/d for control, 6.9 g/d for creatine, and 7.9 g/d for GAA. The 25% increase in creatinine excretion when creatine was provided accounts for 8.3% of the 16.8 g/d creatine infused. Additionally, the 44% increase in creatinine excretion when GAA was provided accounts for 14.3% of the 16.8 g/d creatine that would be produced from 15 g/d GAA. Creatinine forms spontaneously from creatine in a nonenzymatic reaction and represents an irreversible loss of creatine from the body (Brosnan and Brosnan, 2007). Approximately 1.7% of the total body creatine pool is converted to creatinine daily with subsequent quantitative excretion in urine (Wyss and Kaddurah-Daouk, 2000). Increases in plasma creatinine concentrations in response to GAA supplementation have been observed in dairy heifers (Ardalan et al., 2020) and numeric increases have been observed in growing steers in some trials (Speer et al. 2020, 2022, Grant et al. 2021) but not others (Ardalan et al., 2021). Small nonsignificant increases in urinary creatinine excretion have been observed with supplemental GAA in methionine-deficient growing steers (Ardalan et al., 2021) and growing steers fed diets based on corn and alfalfa (Speer et al., 2022; Grant et al., 2021). Similarly, Grant et al. (2021) observed no effect of supplemental GAA or creatine on urinary creatinine excretion. Tossenberger et al. (2016) postulated that excess creatine production or supply, which is expected to increase creatinine production, is a likely source of greater plasma and urinary creatinine. This could suggest that increases in creatine supply from direct supplementation or methylation of GAA in our cattle and cattle of Ardalan et al. (2020, 2021) were large enough to increase the size of the total body creatine pool. Although small in magnitude, nonsignificant increases in plasma concentrations, urinary excretion, or both observed in other trials may also support this theory.

Plasma Amino Acids and Metabolites

Interactions between betaine and MGM were observed for plasma concentrations of Asn, Gln, Tyr, and total AA ($P \leq 0.05$; Table 3.5); however, none of these interactions appear particularly meaningful, so only main effects will be discussed. No interactions were observed between betaine and MGM for plasma concentrations of glucose or urea ($P \geq 0.34$; Table 3.4).

Effects of betaine

Betaine supplementation increased plasma concentrations of Met ($P = 0.03$; Table 3.5), Cys-Cys ($P = 0.04$), and Tyr ($P = 0.01$). Supplemental betaine tended to decrease plasma concentrations of Lys ($P = 0.10$). No effects of betaine were observed for plasma concentrations of other individual AA ($P \geq 0.15$) or total AA ($P = 0.51$). Increased concentrations of plasma Met with supplemental betaine, which, in combination with improved N balance for betaine supplemented steers, could suggest that betaine improved availability of Met in our cattle. Greater Met availability in response to betaine supplementation could be a result of increased remethylation of homocysteine to Met. This might have supported the increased N retention in response to betaine supplementation, but this mechanism was not confirmed by measures of whole-body methionine metabolism (discussed below). Loest et al. (2002) observed no effect of betaine on plasma Met in Met deficient growing steers, which may explain why betaine did not affect N balance in their cattle. In contrast, Robinson et al. (2016, 2018) observed decreases in plasma Met in response to betaine in Met-deficient miniature pigs. Authors suggested that supplemental betaine may have led to increased Met regeneration via BHMT; however, because BHMT is primarily expressed in the liver, Met produced from betaine methyl groups may have been utilized in the liver rather than released into circulation. The tendency for plasma lysine to

decrease in response to supplemental betaine may have resulted from increased uptake of AA for protein deposition. The cause of increased plasma tyrosine in response to betaine is unknown.

Supplemental betaine tended to decrease plasma glucose concentrations ($P = 0.06$; Table 3.4); however, the decrease was small in magnitude (4.83 mM vs. 4.70 mM for control and betaine, respectively). No effect of betaine was observed for plasma urea-N concentrations ($P = 0.90$).

Effects of methyl group modulators

A main effect of MGM was observed ($P \leq 0.05$; Table 3.5) for plasma concentrations of Cys-Cys, Thr, Asn, Ala, Lys, Tyr, Pro, and total AA. Plasma concentrations of other individual AA ($P \geq 0.07$) were not affected by MGM treatment.

Providing GAA or creatine decreased plasma concentrations of Ala, Lys, and total AA ($P \leq 0.05$). Plasma Thr decreased when GAA was provided ($P = 0.003$) and tended to decrease when creatine was provided ($P = 0.08$). Plasma Cys-Cys decreased when creatine was provided ($P = 0.01$) but was unaffected by GAA ($P = 0.26$), the reason for this decrease is not known. Supplemental GAA decreased plasma concentrations of Asn and Pro ($P \leq 0.02$) and tended to decrease plasma Val and total BCAA ($P \leq 0.07$), but creatine did not affect plasma Asn, Pro, Val, or total BCAA ($P \geq 0.14$). Plasma Tyr decreased when GAA was provided ($P = 0.02$) and tended to be greater for creatine-supplemented steers than for those receiving GAA ($P = 0.09$). Overall, decreases in individual and total plasma AA were more consistently observed for GAA than creatine across betaine supplementation. In contrast, Ardalan et al. (2020) observed a quadratic increase in total AA concentrations (maximized at 20 g/d GAA), linear increases in Trp, Tyr, Thr, Asp, and linear decreases in His and Asp. The reasons that plasma AA primarily increased in response to GAA in cattle of Ardalan et al. (2020) and decreased in response to

GAA and creatine for our cattle are unknown. Because retained N was not affected by GAA or creatine, it is unclear why concentrations of BCAA might decrease if not utilized for additional protein deposition.

No effects of MGM treatments on plasma urea N ($P = 0.50$; Table 3.4) or glucose concentrations were observed ($P = 0.49$). This suggests that general energy and protein statuses of our steers were not strongly impacted by supplemental GAA or creatine.

Whole-body Methionine Metabolism

Methionine enters the body Met pool through dietary sources (i.e., ruminally undegraded protein or microbial cell protein), de novo synthesis (i.e., homocysteine remethylation), or protein degradation (Stead et al., 1988). Methionine leaves the free pool through protein synthesis or transmethylation reactions. In our cattle, infused methyl-$^{2}H_3$-L-Met is removed from the Met pool through either protein synthesis or methylation reactions. Conversely, infused 1-^{13}C-L-Met is removed from the Met pool via protein synthesis or transsulfuration, but not transmethylation reactions, because the 1-^{13}C-label is not removed during methylation reactions. Following transmethylation, the resulting homocysteine is either remethylated to resynthesize Met (1-^{13}C-L-Met remains in Met pool) or is lost from the Met pool via transsulfuration (increases flux of 1-^{13}C-L-Met). Methionine synthesis from homocysteine proceeds through one of two remethylation reactions: 1) methionine synthase can transfer a methyl group from 5-methyltetrahydrofolate to homocysteine (a vitamin B_{12} dependent reaction) or 2) betaine-homocysteine methyltransferase can transfer a methyl group from betaine to homocysteine. Although these reactions could not be separated in the present experiment, an increase in

remethylation in response to betaine would likely be attributed to increased betaine-homocysteine methyltransferase activity.

No main effects of MGM nor interactions between betaine and MGM were observed for any measures of whole-body methionine metabolism ($P \geq 0.22$; Table 3.6).

Effects of betaine

Supplemental betaine did not affect flux of 1-^{13}C-L-Met ($P = 0.95$; Table 3.6), which averaged 6.73 mmol/h. Supplemental betaine increased Met deposition (i.e., the protein deposition portion of protein synthesis) but decreased transsulfuration. These effects offset each other, resulting in no net change in 1-^{13}C-L-Met flux (i.e., loss of the Met skeleton through transsulfuration or protein synthesis). Flux of methyl-$^{2}H_3$-L-Met was not affected by betaine ($P = 0.73$) and averaged 7.10 mmol/h. As a methyl group source, betaine has potential to increase Met available for transmethylation or protein synthesis via remethylation of homocysteine, but it did not affect total net loss of Met or Met methyl groups.

As designed, metabolizable Met supply was the same for all steers (1.95 mmol/h; data not analyzed statistically); however, betaine supplementation increased Met deposition by 0.102 mmol/h ($P = 0.03$), which was estimated from N retention. Despite this, protein synthesis and degradation were not affected by betaine ($P = 0.66$ and 0.95, respectively). Although not statistically significant, betaine supplementation led to a small numeric increase in protein synthesis (0.115 mmol/h), whereas protein deposition was negligibly affected by betaine (0.014 mmol/h numeric increase).

Homocysteine production (i.e., transmethylation reactions) was not affected by betaine ($P = 0.70$) and averaged 1.49 mmol/h. As a methyl group source, betaine does not directly impact transmethylation reactions. Rather, betaine donates methyl groups to homocysteine to regenerate

Met following transmethylation, which is then available for further transmethylation reactions. In addition, because betaine is synthesized from choline, endogenous betaine synthesis is also an indirect methyl group consumer. Choline and betaine are rapidly degraded in the rumen (Sharma and Erdman, 1989; Mitchell et al., 1979), so nearly all betaine utilized by ruminants is produced from endogenous choline synthesis, which consumes methyl groups. Supplemental betaine did not affect remethylation of homocysteine ($P = 0.57$), which averaged 0.370 mmol/h Proportionally, only 23% and 27% of homocysteine produced through transmethylation reactions was remethylated to synthesize Met for control and betaine, respectively (data not shown). Continuous infusion of betaine at a rate of 1.99 mmol/h (5.6 g/d) would provide 1.99 mmol/h of betaine methyl groups that could be transferred to homocysteine by betaine-homocysteine methyltransferase to regenerate Met. In addition, two subsequent methyl groups would be available for donation to tetrahydrofolate from dimethylglycine and then sarcosine, for a total of 5.97 mmol/h betaine methyl groups potentially available to betaine supplemented steers. Thus, the lack of an increase in remethylation reactions in response to betaine does not appear to be due to limits in available methyl groups. Moreover, betaine supplementation decreased transsulfuration of homocysteine ($P = 0.03$), which represents a 0.102 mmol/h decrease in irreversible loss of the Met skeleton to produce cysteine. This decrease is similar in magnitude to the increase in Met deposition when betaine was supplemented, which reduced Met available for disposal.

Limited work has investigated the effects of betaine supplementation on methionine metabolism in growing cattle. In Met deficient growing steers, Loest et al. (2002) observed no effect of betaine on flux of methyl-$^{2}H_{3}$-L-Met, which suggests that betaine did not impact the sum of transmethylation reactions and protein synthesis; however, impacts of betaine on

remethylation or transsulfuration were not estimated because only a methyl-labeled Met was utilized. Work in non-ruminants has demonstrated consistent increases in homocysteine remethylation to regenerate methionine in response to supplemental betaine (Finkelstein et al., 1983; Emmert et al., 1996, 1998; Robinson et al., 2018). In part, the difference in response to betaine supplementation in cattle and non-ruminants could be due to the low relative activity of BHMT in ruminants (Xue and Snoswell, 1986). This could explain the lack of an increase in remethylation when betaine was supplemented to our cattle. In addition, the lack of interactions between betaine and MGM for any measures of methionine metabolism could suggest that betaine's efficacy as a methyl donor to replenish Met is not largely affected by methyl group status in growing cattle.

Effects of methyl group modulators

No main effects of MGM were observed for whole-body fluxes of 1-^{13}C-L-Met (P = 0.85; Table 3.6) or methyl-$^{2}H_3$-L-Met (P = 0.47). In addition, no main effects of MGM were observed for Met deposition (P = 0.63), protein synthesis (P = 0.94), or protein degradation (P = 0.85). Because MGM did not affect N retention, it was expected there would be no differences among MGM treatments for Met deposition. This is also reflected in the lack of change in protein synthesis or degradation in response to supplemental GAA or creatine.

Transmethylation reactions (i.e., homocysteine production) were not affected by MGM (P = 0.34); however, a nonsignificant 0.197 mmol/h increase (P = 0.16) in transmethylation reactions was observed when GAA was supplemented. Relative to the 15 g/d supplemental GAA (5.34 mmol/h), this increase represents only 3.7% of infused GAA. Other work from our lab in a similar research model has demonstrated similar small numeric increases in total use of Met for transmethylation reactions in response to GAA (Speer et al., 2022; Grant al., 2022). Methylation

of GAA by SAM to produce creatine is not regulated (Stead et al., 2001), rather, accumulation of creatine inhibits additional GAA synthesis. Thus, methylation of GAA to creatine will occur as long as SAM methyl groups are available (da Silva et al., 2009). In theory, GAA supplementation increases methyl group demand for creatine synthesis, which could decrease availability of Met and methyl groups for other functions (i.e., protein synthesis, other transmethylation reactions). Work in piglets has demonstrated increases in creatine synthesis and subsequent decreases in synthesis of phosphatidylcholine and protein in response to supplemental GAA (McBreairty et al., 2013, 2015). Interestingly, McBreairty et al. (2015) observed no changes in synthesis of creatine, phosphatidylcholine, or protein when creatine was supplemented rather than GAA. In Met deficient cattle, Ardalan et al. (2021) reported no effect of supplemental GAA on transmethylation reactions with or without supplemental Met. In a research model similar to ours, Speer et al. (2022) observed no significant increase in transmethylation reactions in response to supplemental GAA alone; however, total methylation reactions increased when GAA and Met were supplemented together. Speer et al. (2022) attributed this observation to an off-setting decrease in methylation reactions other than creatine, most likely choline synthesis, when GAA was supplemented under Met-limiting conditions (Stead et al., 2006; Brosnan et al., 2007). This is the most likely explanation for the lack of large changes in methylation reactions in response to GAA supplementation in our cattle and those of Ardalan et al. (2021) and Grant et al. (2022). In addition, because creatine supplementation did not affect use of Met for transmethylation reactions, it appears that creatine did not effectively spare methyl groups in our cattle. This insensitivity to supplemental creatine has also been observed in work in growing cattle (Grant et al., 2022), piglets (McBreairty et al., 2015); and rats

(da Silva et al., 2009); however, it is unclear why creatine appears unable to spare Met methyl groups in some cases.

In our cattle, supplemental GAA or creatine did not affect transsulfuration ($P = 0.63$) or remethylation ($P = 0.50$) of homocysteine. The lack of response for GAA agrees with responses observed by Ardalan et al. (2021) in Met-deficient steers, including when GAA and Met were both supplemented. Under a slight methyl deficiency, Speer et al. (2022) observed no effect of GAA alone on remethylation; however, a stark increase in remethylation was observed when GAA and Met were supplemented together. In addition, GAA supplementation tended to slightly increase transsulfuration independent of Met supplementation (Speer et al., 2022). In contrast, Grant et al. (2022) observed a numeric decrease in transsulfuration and corresponding increase in remethylation in response to GAA; creatine did not affect remethylation or transsulfuration. Unlike cattle of Grant et al. (2022), GAA did not affect N retention in our cattle, which could explain the lack of differences observed for methionine remethylation and/or disposal to cysteine. Because creatine supplementation did not affect Met deposition or homocysteine production from Met in our cattle and those of Grant et al. (2022), it is not surprising that homocysteine remethylation and transsulfuration were also unaffected.

Plasma Inflammation and Antioxidant Measures

No betaine × MGM interactions were observed for measures of inflammation and antioxidant capacity ($P \geq 0.14$; Table 3.4).

Effects of betaine

Betaine supplementation did not affect plasma AOP ($P = 0.20$; Table 3.4). In addition, supplemental betaine did not affect plasma concentrations of IL-10 ($P = 0.56$) or haptoglobin (P

= 0.54). Because betaine donates methyl groups to homocysteine to replenish methionine, we expected a potential decrease in inflammation as a result of greater methionine and/or methyl group availability. In addition, providing betaine could spare choline that would otherwise be oxidized to betaine, which would increase choline available for other functions (e.g., lipid transport, phospholipid synthesis). Supplementing choline directly may improve health, inflammatory status, and/or oxidative balance in transition dairy cows (Ardalan et al., 2010; Sun et al., 2016; Vailati-Riboni et al., 2017), bovine immune cells (Abdelmegeid et al., 2017; Garcia et al., 2018), and growing steers (Grant et al., 2021); however, it is unclear if the mechanism of action is related to choline's function as a methyl group source. Betaine supplementation in heat-stressed poultry appears to decrease inflammation by increasing anti-inflammatory signals (e.g., IL-10) while decreasing pro-inflammatory signals in intestinal tissues (e.g., TNF-α, IL-1β, and IL-6; Song et al., 2021; Alhotan et al., 2021). Moreover, betaine supplementation may improve antioxidant capacity in muscle of broilers (Chen et al., 2022). The effects of supplemental betaine on inflammation and antioxidant status in ruminants have been investigated less thoroughly. Under heat stress conditions, ruminally protected betaine supplementation may improve oxidative balance in dairy cows (Zhang et al., 2014; Shah et al., 2020). Positive responses to betaine in poultry and dairy cows have primarily involved situations that induced heat stress or other major physiological stressors. Because our study was not designed to induce such stressors, it is possible that the mechanism by which betaine acts on inflammatory and oxidative balance was not affected.

Effects of methyl group modulators

No main effect of MGM was observed for plasma antioxidant potential (P = 0.55; Table 3.4). In addition, MGM did not affect plasma IL-10 (P = 0.47) or haptoglobin (P = 0.14).

Changes in methyl group status have potential to alter inflammatory and oxidative pathways in the body. Because GAA obligatorily consumes methyl groups to produce creatine, we hypothesized that supplemental GAA may increase inflammation and/or oxidative stress as a consequence of decreased methyl group availability in the body. In part, GAA consumes methyl groups directly from SAM; however, SAM may act as a mediator of oxidative stress in other pathways (Pastor et al., 1996; Cavallaro et al., 2010). In addition, following transmethylation, SAM becomes *S*-adenosylhomocysteine (SAH) and is further converted to homocysteine, both of which actively contribute to oxidative stress and inflammation via homocysteine oxidation if allowed to accumulate in the body (Tyagi et al., 2005; Wu et al., 2007; Tehlivets et al., 2011). Thus, because creatine synthesis from GAA consumes SAM methyl groups, and produces SAH and later homocysteine, we expected GAA supplementation may interfere with oxidative balance in our cattle. Oxidative stress occurs when production of oxidative compounds (i.e., reactive oxygen species; ROS) overwhelms the capacity of available antioxidant mechanisms (e.g., antioxidant enzymes and compounds) to neutralize ROS, and can cause oxidative damage of cells and, as a result, systemic inflammation (Wu et al., 2009; Freitas et al., 2016). Conversely, because direct creatine supplementation is expected to decrease methyl group demand (i.e., decreased GAA production required to support body creatine demands), we hypothesized that supplemental creatine would decrease oxidative stress and inflammation in our cattle.

In the current experiment, plasma AOP was similar among MGM treatments, which suggests that supplemental GAA or creatine did not alter oxidative balance in our cattle. This contrasts with observations of Grant et al. (2021), where supplemental creatine, but not GAA, decreased antioxidant capacity in growing steers. Work in human and murine cell models suggests that GAA supplementation may increase production of ROS and exacerbate oxidative

stress (Mori et al., 1996; Zugno et al., 2007a,b); however, GAA supplementation has not impacted antioxidant capacity in vivo in humans (Ostojic et al., 2013, 2015). Direct creatine supplementation may increase antioxidant capacity in murine and human cell culture models (Sestili et al., 2006; 2007). In contrast, Percário et al. (2012) observed decreased AOP in Brazilian handball players receiving creatine compared with unsupplemented control and placebo athletes. Taken together, it appears that oxidative stress and antioxidant potential responses are variable, but the reason for this variation is unknown. Moreover, because we did not observe differences in inflammatory markers, nor did cattle of Grant et al. (2021) or Speer et al. (2022), it appears unlikely that GAA and creatine supplementation largely impact inflammation in growing cattle.

Blood cell profile and neutrophil functionality

An interaction between betaine and MGM was observed for fibrinogen ($P = 0.03$; Table 3.7); no other interactions between betaine and MGM were observed for complete blood count measures. When betaine was provided, creatine decreased fibrinogen relative to control ($P = 0.02$), but not when creatine was supplemented alone ($P = 0.21$); fibrinogen was not affected by GAA with or without betaine ($P \geq 0.40$). No main effects of betaine or MGM were detected for fibrinogen ($P \geq 0.46$), and treatments were near or slightly greater than the upper bound of the reference range for cattle (100 – 600 mg/dL), which is consistent with observations of Grant (2020). No interactions between betaine and MGM were observed for measures of PMN function ($P > 0.05$; Table 3.8).

Effects of betaine

Betaine supplementation alone did not affect any parameters in the CBC analysis ($P \geq 0.32$; Table 3.7). In addition, no effects of betaine were observed for PMN oxidative burst and phagocytosis in the presence or absence of LPS ($P \geq 0.31$; Table 3.8). This agrees with work of Al-Qaisi et al. (2022), who reported no effects of betaine on hematological indices in heat tressed dairy heifers.

Effects of methyl group modulators

A main effect of MGM was observed for mean cell hemoglobin ($P = 0.04$; Table 3.7). In addition, tendencies were observed for main effects of MGM on blood hemoglobin concentration ($P = 0.08$), mean cell hemoglobin concentration ($P = 0.08$), and plasma protein concentration ($P = 0.07$). No other hematology parameters were affected by MGM ($P \geq 0.14$).

Creatine supplementation increased ($P = 0.01$) mean cell hemoglobin, whereas GAA did not ($P = 0.21$). Similarly, mean cell hemoglobin concentration was greater for creatine relative to control ($P = 0.03$). Total blood hemoglobin concentrations were greater for creatine ($P = 0.03$) and tended to increase for GAA ($P = 0.09$); creatine and GAA did not differ ($P = 0.63$). It is unclear why supplemental creatine or GAA would increase blood parameters associated with hemoglobin. In a similar experiment, Grant (2020) observed no differences in hemoglobin concentration, mean cell hemoglobin weight, or mean cell hemoglobin concentration in growing cattle supplemented with GAA or creatine. These parameters also appear to be unaffected by GAA in non-ruminants (Robinson et al., 2000; Kreider et al., 2003; Eijnde et al., 2003; Joy et al., 2014; Iqbal et al., 2013). In addition, all treatment means were within the normal reference range for total hemoglobin concentration (8 – 15 g/dL) and mean cell hemoglobin (11 – 17 pg; Fielder et al., 2022). In contrast, mean cell hemoglobin concentration averaged 36.7, 36.8, and 37.0 g/dL

for control, GAA, and creatine, respectively, and was slightly elevated for all treatments (normal range: 30 – 36 g/dL; Merck Veterinary Manual, 2022). Total plasma protein concentrations decreased with GAA supplementation ($P = 0.04$) and tended to decrease for creatine ($P = 0.06$); GAA and creatine did not differ ($P = 0.82$). Plasma protein concentrations averaged 7.8, 7.6, and 7.6 g/dL for control, GAA, and creatine, respectively, and were all within the normal range for cattle (6.0 to 8.0 g/dL; Fielder et al., 2022). Differences among MGM treatments were primarily related to red blood cell measures rather than shifts in the proportion of individual or total leukocytes. This agrees with work of Grant (2020), where supplemental GAA or creatine did not impact the immune cell profile in growing steers. Thus, it appears that short term changes in methyl group status may not largely alter the profile of immune cells in cattle.

No main effects for MGM treatment on PMN oxidative burst or phagocytosis with or without LPS present were observed ($P \geq 0.15$; Table 3.8). This further suggests that modulated methyl status in our steers may not have directly affected the immune cell profile or functionality.

Conclusions

Previous work in beef cattle has demonstrated mixed results when studying the effects of betaine on growth performance under different dietary conditions and stages of production. In our study, supplemental betaine improved N retention in growing steers fed a corn-based diet previously identified to be deficient in Met. In our experiment, post-ruminal GAA or creatine supplementation did not improve N retention independent of betaine supplementation. Betaine supplementation increased Met deposition, which led to a decrease in Met loss in transsulfuration; however, betaine did not affect Met transmethylation reactions or homocysteine remethylation. Use of Met for methylation reactions was not affected by GAA, which suggests that methylation of GAA to creatine likely decreased use of Met for other transmethylation reactions, such as choline synthesis. In addition, creatine did not spare Met use for transmethylation reactions. Measures of inflammation, antioxidant capacity, and immune cell function were largely unaffected by betaine or MGM, which suggests that methyl groups status may not greatly alter immune function in growing steers under our experimental conditions. Our data suggest that betaine supplementation improves protein deposition in growing steers fed a corn-based diet independent of methyl groups status.

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Table 0.1. Composition of diet fed to growing steers

Item	% of dietary dry matter
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	
Dry matter, % of as is	86.9
Organic matter	95.0
Crude protein	12.5
Neutral detergent fiber[3]	12.9
Acid detergent fiber[4]	6.1

[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 0.2. Effects of betaine on diet digestibility and nitrogen balance in steers supplemented with guanidinoacetic acid (GAA) or creatine

	Betaine, g/d									
	0			5.6						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Betaine	MGM	Betaine × MGM
n	7	6	7	7	7	6				
Intake, kg/d[3]										
DM	3.49	3.49	3.49	3.49	3.49	3.49	–	–	–	–
OM	3.32	3.32	3.32	3.32	3.32	3.32	–	–	–	–
Apparent digestibility, %										
DM	76.8	77.9	77.2	76.7	77.6	76.6	1.49	0.70	0.62	0.96
OM	77.4	78.7	77.9	77.5	78.3	77.2	1.54	0.71	0.61	0.93
Nitrogen, g/d										
Feed	68.9	69.0	69.4	69.1	68.9	69.0	–	–	–	–
Infused	0.00	5.37	5.37	0.67	6.04	6.03	–	–	–	–
Intake[4]	68.9	74.4	74.8	69.8	74.9	75.1	–	–	–	–
Urinary	24.1	26.9	25.9	24.0	25.8	26.0	1.15	0.67	0.08[5]	0.84
Urinary urea-N	10.2	12.0	13.3	12.0	12.5	12.8	1.24	0.50	0.19	0.52
Fecal	20.8	25.0	27.2	20.0	23.6	23.8	1.85	0.16	0.01[6]	0.16
Retained	24.1	22.5	21.3	25.7	25.7	25.4	1.99	0.03	0.63	0.71

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments

[3] Excludes abomasal infusions

[4] Feed N + infused N

[5] Pairwise means separated within MGM as control < GAA = creatine; $P \leq 0.10$

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

Table 0.3. Effects of betaine on plasma concentrations and urinary excretion on guanidinoacetic acid (GAA), creatine, and creatinine in growing steers supplemented with GAA or creatine

	Betaine, g/d									
	0			5.6						
	Methyl Group Modulator (MGM)							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[1]	Betaine	MGM	Betaine × MGM
n	7	6	7	7	7	6				
Plasma, mg/L										
GAA	0.440	0.343	0.732	0.403	0.368	0.755	0.0630	0.92	<0.01[3]	0.75
Creatine	9.84^{z}	10.81^{y}	12.85^{x}	9.50^{z}	12.30^{x}	13.04^{x}	0.692	0.18	<0.01[4]	0.08
Creatinine	18.9^{bc}	20.1^{b}	22.5^{a}	18.3^{c}	22.4^{a}	22.8^{a}	1.11	0.14	<0.01[4]	0.03
Urine, g/d										
GAA	0.157	0.195	0.450	0.183	0.267	0.441	0.0777	0.61	<0.01[3]	0.85
Creatine	2.36	5.00	6.04	2.09	4.12	6.94	0.704	0.88	<0.01[4]	0.39
Creatinine	5.55	7.57	7.57	5.42	6.18	8.14	0.668	0.51	<0.01[3,5]	0.25

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments

[3] Pairwise means separated within MGM as GAA > Creatine = Control, $P \leq 0.05$

[4] Pairwise means separated within MGM as GAA > Creatine > Control, $P \leq 0.05$

[5] Pairwise means separated within MGM as GAA > Creatine, $P \leq 0.10$

[a-c] Means without a common superscript differ, $P \leq 0.05$

[x-z] Means without a common superscript differ, $P \leq 0.10$

Table 0.4. Effects of betaine on plasma glucose, urea, antioxidant capacity, and inflammatory biomarkers in growing steers supplemented with guanidinoacetic acid (GAA) or creatine

	Betaine, g/d									
	0			5.6						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Betaine	MGM	Betaine × MGM
n	7	6	7	7	7	6				
Glucose, mM	4.82	4.78	4.88	4.81	4.67	4.62	0.104	0.06	0.50	0.31
Urea, mM	3.31	3.30	3.15	3.09	3.42	3.20	0.176	0.90	0.49	0.57
Trolox-equivalent AOP, μL^{-1}	61.1	65.7	62.0	64.4	63.1	68.0	2.18	0.20	0.55	0.14
Haptoglobin, µg/mL	539	900	450	449	693	477	193.9	0.54	0.14	0.81
Interleukin-10, pg/mL	230	264	206	348	292	181	116.0	0.56	0.47	0.69

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments

Table 0.5. Effects of betaine on plasma amino acids in steers supplemented with guanidinoacetic acid (GAA) or creatine

	Betaine, g/d									
	0			5.6						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Betaine	MGM	Betaine × MGM
n	7	6	7	7	7	6				
AA, μM										
Met	26.4	23.5	26.4	26.9	27.7	27.4	1.65	0.03	0.41	0.17
Cys-Cys	13.2	11.8	12.6	14.3	12.6	13.6	0.58	0.04	0.03	0.97
Tau	43.7	40.2	42.7	43.1	43.1	43.6	2.38	0.53	0.68	0.71
Ser	78.1	71.0	71.1	75.3	77.8	74.2	3.85	0.24	0.27	0.16
Gly	258.4	222.0	243.6	249.7	248.4	231.9	17.92	0.80	0.12	0.12
Arg	95.9	86.6	89.9	91.6	89.9	89.7	5.79	0.89	0.25	0.52
Thr	96.7	85.1	83.6	91.8	91.4	83.4	5.75	0.89	0.01	0.24
Ile	81.6	74.2	73.5	76.9	77.2	72.4	4.89	0.71	0.13	0.44
Asn	33.4[a]	29.2[b]	30.5[b]	31.1[ab]	33.2[a]	29.4[b]	2.16	0.77	0.05	<0.01
Glu+Cit	274.3	272.8	280.6	275.7	280.0	290.0	12.97	0.32	0.32	0.84
Gln	204.5[a]	189.6[b]	196.5[ab]	196.5[ab]	206.6[a]	199.5[ab]	10.32	0.30	0.83	0.04
Val	152.1	142.2	138.1	145.2	142.1	136.2	6.75	0.45	0.07	0.76
Ala	159.7	137.4	141.7	156.1	154.0	139.1	8.42	0.44	0.01	0.14
Lys	70.9	58.3	58.3	61.3	59.7	52.3	5.17	0.10	0.01	0.28
Tyr	54.7[ab]	46.6[b]	49.3[b]	60.5[a]	63.7[a]	49.1[b]	4.16	0.01	0.05	0.05
Phe	55.3	53.0	52.9	55.3	53.1	52.2	2.50	0.88	0.22	0.97
Trp	27.9	26.9	28.4	27.7	29.0	27.4	1.59	0.69	0.99	0.32
Orn	51.3	46.9	48.2	48.3	42.9	44.8	4.29	0.15	0.23	0.99
His	66.6	59.8	61.6	63.2	60.6	59.3	3.38	0.49	0.20	0.76
Leu	97.5	92.7	89.3	96.9	91.5	91.9	7.02	0.94	0.38	0.92
Asp	11.8	11.6	11.7	11.7	12.0	12.3	0.74	0.46	0.90	0.76
Pro	74.8	67.0	68.5	74.3	74.7	67.2	3.84	0.32	0.04	0.14
HyPro	40.9	37.4	39.6	39.7	42.5	39.7	2.57	0.32	0.92	0.13
Total BCAA	331	309	301	319	311	300	17.4	0.69	0.10	0.78
Total AA	2070	1882	1938	2012	2013	1923	74.8	0.51	0.01	0.04

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments

[a-c] Means without a common superscript differ, $P < 0.05$

Table 0.6. Effects of betaine on whole-body methionine metabolism in growing steers supplemented with guanidinoacetic acid (GAA) or creatine

	Betaine, g/d									
	0			5.6						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Betaine	MGM	Betaine × MGM
n	7	6	7	7	7	6				
Flux, mmol Met/h										
1-^{13}C-L-methioinine	6.62	6.72	6.83	6.71	6.68	6.81	0.302	0.95	0.85	0.97
Methyl-$^{2}H_3$-L-methionine	7.00	6.91	7.29	6.98	7.16	7.28	0.284	0.73	0.47	0.84
Metabolizable methionine	1.95	1.95	1.95	1.95	1.95	1.95	–	–	–	–
Met deposition	0.842	0.787	0.744	0.895	0.895	0.888	0.069	0.03	0.63	0.71
Protein synthesis	5.51	5.55	5.62	5.65	5.62	5.74	0.310	0.66	0.94	0.99
Protein degradation	4.67	4.77	4.88	4.76	4.73	4.87	0.302	0.95	0.85	0.97
Homocysteine production	1.49	1.37	1.67	1.32	1.54	1.54	0.143	0.70	0.34	0.41
Transsulfuration	1.11	1.16	1.21	1.05	1.05	1.06	0.065	0.03	0.63	0.71
Remethylation	0.382	0.183	0.460	0.263	0.482	0.453	0.129	0.57	0.50	0.22

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM among treatments

Table 0.7. Effects of betaine on complete blood count parameters in growing steers supplemented with guanidinoacetic acid (GAA) or creatine

	Betaine, g/d									
	0			5						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Betaine	MGM	Betaine × MGM
n	7	6	7	7	7	6				
Leukocyte count, 10^3/uL	13.0	12.6	12.6	12.8	12.9	12.8	0.38	0.70	0.60	0.60
Erythrocyte conc, 10^6/uL	11.0	9.6	9.5	9.6	9.6	9.6	0.70	0.39	0.40	0.39
Hemoglobin, g/dL	11.9	12.4	12.1	11.9	12.1	12.3	0.43	0.62	0.08[4]	0.18
Cellular hemoglobin, g/dL	11.8	12.3	12.0	11.8	11.9	12.1	0.44	0.45	0.11	0.16
Hematocrit, % (calc)	32.7	33.5	32.8	32.6	32.6	33.5	1.09	0.82	0.39	0.20
Mean cell volume, fL	34.1^{xy}	34.5^{x}	34.0^{y}	34.1^{xy}	34.1^{xy}	34.4^{x}	0.41	0.91	0.41	0.08
Mean cell hemoglobin, pg	12.5^{y}	12.8^{x}	12.5^{y}	12.5^{y}	12.6^{xy}	12.7^{x}	0.18	0.83	0.04[3]	0.10
Mean cell hemoglobin conc, g/dL	36.8	37.0	36.8	36.7	37.0	36.8	0.16	0.73	0.08[3]	0.97
Cell hemoglobin conc mean, g/dL	36.3	36.5	36.5	36.3	36.4	36.4	0.21	0.32	0.04	0.98
RBC distribution width, %	19.9	20.0	19.9	19.8	20.0	19.9	0.44	0.81	0.58	0.97
Platelet, 10^3/uL	485	555	556	525	531	507	69.4	0.70	0.53	0.37
Segmented neutrophil conc, 10^3/uL	4.76	4.04	4.11	4.10	4.45	3.96	0.448	0.69	0.64	0.45
Band neutrophil conc, 10^3/uL	0.00	0.00	0.01	0.00	0.00	0.00	0.006	0.34	0.41	0.38
Lymphocyte conc., 10^3/uL	6.90	7.10	6.95	7.39	6.98	7.34	0.537	0.39	0.95	0.66
Monocyte conc., 10^3/uL	0.77	0.83	0.89	0.73	0.90	0.89	0.163	0.91	0.61	0.93
Eosinophil conc., 10^3/uL	0.46	0.58	0.54	0.43	0.53	0.53	0.115	0.64	0.39	0.96
Basophil conc., 10^3/uL	0.16	0.12	0.11	0.15	0.02	0.13	0.044	0.50	0.14	0.33
Hematocrit, % (spun)	34.3	34.9	34.4	33.9	34.3	34.8	1.23	0.45	0.35	0.44
Plasma protein (refractom.), g/dL	7.92	7.67	7.61	7.73	7.62	7.63	0.146	0.35	0.07[5]	0.51
Fibrinogen (heat ppt), mg/dL	533^{ab}	609^{ab}	558^{ab}	650^{a}	502^{b}	626^{a}	53.5	0.46	0.62	0.03

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2]Average SEM across all treatments.

[3] Pairwise means separated as: Control = GAA < Creatine, $P \leq 0.05$

[4] Pairwise means separated as: Control < GAA = Creatine, $P \leq 0.10$

[5] Pairwise means separated as: Control > GAA = Creatine, $P \leq 0.10$

[y,z] Means without a common superscript differ, $P \leq 0.10$

[a,b] Means without a common superscript differ, $P \leq 0.05$

Table 0.8. Effects of betaine on immune cell function in growing steers supplemented with guanidinoacetic acid (GAA) or creatine

	Betaine, g/d									
	0			5.6						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Betaine	MGM	Betaine × MGM
n	7	6	7	7	7	6				
Oxidative burst, %										
−LPS[3]	40.3	42.5	43.5	41.7	47.1	42.0	3.12	0.45	0.33	0.49
+LPS	36.4	39.1	40.2	38.4	43.3	40.8	2.96	0.31	0.33	0.81
Phagocytosis, %										
−LPS	33.1	33.9	34.9	34.4	37.2	34.6	3.27	0.36	0.64	0.67
+LPS	29.7	31.4	31.4	26.8	34.4	32.1	3.18	0.89	0.20	0.53
Reactive oxygen species, neutrophil^{-1}†										
−LPS	1982	1995	2138	1997	2095	1981	162.2	0.90	0.86	0.64
+LPS	1947	1903	2040	1846	2074	1944	132.3	0.93	0.67	0.45
Bacteria phagocytized, neutrophil^{-1}‡										
−LPS	568	533	608	587	549	545	32.0	0.58	0.15	0.10
+LPS	537	515	570	527	534	525	28.3	0.42	0.43	0.23

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments

[3] LPS = lipopolysaccharide

† Mean fluorescence intensity of samples positive for oxidative burse (i.e., green fluorescence)

‡ Mean fluorescence intensity of samples positive for phagocytosis (i.e., red fluorescence)

Chapter 4 - Effects of methionine on nitrogen balance, methionine metabolism, and immune function in growing steers with altered methyl group status[1]

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Abstract

Methionine 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, methyl group metabolism, and immune function in growing cattle. Six ruminally cannulated Holstein steers (161 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 methionine (0 or 7.2 g/d). Supplemental GAA increases body creatine supply by consuming methyl groups to produce creatine, whereas supplemental creatine increases body creatine supply and may spare methyl groups that could otherwise be used to produce creatine. Steers were fed 3.4 kg/d (dry matter basis) of a corn-based diet. Total collection of urine and feces was used to measure N retention. Jugular blood was collected on d 10. No effects of Met or MGM were observed for N retention ($P \geq 0.29$), but supplemental GAA or creatine increased urinary N excretion ($P < 0.0001$) and urinary urea-N excretion ($P \leq 0.05$). Plasma concentrations of GAA increased with supplemental GAA ($P < 0.01$) but not creatine ($P = 0.88$). Plasma concentrations of GAA and creatine were not affected by Met ($P \geq 0.14$), whereas Met decreased plasma creatinine ($P = 0.02$). Supplemental GAA or creatine increased plasma concentrations of creatine and creatinine ($P < 0.01$), and the increase was larger for GAA than creatine ($P < 0.01$). Urinary creatine excretion was increased by GAA and creatine supplementation ($P < 0.01$), and the increase was greater for GAA than creatine ($P < 0.01$). Urinary GAA output was greater for GAA than creatine and control ($P < 0.01$). No effects of Met or MGM on plasma antioxidant potential, haptoglobin, or interleukin-10 ($P \geq 0.16$) were observed. Supplemental Met tended to increase blood neutrophil counts ($P = 0.08$) and tended to decrease ex vivo neutrophil

phagocytosis in the presence of LPS ($P = 0.08$). Ex vivo neutrophil phagocytosis without LPS ($P = 0.22$) and oxidative burst with or without LPS ($P \geq 0.46$) were not affected by Met. Supplemental GAA or creatine decreased blood leukocyte counts ($P \leq 0.04$) and GAA decreased blood neutrophil counts ($P \leq 0.02$). In the presence of LPS, GAA decreased neutrophil phagocytosis ($P = 0.03$), but creatine did not ($P = 0.13$). No effects of GAA or creatine were observed for neutrophil phagocytosis without LPS ($P = 0.14$) or for neutrophil oxidative burst ($P \geq 0.40$). Our data suggest that, regardless of methyl group status, our cattle were not limited by Met supply. In addition, some immunomodulatory effects of Met and GAA may exist independent of their functions in methyl group metabolism.

Introduction

Methionine is an essential amino acid and can limit growth in cattle when microbial protein is the primary source of metabolizable protein (Richardson and Hatfield, 1978). Beyond its role in protein synthesis, Met can be converted to *S*-adenosylmethionine (SAM), which is the most widely utilized methyl donor in the body (Finkelstein, 1990). As SAM, Met participates in over one hundred transmethylation reactions, and two of the most quantitatively important reactions are phosphatidylcholine and creatine synthesis (Lobley et al., 1992). Phosphatidylcholine is incorporated into cell membranes, aids in hepatic lipid transport via very light density lipoproteins, and can be cleaved to produce free choline (Zeisel, 2006). After donating its methyl group, SAM becomes *S*-adenosylhomocysteine, which is further converted to homocysteine. Homocysteine can either be remethylated to regenerate Met, or undergo transsulfuration to produce cysteine (Finkelstein, 1998). If remethylated, homocysteine accepts a methyl group from either 5-methyltetrahydrofolate (5-MTHF) in a reaction catalyzed by methionine synthase and vitamin B12 or betaine (a product of choline oxidation) in a reaction catalyzed by betaine-homocysteine methyltransferase (BHMT) and pyridoxine. If not remethylated, homocysteine undergoes transsulfuration in an irreversible two-step process, which yields cysteine (Finkelstein, 1998). Cysteine can be further metabolized to form essential antioxidants glutathione and taurine (Brosnan and Brosnan, 2006). Poor regulation of oxidative balance or lipid transport can lead to inflammation (Drackley et al., 1999; Sordillo and Aitken, 2009). Because Met is essential for both choline synthesis and antioxidant production, adequate Met may control inflammation and oxidative balance, which has been demonstrated in transition dairy cows (Osorio et al., 2014; Zhou et al., 2016a,b; Batistel et al., 2018).

Creatine is essential for maintenance of energy homeostasis in muscle tissue and serves as a shuttle of high-energy phosphate bonds to replenish ATP when cellular energy demand is high (Wyss and Kaddurah-Daouk, 2000). Guanidinoacetic acid (GAA) is the sole precursor to creatine and is produced in the kidney from arginine and glycine in a reaction that yields GAA and ornithine (Brosnan and Brosnan, 2007). GAA is released into circulation and can be taken up by the liver, where guanidinoacetate *N*-methyltransferase (GAMT) transfers a methyl group from SAM to produce creatine and homocysteine. Creatine supply does not inhibit GAMT activity, but rather inhibits GAA production. Thus, an increased supply of GAA will consume methyl groups to produce creatine as long as SAM is available, and this can deplete methyl groups or divert methyl groups away from other biological processes if methionine supply is not adequate (Stead et al., 2001; Setoue et al., 2008). Co-supplementation of GAA and methyl donors (e.g., methionine, choline, or betaine) may be an appropriate strategy to improve creatine status without depleting methyl groups supply (Tehlivets et al., 2013).

Recent work in our lab has demonstrated efficacy of GAA to improve creatine status in growing cattle (Ardalan et al., 2020, 2021; Speer et al., 2020, 2022; Grant et al., 2021). Ardalan et al. (2021) reported a tendency for GAA to improve protein deposition, measured by N retention, when Met was adequate, but not when steers were Met deficient. In contrast, Speer et al. (2022) fed growing steers a corn-based diet and observed no effect of supplemental GAA on N retention; however, supplemental Met increased N retention, demonstrating that the diet was deficient in Met. Across two experiments similar to the present study, we evaluated supplemental GAA or creatine alone or in combination with choline (Grant et al., 2021) or betaine (Grant et al., 2022) in steers fed corn-based diets. Grant et al. (2021) observed an improvement in protein deposition when steers received GAA regardless of supplemental choline, whereas GAA did not

affect protein deposition in cattle of Grant et al. (2022); creatine supplementation did not affect N balance in either experiment. Although Speer et al. (2022) investigated GAA and Met in steers fed corn-based diets, Met has not been investigated in combination with creatine, nor have impacts on immune response been documented in growing cattle supplemented with GAA and Met. Our objective was to evaluate the effects of supplemental Met alone or in combination with GAA or creatine on protein deposition, whole-body Met metabolism, 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 (161 ± 9.4 kg) were used in a 6 × 6 Latin square design with 10-d periods. Treatments were arranged in a 2 × 3 factorial and treatment sequence was balanced for carryover effects. All treatments were infused abomasally and included 3 levels of methyl group status by providing either a saline solution (5.3 g/d NaCl; control), 15 g/d GAA (which consumes methyl groups), or 16.8 g/d creatine (which spares methyl groups; equimolar with the GAA treatment) in combination with either 0 or 7.2 g/d of Met.

Cattle were housed in a temperature-controlled room (20 °C) with ad libitum access to water and were limit-fed a corn-based diet (3.5 kg/d DM) in 2 equal portions at 12-h intervals (0700 and 1900 h) daily. The basal diet (Table 4.1) was formulated to be identical to that of Speer et al. (2022) and was similar to a diet that could be fed in a production-type setting. Three weeks prior to the experiment, steers were adapted to the diet while housed in tie-stalls. Three days prior to initiation of treatment infusion, calves were placed in individual metabolism stalls that allowed for total collection of urine and feces for the duration of the experiment. A flexible Tygon infusion line (2.38 mm i.d.; Fisher Scientific, Pittsburgh, PA) was placed in the abomasum of each steer via the ruminal cannula prior to the study. A rubber flange (10 cm diameter) affixed near the end of the infusion line was used to hold the line in the abomasum. Treatments were infused continuously using a peristaltic pump (Ismatec MCP Standard ISM404-

0121; Cole-Parmer Instrument Company, Vernon Hills, IL) at a rate of 2.77 mL/min; total infusion amounts were 4 kg daily. Periods were 10 d in length with 6 d for treatment adaptation, 3 d for total collection of urine and feces, and 1 d for blood sampling.

The GAA treatment solution was prepared as described by Speer et al. (2020). Briefly, 91 g GAA (CreAmino®, AlzChem Group, Trostberg, Germany) was dissolved in 17,450 g water containing 218.4 g 92.5% wt/wt HCl. After all GAA had dissolved, approximately 436 g 5% wt/wt NaOH was added to increase final solution pH between 6 to 7. Water was then added to reach a final solution weight of 18.2 kg, resulting in a 5 g/kg GAA solution. The creatine treatment solution was prepared by dissolving 102 g creatine (116 g creatine monohydrate CreaPure®, AlzChem Group, Trostberg, Germany) and 32 g NaCl in 17,500 g water. After creatine and NaCl were dissolved, water was added to bring final solution weight to 18.2 kg, for a final concentration of 5.56 g/kg creatine and 1.76 g/kg NaCl. For the control (saline) solution, 32 g NaCl was added to 17,500 g water. After NaCl was dissolved, water was added to a final solution weight of 18.2 kg, for a final concentration of 1.76 g/kg NaCl. For the creatine and control treatment solutions, NaCl was added to equalize electrolyte content across treatments; solutions were balanced specifically for Na content. The Met treatment solution was prepared by dissolving 72 g L-Met into a 2-L solution with deionized water, creating a 3.6% wt/vol Met solution. The GAA, creatine, and Met solutions were stored at 4 °C until use; control (saline) solution was stored at room temperature. Infusion bottles (for 12 h of infusion) were prepared daily by adding 1.5 kg of the respective methyl group modulator treatment (i.e., saline, GAA, or creatine) to each bottle (3 kg/d per steer). For steers receiving 7.2 g/d Met, 100 mL Met solution was added to each bottle (200 mL/d per steer). Infusion bottles were filled to a final weight of 2

kg with water (total infusion weight 4 kg/d per steer) and stored at 4 °C until use; infusion bottles were changed at 12-h intervals (0700 and 1900 h).

Sample Collection

At diet mixing, representative samples of individual feed ingredients were collected and stored at −20 °C until analysis. On d 6 to 8 of each period, a representative diet sample was collected and frozen at −20 °C. Within period, diet samples were composited by day, 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. Within period, orts were composited by steer, subsampled, and frozen at −20 °C until analysis. From d 7 to 9 of each period, total outputs of urine and feces were collected and weighed daily for each steer. Urine was collected into plastic buckets containing 900 mL of 10% (wt/wt) H_2SO_4 to decrease urine pH to ≤ 3 and prevent ammonia volatilization. Urine buckets were mixed thoroughly prior to sampling. Feces were collected into plastic-lined metal pans and mixed thoroughly prior to sampling. After total output was weighed, a representative sample of urine and feces was collected from each steer daily and frozen at −20 °C. Within period, daily urine and fecal samples were composited by steer in proportion to total daily outputs and frozen at −20 °C until analysis.

On d 10 of each period, jugular blood was collected into vacutainer tubes (Becton, Dickinson, and Company, Franklin Lakes, NJ) containing sodium heparin (50 mL) or potassium EDTA (10 mL) 1 h after morning feeding (0800 h). Tubes were immediately inverted several times and placed on ice. For blood containing sodium heparin, 20 mL was centrifuged at 1,200 × *g* at 4 °C for 20 min, and plasma was harvested and stored at −20 °C until analysis. Frozen

plasma was used to measure plasma GAA, creatine, creatinine, amino acids, urea, glucose, haptoglobin, Trolox-equivalent antioxidant capacity, and Interleukin-10. The remaining 30 mL of heparinized whole blood was stored on ice until use for polymorphonuclear cell isolation and phagocytosis and oxidative burst analyses. Blood containing potassium EDTA (10 mL) was analyzed for complete blood count with differential at the Kansas State University Veterinary Diagnostic Lab (Manhattan, KS).

On d 10 of each period, whole-body Met flux was measured. Plasma collected at 0800 h was used to measure background enrichment of methyl-$^{2}H_3$-L-Met and 1-^{13}C-L-Met. At 0900 h on d 10, an indwelling jugular catheter (MILACATH #LA1420; MILA International Inc., Florence, KY) was placed for continuous infusion of methyl-$^{2}H_3$-L-Met and 1-^{13}C-L-Met. Catheters were placed using sterile technique in the jugular vein contralateral to site of jugular blood collection. Immediately after catheter placement, a 76.2-cm catheter extension (Hospira Inc., Lake Forest, IL) was attached, and catheter and extension were flushed with heparinized saline (4 U/mL heparin in 0.9% sterile saline solution). After catheters were placed in all steers, a line of flexible Tygon tubing (1.5 mm i.d.; Fisher Scientific, Pittsburgh, PA) was attached to each catheter extension. Immediately prior to initiation of label infusions, a pulse dose of 0.04 g each of methyl-$^{2}H_3$-L-Met and 1-^{13}C-L-Met was administered intrajugularly through a 0.22-µm membrane syringe filter (Millex-GV; MilliporeSigma, Burlington, MA). The infusion line with filter was connected to a 60-mL syringe attached to a programmable syringe pump (BS-9000-6 Multi-Phaser; Braintree Scientific, Inc., Braintree, MA). A solution containing methyl-$^{2}H_3$-L-Met and 1-^{13}C-L-Met was continuously infused at a rate of 10 mL/h (0.04 g/h each of methyl-$^{2}H_3$-L-Met and 1-^{13}C-L-Met) for 4 h (1100 h to 1500 h). At 1500 h, blood (20 mL) was collected from the jugular vein contralateral to the infusion catheter into vacutainer tubes (Becton,

Dickinson, and Company, Franklin Lakes, NJ) containing sodium heparin. Samples were immediately placed on ice and centrifuged at 1,200 × *g* at 4 °C for 20 min. Plasma was isolated and frozen at −20 °C until analysis for plasma enrichment of methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met.

The methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met solution was prepared in a laminar flow hood using aseptic technique. Methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met (98% and 99% purity, respectively; Cambridge Isotope Laboratories, Inc., Andover, MA) were dissolved in sterile saline (0.9% NaCl) to produce a solution containing 4 g/L each of both methyl-$^{2}H_{3}$-L-Met and 1-^{13}C-L-Met . Then, the solution was sterilized by filtration through a 0.22-µm syringe filter (Millex-GV; MilliporeSigma, Burlington, MA) into a 500-mL serum bottle. After filtration, the bottle was sealed with a crimped rubber septum and stored at 4 °C until use.

Laboratory Analyses

Diet and orts subsamples were dried in a forced air oven at 55 °C for 48 h, air equilibrated for 24 h, and weighed to calculate partial dry matter. Samples were then ground through a 1-mm screen using a Thomas Wiley Mill (Thomas Scientific, Swedesboro, NJ). Dried and ground feed and orts subsamples and wet fecal samples were dried at 105 °C in a forced-air oven for 24 h to determine dry matter content and heated to 450 °C in a muffle oven for 8 h to determine organic matter content. Feed samples were sequentially analyzed for NDF (with α-amylase and sodium sulfite) and ADF using a ANKOM Fiber Analyzer (ANKOM Technology, Macedon, NY). Feed and orts samples, wet fecal samples, and urine samples were analyzed for N content by combustion using a LECO TruMac N Analyzer (LECO Corporation, Saint Joseph, MI). For feed samples, crude protein content was calculated by multiplying N content × 6.25.

Plasma and urine samples were analyzed for urea concentration according to methods of Marsh et al. (1965). Urinary urea-N excretion was calculated by multiplying urea concentration by total urine output. Plasma glucose concentration was measured using a commercially available kit 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 haptoglobin concentrations were measured using modified methods of Cooke and Arthington (2013) as a biomarker for inflammation status; modifications to these methods are described in Chapter 2 and samples were analyzed in quintuplet and the median value was utilized. Plasma antioxidant potential (AOP) was measured using Trolox-equivalent antioxidant capacity as described by Re et al. (1999). Plasma antioxidant potential was determined by comparison to standardized reduction of Trolox, a 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). Total plasma AOP estimates total circulating antioxidant activity. Plasma concentrations of interleukin-10 were measured using a commercially available ELISA kit (RayBio® Bovine IL-10 ELISA Kit; RayBiotech, Norcross, GA) at the University of Minnesota Cytokine Reference Laboratory (Minneapolis, MN). Plasma and urinary GAA, creatine, and creatinine concentrations were measured using procedures similar to those described by Ardalan et al. (2020), which were based on the HPLC method developed by Shingfield and Offer (1999).

Neutrophil phagocytic and oxidative burst activities were measured by first isolating polymorphonuclear cells (PMN; primarily neutrophils) as described by Garcia et al. (2018). Heparinized whole blood (30 mL) was diluted with 30 mL PBS (4 °C) and centrifuged with Ficoll-paque PLUS (20 mL; GE Healthcare; Pittsburg, PA) at 1,700 × *g* at 4 °C for 30 min. After

centrifugation, plasma, mononuclear cells, and Ficoll layers were removed with a vacuum pump; red blood cells and PMN remained in the tube. Remaining cells were subjected to red blood cell lysis with ACK lysing buffer (5 mL; 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 94%.

Immediately following isolation and cell counting, PMN were diluted to 3×10^5 live cells/mL, 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 min. Next, dihydrorhodamine (DHR; 100 µM final concentration; Invitrogen, Waltham, MA) was added and cells were incubated at 37 °C for 10 min. Then, *E. coli* covalently labeled with Texas Red (Molecular Probes Inc., Eugene, OR) were added to cells at a ratio of 20:1 (bacteria:neutrophil) and incubated at 37 °C for 40 min. Following incubation, PMN were cold shocked to halt activity and then washed with PBS and pelleted thrice by centrifugation at 500 × g at 4 °C for 5 min. Finally, PMN were reconstituted with 200 µL PBS for fluorescence measurement. Activity of PMN was measured using a capillary flow cytometer (Guave EasyCyte Mini Clow Cytometry System; MilliporeSigma, Billerica, MA). Five thousand cells were counted from each sample and PMN were gated based on side scatter- and forward scatter-area. Cells incubated with DHR but without Texas Red-labeled *E. coli* served as negative controls to correct for nonspecific fluorescence. The proportion of neutrophils containing red fluorescence represented phagocytosis of Texas Red-labeled *E. coli*. The proportion of neutrophils with green fluorescence represented oxidative burst.

Calculations

Retained N was calculated by 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 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). The model included period and treatment (Met, MGM, and Met × 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-tests when the overall 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 one steer was removed from period 5 as a result of excessive digesta loss from the ruminal cannula.

Results and Discussion

Nutrient Intake and Digestibility

Dry matter (DM) and organic matter (OM) intakes were similar among treatments ($P \geq 0.28$) and averaged 3.4 kg/d and 3.3 kg/d, respectively (Table 4.2). No differences among treatments were observed for apparent DM or OM digestibility ($P \geq 0.52$), which averaged 75.4% and 75.8%, respectively.

Nitrogen Retention

No interactions between Met and MGM were observed for any measures associated with measures of N retention ($P \geq 0.14$; Table 4.2). Total N intakes were greater for steers supplemented with Met, GAA, or creatine compared to unsupplemented controls ($P < 0.0001$), which was expected due to the treatment structure.

Effects of methionine

Neither total urinary N excretion nor urinary urea-N were affected by Met supplementation ($P \geq 0.19$; Table 4.2). The proportion of urinary N excreted as urea-N was not affected by Met and averaged 59% ($P = 0.40$; data not shown). Fecal N excretion was unaffected by Met ($P = 0.18$). Retained N did not differ between steers provided 0 or 7.2 g/d Met ($P = 0.20$), suggesting that our cattle were not deficient in Met.

The lack of response to Met is not unexpected, because corn-based diets are typically first-limiting in lysine rather than Met (Burris et al., 1976; Hill et al., 1980; Titgemeyer et al., 1988). However, Speer et al. (2022), observed a 6 g/d increase in retained N when 5 g/d Met was supplemented to growing steers fed a diet formulated identically to ours. Cattle of Speer et al. (2022) were heavier (256 kg vs. 161 kg) than our steers but had similar dry matter intake relative

to body weight (2.1% for both studies). Based on NASEM (2016) recommendations, Speer et al. (2022) estimated the diet to be more limiting in Lys than Met (55% vs. 65% of requirement provided, respectively) for maximal protein deposition. Nevertheless, their reported increase in N retention when 5 g/d Met was supplemented suggests that Met was first limiting. Speer et al. (2022) observed an incremental efficiency of Met deposition of 14%, which is low relative to the estimated efficiency (54%; NASEM, 2016), which suggests that the requirement was substantially less than 5 g/d supplemental Met. Based on NASEM (2016) predictions and net protein of 189 g/d (6.25 × average retained N for all steers), metabolizable Met supply (6.4 g/d) was 69% of the requirement (9.3 g/d), and metabolizable lysine supply (17.4 g/d) was 59% of the requirement (29.6 g/d). Similar to Speer et al. (2022), lysine was calculated to be more limiting than Met, which agrees with our observation that Met did not improve retained N. Although it is not clear why our cattle responded differently to Met than did cattle of Speer et al. (2022), our hypothesis is that lysine was more limiting than Met in our cattle.

Effects of methyl group modulators

We observed a main effect of MGM on urinary N excretion ($P < 0.001$; Table 4.2) and urinary urea N excretion ($P = 0.05$). Relative to control, providing GAA or creatine increased urinary N excretion by 3.6 and 3.3 g/d, respectively ($P < 0.001$), without difference between GAA and creatine ($P = 0.66$). Urinary urea-N excretion increased by 2.6 g/d when creatine was provided ($P = 0.02$) and tended to increase by 1.9 g/d when GAA was provided ($P = 0.09$); GAA and creatine did not differ ($P = 0.48$). Fecal N was not affected by MGM ($P = 0.33$), and averaged 23.2, 24.3, and 24.7 g/d for control, GAA, and creatine, respectively. Retained N was similar among MGM treatments ($P = 0.83$) and averaged 29.9, 30.5, and 30.5 g/d for control, GAA, and creatine, respectively.

Recent work investigating post-ruminal GAA supplementation in growing cattle has yielded variable results. In severely Met-deficient growing steers, Ardalan et al. (2021) observed no change in retained N when GAA was supplemented alone, but they observed a tendency (P = 0.10) for GAA to increase retained N when supplemented with 6 g/d Met. In contrast, under experimental conditions similar to our study, Speer et al. (2022) observed a numeric linear decrease (P = 0.12) in N retention with increasing GAA supplementation (0, 7.5, or 15 g/d GAA) to growing steers fed a corn-based diet. As discussed previously, cattle of Speer et al. (2022) were mildly deficient in Met, but authors reported similar responses to supplemental GAA regardless of Met supplementation. In other work from our laboratory using a similar research model, Grant et al. (2021) observed a modest increase in N retention when 15 g/d GAA was supplemented, whereas Grant et al. (2022) observed no improvements in N retention when 15 g/d GAA was supplemented. It is unclear why observed responses to GAA supplementation are variable among steers managed under nearly identical conditions. As mentioned previously, cattle of Speer et al. (2022; 256 kg) were heavier than our cattle (161 kg), but also those of Grant et al. (2021, Chapter 2; 149 kg and 189 kg, respectively). In addition, control cattle of Speer et al. (2022) had greater N retention (38.5 g/d) compared with our control cattle (30.6 g/d) and those of Grant et al. (2021; 27.2 g/d) and Grant et al. (2022; 24.1 g/d). However, N retention relative to body weight was greater for cattle of Grant et al. (2021; 0.19 g/kg BW) and our cattle (0.18 g/kg BW) compared to cattle of Speer et al. (2022; 0.15 g/kg BW) and Grant et al. (2022; 0.13 g/kg BW). Creatine requirements are greater for growing animals than mature animals, as protein deposition and tissue development are still dominant processes during the growing phase (Walker, 1979). Thus, the cattle of Grant et al. (2021) may have been most responsive to supplemental GAA due to increased need for creatine synthesis. In our cattle and those of Speer

et al. (2022) and Grant et al. (2021, 2022), large amounts of GAA were supplemented with the primary goal of creating a methyl group deficiency. Negative responses to supplemental GAA reported by Speer et al. (2022) may suggest that a methyl group deficiency was induced in their cattle. The lack of a similar response in our cattle and those of Grant et al. (2021; 2022) could suggest that cattle of lighter BW may be more resistant to deleterious effects of GAA on methyl group availability or that other animal factors play a larger role in the efficacy of GAA in growing cattle than methyl group availability alone. Together, these data could suggest that post-ruminal GAA supplementation has a limited impact on protein deposition in growing steers fed corn-based diets, particularly as animals mature.

The lack of response to post-ruminal supplementation with creatine is consistent with other work conducted in growing steers in a similar model (Grant et al. 2021; 2022). Because responses to GAA were neutral or slightly positive in these experiments, it is not overly surprising that cattle also did not respond to creatine supplementation. Previous work has demonstrated increases in fecal N in response to creatine supplementation (Grant et al., 2021, 2022), which could be attributed to poor intestinal creatine absorption, which would prevent its utilization in the body. In our cattle, a numeric increase in fecal N was observed with supplemental creatine (1.5 g/d increase; $P = 0.15$), which likely suggests incomplete intestinal absorption of creatine. Because neither GAA nor creatine supplementation affected protein deposition, supplemental GAA did not likely create a methyl group deficiency and our cattle likely could support creatine needs through de novo synthesis alone.

Other recent work has investigated performance responses to feeding GAA to Chinese Angus bulls. Li et al. (2020) observed a stark 0.39 kg/d increase in average daily gain and 9.4% increase in feed efficiency when GAA was fed to Angus bulls at 0.6 g/kg dietary DM

(approximately 7 g/d). Similarly large increases in daily gain and feed efficiency were observed in other experiments feeding GAA at 0.6 g/kg dietary DM to Angus bulls (7 to 8 g/d GAA; Liu et al., 2021a,b). In another study, performance was maximized when GAA was provided at 0.20% of diet DM (approximately 17 g/d GAA), which increased daily gain by 0.34 kg/d and feed efficiency by 35% (Li et al., 2021). In finishing Angus steers, Yi et al. (2024) observed a 0.15 kg/d improvement in average daily gain when GAA was supplemented at either 0.8 or 1.6 g/kg diet DM (approximately 8 and 16 g/d GAA, respectively). The large positive performance responses to GAA in Angus bulls and steers are in contrast to our observations supplementing GAA via abomasal infusion in the present study and other work from our lab (Speer et al., 2022; Grant et al., 2021, 2022). Some possible explanations for inconsistent responses to GAA supplementation include differences in 1) GAA dosage and delivery, 2) duration of supplementation, and 3) weight, breed, and maturity. Li et al. (2020, 2021), Liu et al. (2020, 2021), and Yi et al. (2024) provided GAA in the feed, whereas we infused GAA abomasally. Interestingly, studies in Angus bulls observed greater dry matter and nutrient digestibilities when GAA was supplemented (Li et al., 2020; Liu et al., 2020, 2021). In our cattle, GAA did not affect apparent dry matter digestibility, but because we provided GAA abomasally, GAA supplementation would not affect ruminal fermentation. Our cattle received 15 g/d GAA, whereas studies in Angus bulls observed maximal responses at 6.7 to 7.8 g/d GAA when supplemented at 0.6 g/kg dietary dry matter (Li et al., 2020; Liu et al., 2020, 2021), 17 g/d GAA when supplemented at 0.2% dietary dry matter (Li et al., 2021), or 8 or 16 g/d at 0.8 or 1.6 g/kg diet DM (Yi et al., 2024). Previous work from our laboratory suggests that ruminal degradation of GAA is approximately 50%, which further separates GAA dosage among our cattle and work in Angus bulls and steers. In addition, our cattle underwent short-term GAA supplementation (10

d), whereas other experiments were much longer in duration (60 to 130 d). Although it is possible that long-term GAA supplementation is required to demonstrate increased growth performance, Liu et al. (2021) observed improved growth performance within 30 d of supplementing GAA.

Plasma and Urinary Creatine Metabolites

A Met × MGM interaction was observed for urinary creatine excretion ($P \leq 0.02$; Table 4.3). Regardless of Met supplementation, both GAA and creatine increased ($P < 0.01$) urinary creatine excretion relative to control. When Met was not supplemented, GAA increased urinary creatine excretion to a greater degree than creatine (8.5 g/d vs. 3.4 g/d increase relative to control for GAA and creatine, respectively; $P < 0.0001$). However, for GAA-supplemented steers, supplementation with Met reduced urinary creatine excretion (to 6.2 g/d), but this effect of Met was not observed for creatine-supplemented steers. A similar treatment response pattern was observed for plasma creatine concentrations. In addition, a Met × MGM interaction was observed for plasma creatine ($P = 0.01$) and creatinine ($P = 0.02$) concentrations, which followed a treatment response pattern similar to observations for urinary creatine excretion. Ardalan et al. (2021) observed similar responses in Met-deficient growing steers where Met tended to decrease urinary creatine excretion up to 7.5 g/d GAA, but not 15 g/d GAA (Met × quadratic GAA interaction). In contrast, in a model similar to the present study, Speer et al. (2022) observed no impact of supplemental Met on urinary creatine excretion. Ardalan et al. (2021) suggested that in the presence of adequate Met, creatine uptake by muscle could increase with increased Met availability, which is reasonable because Met increased N retention in their cattle. However, this explanation is unlikely valid for our study, because N retention was not affected by Met. No

additional interactions between Met and MGM treatments were observed for other plasma or urinary creatine metabolites measured ($P \geq 0.13$).

Effects of methionine

Supplemental Met decreased plasma concentrations of creatinine ($P = 0.02$), but did not affect plasma concentrations of GAA or creatine ($P \geq 0.14$). Supplemental Met did not affect urinary excretion of GAA or creatinine ($P \geq 0.32$). It is unclear why supplemental Met alone decreased plasma creatinine concentrations; however, it could potentially be related to muscle creatine uptake as suggested by Ardalan et al. (2021) as described above.

No effects of Met on plasma urea-N ($P = 0.30$; Table 4.4) or glucose ($P = 0.34$) concentrations were observed, suggesting that general energy and protein status were not affected by methionine supplementation.

Effects of methyl group modulators

Plasma concentrations and urinary excretion of GAA, creatine, and creatinine were affected by MGM ($P < 0.01$). Plasma GAA concentrations and urinary GAA excretion were greater for GAA than control or creatine ($P < 0.01$); creatine did not differ from control ($P \geq 0.25$). Urinary GAA excretion averaged 0.24, 0.28, and 0.68 g/d for control, creatine, and GAA, respectively. Increases in urinary GAA excretion when steers received GAA represented about 3% of the 15 g/d GAA infused. Previous research has demonstrated increases in plasma concentrations and urinary excretion of GAA when GAA was provided post-ruminally (Ardalan et al., 2020; Ardalan et al., 2021; Speer et al., 2020). In addition, more recent work from our lab in similar research models to the present experiment consistently demonstrate a two- to threefold increase in urinary GAA excretion when supplemented at 15 g/d GAA (1.5 to 2.5% of infused GAA; Speer et al., 2022; Grant et al., 2021; Grant et al., Chapter 3). Among experiments in

growing steers fed corn-based diets, increases in urinary GAA excretion have ranged from 1.5 to 2.5% of infused GAA.

Supplemental GAA or creatine increased plasma creatine concentrations and urinary creatine excretion ($P < 0.01$), and excretion was greater for GAA than creatine ($P < 0.01$). Urinary creatine excretion averaged 1.67, 5.41, and 9.0 g/d for control, creatine, and GAA, respectively. The approximately fivefold increase in creatine excretion for GAA supplemented steers represents 43% of the 16.8 g/d creatine that could be produced from the 15 g/d GAA infusion. In addition, the threefold increase in urinary creatine excretion with creatine supplementation represents 22% of the 16.8 g/d creatine infused. Our observation that supplemental GAA increases plasma concentration and urinary excretion of creatine is consistent with other work investigating post-ruminal GAA supplementation to cattle (Ardalan et al., 2020, 2021; Speer et al., 2020, 2022; Grant et al., 2021; Grant et al., Chapter 3). In addition, Li et al. (2020; 2021) and Liu et al. (2020; 2021) observed increases in plasma creatine concentrations with GAA supplementation to Angus bulls. The larger increase in urinary creatine excretion for GAA-supplemented steers compared with creatine-supplemented steers is consistent with other work in growing cattle (Grant et al., 2021; Grant et al., Chapter 3). Moreover, Ostojic et al. (2016) demonstrated in humans that GAA increased creatine concentrations in muscle and brain tissues than did creatine. In rats, Stead et al. (2001) reported that GAA and creatine increased muscle creatine concentrations similarly; however, total muscle creatine stores (i.e., including creatine and phosphocreatine) were greater for supplemental creatine compared with GAA. In Yucatan miniature pigs, supplemental GAA increased hepatic creatine concentrations to a larger degree than did creatine, whereas significant increases in muscle creatine were observed for supplemental GAA but increases were only numeric for supplemental creatine (McBreairty et al.,

2015). Ostojic et al. (2016) suggested that GAA may be more effective at improving creatine status because GAA is more readily taken up in tissues than creatine, possibly due to limited transporter availability for creatine (Braissant, 2012). Creatine uptake relies on transporter SLC6A8 for cellular uptake, whereas GAA is taken up by multiple transporters (SLC6A6, SLC6A8, and SLC6A13) and passive diffusion (Tachikawa et al., 2011, 2012). This could partially explain the differences in efficacy between GAA and creatine supplementation to increase creatine supply. Taken together, it is clear that GAA or creatine supplementation can improve body creatine status, and GAA may be more effective in this than supplemental creatine, possibly due to incomplete intestinal absorption of creatine.

Plasma concentrations and urinary excretion of creatinine increased ($P \leq 0.02$) when GAA or creatine were supplemented, and this increase was greater for GAA than creatine ($P \leq 0.02$). Creatinine excretion averaged 6.6, 7.6, and 8.7 g/d for control, creatine, and GAA. The 2.1 g/d increase in urinary creatinine when GAA was supplemented represents 14.5% of the 16.8 g/d creatine that could be produced from 15 g/d supplemental GAA. In addition, the 1.0 g/d increase in urinary creatinine excretion when creatine was supplemented represents 6.9% of the 16.8 g/d creatine provided. Creatinine forms spontaneously in the body from creatine and phosphocreatine through an irreversible, nonenzymatic reaction (Brosnan and Brosnan, 2007) and is lost through urinary excretion at a constant rate of about 1.7% of total body creatine daily (Wyss and Kaddurah-Daouk, 2000). Ardalan et al. (2020) observed linear increases in plasma and urinary creatinine concentrations when up to 40 g/d GAA was supplemented to Holstein heifers. In growing steers, Grant et al. (Chapter 3) demonstrated similar increases in plasma concentrations and urinary excretion of creatinine in response to 15 g/d GAA supplementation. In contrast, other experiments in growing steers have shown no impact of supplementing up to

15 g/d GAA on plasma concentrations or urinary excretion of creatinine (Ardalan et al., 2021; Speer et al., 2022; Grant et al., 2021), although increases may have been present but too small to detect statistically. Because creatinine excretion is considered a relatively static measure based on creatine status, it is somewhat puzzling that increases in creatinine excretion differ among similar experiments in growing steers. Tossenberger et al. (2016) suggested that excess creatine synthesis and/or supply, which is expected to proportionately increase conversion of creatine to creatinine, may lead to additional circulating and excreted creatinine. This could suggest that the total body creatine pool increased in proportion to increased creatine availability resulting from GAA or creatine supplementation in our cattle and those of Ardalan et al. (2020) and Grant et al. (Chapter 3).

Plasma glucose concentration tended to be affected by MGM ($P = 0.10$; Table 4.4); GAA supplementation increased ($P = 0.05$) plasma glucose relative to control, and plasma glucose tended to be greater for GAA than creatine ($P = 0.08$); control and creatine did not differ ($P = 0.71$). This could suggest improved energy homeostasis in GAA supplemented cattle; however, GAA supplementation has not affected plasma glucose concentrations in other experiments in growing cattle (Grant, 2020; Chapter 3) or finishing cattle (Li et al. 2020, 2021 and Liu et al., 2020, 2021). Plasma urea-N was not affected by MGM ($P = 0.74$), which is not surprising because N retention was not affected by GAA or creatine supplementation. In addition, creatine metabolism and excretion does not intersect with the urea cycle, so GAA or creatine supplementation is not otherwise expected to affect N metabolism.

Plasma Inflammation and Antioxidant Measures

No Met × MGM interactions were observed for measures of inflammation and antioxidant capacity ($P \geq 0.14$; Table 4.4).

Effects of methionine

Methionine supplementation did not affect plasma AOP ($P = 0.31$; Table 4.4). In addition, Met did not affect plasma haptoglobin ($P = 0.98$) or IL-10 ($P = 0.59$). Oxidative stress is caused by an imbalance in the production and accumulation of oxidative compounds (i.e., reactive oxygen species; ROS) and availability of antioxidant systems (e.g., antioxidant enzymes and compounds) to neutralize ROS (Wu et al., 2009, Freitas et al., 2016). Methionine is a precursor to intracellular antioxidants glutathione and taurine, which are both products of cysteine metabolism (Brosnan and Brosnan, 2006). Thus, we hypothesized that supplemental Met would increase plasma AOP in our steers, but the data do not support this hypothesis. In transition dairy cows, Met can increase plasma and hepatic concentrations of glutathione and taurine (Osorio et al., 2014; Zhou et al., 2018; Batistel et al., 2018) and total plasma antioxidant capacity (Osorio et al., 2014; Batistel et al., 2018). Although we cannot conclude if supplemental Met affected glutathione specifically, the lack of increase in total AOP suggests that Met did not quantitatively alter total antioxidant activity in our cattle.

For biomarkers of inflammation, we hypothesized that Met would decrease plasma haptoglobin and increase plasma IL-10. Haptoglobin is a positive acute phase protein that is produced in the liver and released into circulation in response to inflammatory stimuli, whereas IL-10 is an anti-inflammatory cytokine produced primarily in monocytes and works to control inflammatory responses (Tizard, 2018). In transition dairy cows, decreases in haptoglobin have been observed in response to peripartal ruminally protected Met supplementation in some

experiments (Zhou et al., 2016; Batistel et al., 2018), but not others (Osorio et al., 2014; Vailati-Riboni et al., 2017). In newly received beef heifers, Grant et al. (2022) observed a linear increase in plasma haptoglobin for control cattle over the 60-d experiment, but haptoglobin did not increase for heifers receiving ruminally protected Met, which could suggest that Met mitigated inflammation. Supplemental Met has demonstrated variable responses in IL-10 gene expression in bovine PMN. Zhou et al. (2018) observed a trend for IL-10 expression to decrease in PMN isolated from cows supplemented with Met, whereas Abdelmegeid et al. (2017) observed increased IL-10 expression in response to in vitro Met supplementation in PMN isolated from bovine neonates. The lack of response in antioxidant capacity, acute phase protein response, and anti-inflammatory cytokine concentrations suggests that Met did not alter oxidative balance or inflammatory signaling in our cattle.

Effects of methyl group modulators

No effects of MGM were observed for plasma AOP ($P = 0.16$; Table 4.4). In addition, MGM did not affect haptoglobin or IL-10 concentrations ($P \geq 0.37$). Because GAA consumes methionine methyl groups in its conversion to creatine, we thought GAA might increase oxidative stress and/or inflammation due to reduced availability of methyl groups for other purposes. Creatine synthesis from GAA obligatorily consumes SAM methyl groups, and a reduction in methyl group availability may be associated with increased oxidative stress (Zhao et al., 2018; Cavallaro et al., 2010). In addition, after SAM donates its methyl group to GAA, it becomes *S*-adenosylhomocysteine (SAH) and subsequently homocysteine, which both increases oxidative stress and contributes to inflammation (Wu et al., 2007; Tehlivets, 2011). Thus, we hypothesized that GAA supplementation might disrupt oxidative balance in our cattle. Because direct creatine supplementation was expected to decrease demand for methyl groups that would

otherwise be used for its synthesis, we expected creatine to possibly improve oxidative balance. In contrast, Grant (2020) reported a decrease in plasma AOP when creatine, but not GAA, was supplemented to growing steers. Grant et al. (Chapter 3) observed no effect of GAA or creatine supplementation on plasma AOP. In nonruminants, responses to GAA or creatine are also inconsistent. Large amounts of supplemental GAA may promote formation of reactive oxygen species in cell culture models (Mori et al., 1996; Zugno et al., 2007a,b), but this response has not been observed in vivo (Ostojic et al., 2013, 2015). In addition, creatine supplementation appears to improve antioxidant status in cell culture models (Sestili et al., 2006, 2007), but negative impacts of creatine on antioxidant status have been observed in handball athletes (Percário et al., 2012). The lack of response in AOP agrees with the lack of effect of GAA or creatine on plasma haptoglobin or IL-10 concentrations. Similarly, Grant (2020) and Grant et al. (Chapter 3) observed no effects of GAA or creatine supplementation on markers of inflammation in growing steers in a similar model. Moreover, Speer et al. (2022) did not observe effects of GAA supplementation on plasma haptoglobin concentrations. Taken together, it appears that GAA or creatine supplementation may not greatly alter antioxidant status and inflammatory signals in growing steers.

Blood Cell Profile and Neutrophil Functionality

An interaction between Met and MGM was observed for blood monocyte concentration ($P = 0.04$; Table 4.5); however, main effects of Met and MGM were not observed ($P \geq 0.71$). In creatine supplemented steers, providing Met decreased monocyte concentration by nearly 50% (641 vs. 322 cells/μL; $P = 0.02$), whereas, for GAA supplemented steers, providing Met tended to increase monocyte concentration by 38% (450 vs. 619 cells/μL; $P = 0.08$); providing Met to

control steers did not affect monocyte counts ($P = 0.61$). The elevation in blood monocytes for GAA-supplemented calves receiving Met could suggest increased immune response, whereas the decrease for creatine supplemented calves when Met was provided could suggest a suppression of immune activity (Ackerman, 2017). Nevertheless, mean monocyte counts for all treatments were within the normal reference range for cattle (0 to 900 cells/µL). No additional interactions were detected for other CBC parameters ($P \geq 0.13$) or measures of PMN function ($P \geq 0.33$; Table 4.6).

Effects of methionine

Supplemental Met tended to increase segmented neutrophil counts ($P = 0.08$; Table 4.5), which averaged 4,680 and 5,500 cells/µL for steers receiving 0 or 7.2 g/d Met, respectively. Band neutrophil counts averaged 13 cells/µL were unaffected by Met ($P = 0.99$), and were within the normal reference range for cattle (0 to 100 cells/µL). Following production in bone marrow, immature band neutrophils are released into circulation in response to inflammation or pathogen exposure (Jones and Allison, 2007). Circulating band neutrophils mature into segmented neutrophils when activated or exposed to pathogens. Band neutrophils rarely accumulate in blood except in cases of severe inflammation or sepsis (Jones and Allison, 2007), so it is not surprising that Met did not affect band neutrophil counts. The tendency for Met to increase segmented neutrophil counts could suggest a greater inflammatory state for Met supplemented calves; however, mean segmented neutrophil counts for both 0 and 7.2 g/d Met were greater than the reference range for cattle (600 to 4,000 cells/µL; Fielder, 2022), which could suggest mild inflammation for all cattle. Met supplementation did not affect any other CBC analysis parameters ($P \geq 0.13$).

Methionine supplementation tended to decrease phagocytosis for PMN treated with LPS ($P = 0.08$; Table 4.6) and averaged 49.7% and 44.9% for steers provided 0 or 7.2 g/d Met, respectively (Table 4.8). Neutrophil phagocytosis without LPS was not affected by Met ($P = 0.22$). Supplemental Met did not affect PMN oxidative burst with or without LPS ($P \geq 0.43$). Mean fluorescence intensity of oxidative burst and phagocytosis, which represent the mean number of ROS produced per neutrophil and mean number of bacteria phagocytosed per neutrophil, respectively, were not affected by Met ($P \geq 0.14$). The tendency for Met to decrease PMN phagocytosis in cells treated with LPS may suggest that Met altered the innate immune response when subjected to inflammatory stimuli. These data contrast with work in transition dairy cattle, where increased neutrophil phagocytosis in response to supplemental Met has been observed consistently (Osorio et al., 2013; Zhou et al., 2018; Batistel et al., 2018). It is unclear why our cattle responded oppositely to Met relative to observations in transition cows; however, differences in stage of production and physiological stress (e.g., depressed pre- and post-partum intake, calving, initiation of lactation) may contribute to differences in immune cell responses.

Effects of methyl group modulators

We observed a main effect of MGM on blood leukocyte count ($P = 0.05$; Table 4.5), which decreased when GAA or creatine were supplemented relative to control ($P \leq 0.04$); GAA and creatine led to similar responses ($P = 0.83$). Blood leukocyte counts averaged 13.4, 11.8, and 11.7 × 10^3 cells/μL for control, creatine, and GAA, respectively, and for control cattle were slightly elevated above the normal range (4.0 to 12.0 × 10^3 cells/μL; Fielder et al., 2022). Segmented neutrophil counts averaged 5.9, 5.0, and 4.4 × 10^3 cells/μL for control, creatine, and GAA, respectively, and counts were less for supplemental GAA than control ($P = 0.02$), whereas creatine was intermediate and did not differ from either control ($P = 0.14$) or GAA ($P = 0.26$).

For all treatments, segmented neutrophil counts were greater than the normal range (0.6 to 4.0 × 10^3 cells/μL; Fielder et al., 2022). Because neutrophils are a class of leukocytes (i.e., white blood cells), their decrease when GAA was supplemented and numeric decrease when creatine was supplemented correlates well with decreases in total leukocyte count. Elevated total leukocyte and neutrophil counts could suggest that our cattle were in a mild inflammatory state, and the decreases observed with GAA and creatine supplementation may suggest that their supplementation mitigated inflammation. Other work in our lab has not demonstrated effects of GAA or creatine on leukocyte or neutrophil counts in growing cattle (Grant, 2020; Chapter 3). No main effects of MGM were observed for other CBC parameters ($P \geq 0.19$).

In the presence of LPS, we observed a tendency for a main effect of MGM on PMN phagocytosis ($P = 0.07$; Table 4.6), which averaged 51.7% for control, 46.7% for creatine, and 43.5% for GAA. Relative to control, GAA decreased neutrophil phagocytosis when LPS was present ($P = 0.03$), but creatine did not ($P = 0.14$); GAA and creatine did not differ ($P = 0.40$). In contrast, we did not detect a main effect of MGM on PMN phagocytosis without LPS ($P = 0.14$). No main effects of MGM were observed for PMN oxidative burst with or without LPS treatment ($P \geq 0.32$). In addition, mean ROS produced per neutrophil and mean number of bacteria phagocytosed per neutrophil were unaffected by MGM ($P = 0.60$). The reasons for our observed trends and numeric decreases in PMN phagocytosis for GAA and creatine supplementation are unknown. However, if paired with our observed decreases in leukocyte and neutrophil counts for GAA, these data further suggest that GAA supplementation suppressed an inflammatory response in the presence of immune stimulation (i.e., LPS treatment). Effects of GAA or creatine oxidative burst and phagocytosis have not been observed in other experiments in growing steers (Grant, 2020; Chapter 3). In addition, the lack of change in oxidative balance or inflammatory

signals could suggest that small changes in neutrophil function with creatine or GAA did not alter systemic inflammation.

Conclusions

Supplementation with GAA or creatine did not affect N retention of growing cattle. As well, Met supplementation did not affect N retention in this experiment, which suggests that our diet was not first-limiting in Met. As observed in previous experiments, GAA and creatine supplementation effectively improved creatine supply, and GAA was more effective in doing so than creatine. Methionine did not affect antioxidant balance or inflammatory markers in our cattle, nor did GAA or creatine. Supplemental Met tended to increase neutrophil counts, but tended to decrease PMN phagocytosis, which could suggest an immunomodulatory effect of Met. In contrast, supplemental GAA decreased neutrophil counts and decreased PMN phagocytosis in the presence of an in vitro LPS challenge. Some immunomodulatory effects of methionine or GAA may exist; however, the meaning of these effects is unknown.

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Table 4.1. Composition of diet fed to growing 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	
Dry matter, % of as is	86.0
Organic matter	95.1
Crude protein	12.6
Neutral detergent fiber[3]	14.6
Acid detergent fiber[4]	7.0

[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 4.2. Effects of methionine on diet digestibility and nitrogen balance in steers supplemented with guanidinoacetic acid and creatine

	Methionine, g/d									
	0			7.2						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Met	MGM	Met × MGM
n	6	6	5	6	6	6				
Intake, kg/d[3]										
DM	3.44	3.44	3.43	3.44	3.44	3.44	0.004	0.28	0.46	0.46
OM	3.29	3.29	3.28	3.29	3.29	3.29	0.004	0.28	0.46	0.46
Apparent digestibility, %										
DM	74.7	75.3	76.0	75.1	75.8	74.1	1.46	0.73	0.81	0.55
OM	75.1	75.8	74.6	75.6	76.3	74.6	1.47	0.74	0.80	0.52
N, g/d										
Feed	69.2	69.2	69.0	69.2	69.2	69.2	0.08	0.28	0.46	0.46
Infused	0.00	5.26	5.35	0.80	6.18	6.13	0.041	<0.01	<0.01[5]	0.16
Intake[4]	69.2	74.5	74.4	70.0	75.4	75.3	0.09	<0.01	<0.01[5]	0.74
Urinary	15.8	19.3	20.1	17.2	20.3	20.1	0.92	0.19	<0.01[5]	0.63
Urinary urea N	8.5	12.2	11.6	11.1	12.6	11.7	1.25	0.21	0.05[6,7]	0.43
Fecal	22.7	25.8	22.5	23.8	24.3	26.0	1.32	0.28	0.35	0.14
Retained	30.6	29.5	31.7	28.9	30.8	29.3	1.34	0.29	0.82	0.19

[1] Methyl group modulator treatments included: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments.

[3] Excludes abomasal infusions.

[4] Feed N + infused N.

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

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

[7] Pairwise means separated within MGM as control < GAA; $P \leq 0.10$

Table 4.3. Effects of methionine on plasma concentrations and urinary excretion of guanidinoacetic acid, creatine, and creatinine in growing steers supplemented with GAA or creatine

	Methionine, g/d									
	0			7.2						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Met	MGM	Met × MGM
n	6	6	5	6	6	6				
Plasma, mg/L										
GAA	0.76	1.27	2.14	1.45	0.86	2.38	0.590	0.41	<0.01[3]	0.13
Creatine	14.7^{c}	17.7^{b}	22.0^{a}	14.3^{c}	19.0^{b}	18.7^{b}	0.79	0.14	<0.01[4]	0.01
Creatinine	18.5^{c}	20.5^{b}	23.4^{a}	17.4^{c}	21.2^{b}	20.8^{b}	1.08	0.02	<0.01[4]	0.02
Urine, g/d										
GAA	0.243	0.308	0.701	0.230	0.261	0.660	0.0545	0.32	<0.01[3]	0.91
Creatine	1.89^{d}	5.30^{c}	10.39^{a}	1.46^{d}	5.52^{c}	7.62^{b}	0.700	0.02	<0.01[4]	0.02
Creatinine	6.66	7.56	8.92	6.55	7.61	8.54	0.476	0.65	<0.01[4]	0.87

[1] Methyl group modulator treatments included: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments

[3] Pairwise means separated within MGM as GAA > Creatine = Control, $P \leq 0.05$

[4] Pairwise means separated within MGM as GAA > Creatine > Control, $P \leq 0.05$

[a-d] Means without a common superscript differ, $P \leq 0.05$

Table 4.4. Effects of methionine on plasma glucose, urea, antioxidant capacity, and haptoglobin concentrations in growing steers supplemented with GAA and creatine

	Methionine, g/d									
	0			7.2						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Met	MGM	Met × MGM
n	6	6	5	6	6	6				
Glucose, mM	4.33	4.28	4.60	4.26	4.37	4.38	0.132	0.34	0.10[3]	0.26
Urea, mM	2.87	2.81	3.21	3.21	3.12	2.96	0.238	0.30	0.74	0.15
Trolox-equivalent AOP, µM[4]	97.9	100.6	98.7	97.4	100.2	94.0	3.28	0.31	0.16	0.46
Haptoglobin, µg/mL[5]	430	485	423	419	421	498	78.7	0.98	0.74	0.89
Interleukin-10, pg/mL	748	692	563	828	887	630	266.7	0.59	0.37	0.81

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments

[3] Pairwise means separated within MGM as Control = Creatine < GAA; $P \leq 0.10$.

[4] One observation was identified as an outlier and removed from the Control + 7.2 g/d Met treatment due to unusually low AOP

[5] Four observations were removed due to hemolysis: one observation each from Control + 7.2 g/d Met, GAA + 0 g/d Met, GAA + 7.2 g/d Met, and Creatine + 7.2 g/d Met

Table 4.5. Effects of methionine on complete blood count parameters in growing steers supplemented with guanidinoacetic acid or creatine

	Methionine, g/d									
	0			7.2						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Met	MGM	Met × MGM
n	5	6	5	5	6	5				
Leukocyte count, cells/nL	12.7	12.1	11.4	14.2	11.4	11.9	1.15	0.51	0.05[3]	0.37
Erythrocyte conc, cells/pL	9.5	9.6	9.4	8.7	9.5	9.3	0.56	0.14	0.29	0.35
Hemoglobin, g/dL	11.7	11.5	11.2	11.1	11.5	11.2	0.31	0.13	0.22	0.25
Mean cell volume, fL	33.7	33.7	33.6	33.5	33.5	33.7	1.16	0.53	0.90	0.57
Mean cell hemoglobin, pg	12.3	12.2	12.2	12.1	12.2	12.2	0.44	0.68	0.95	0.79
Mean cell hemoglobin conc., g/dL	36.3	36.2	36.2	36.1	36.5	36.0	0.34	0.95	0.69	0.65
Red blood cell distribution width, %	22.0	21.7	21.5	21.6	22.0	21.9	0.57	0.87	0.88	0.64
Platelets, cells/nL	613	595	514	549	609	586	77.3	0.79	0.43	0.21
Segmented neutrophils, cells/nL	4.77	4.89	4.37	6.99	5.15	5.35	0.731	0.08	0.05[4]	0.13
Band neutrophils, cells/nL	0.010	0.001	0.027	0.042	0.000	0.000	0.0226	0.99	0.46	0.41
Lymphocytes, cells/nL	6.75	6.11	6.14	6.09	5.57	6.27	0.512	0.20	0.24	0.50
Monocytes., cells/nL	0.459[ab]	0.641[a]	0.450[ab]	0.525[ab]	0.322[b]	0.619[a]	0.1496	0.71	0.84	0.04
Eosinophils, cells/nL	0.611	0.391	0.360	0.404	0.249	0.531	0.2356	0.71	0.61	0.62
Basophils, cells/nL	0.116	0.059	0.133	0.134	0.179	0.070	0.0623	0.58	0.92	0.30
Hematocrit, % (calculated)	32.2	32.0	30.9	30.6	31.6	31.0	1.02	0.22	0.47	0.45
Hematocrit, % (spun)	34.1	33.4	33.1	32.5	34.1	33.4	1.12	0.75	0.79	0.29
Plasma protein (refract.), g/dL	7.56	7.67	7.37	7.46	7.58	7.43	0.185	0.92	0.16	0.81
Fibrinogen (heat ppt.), mg/dL	604	665	583	684	654	696	63.2	0.16	0.92	0.47

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments.

[3] Pairwise means separated within MGM as Control > Creatine = GAA; $P \leq 0.05$.

[4] Pairwise means separated within MGM as Control > GAA, $P \leq 0.05$;

[a,b] Within row, means without a common superscript differ, $P \leq 0.05$.

Table 4.6. Effects of methionine on immune cell function in growing steers supplemented with guanidinoacetic acid and creatine

	Methionine, g/d									
	0			7.2						
	Methyl Group Modulator (MGM)[1]							*P*-value		
Item	Control	Creatine	GAA	Control	Creatine	GAA	SEM[2]	Met	MGM	Met × MGM
n	6	6	5	6	6	6				
Oxidative burst, %										
−LPS[3]	37.5	38.9	39.8	38.3	31.2	40.4	5.02	0.42	0.32	0.33
+LPS	35.3	36.4	34.8	37.8	25.7	35.0	6.48	0.53	0.57	0.42
Phagocytosis, %										
−LPS	50.6	47.9	52.5	52.3	40.0	49.5	5.70	0.22	0.14	0.60
+LPS	53.6	50.4	45.1	49.8	43.0	41.8	4.47	0.08	0.07[4]	0.80
Reactive oxygen species, neutrophil$^{-1\dagger}$										
−LPS	2,228	2,386	2,419	2,196	2,212	2,219	234.3	0.26	0.76	0.84
+LPS	2,213	2,293	2,351	2,097	1,961	2,013	229.4	0.14	0.92	0.64
Bacteria phagocytized, neutrophil$^{-1\ddagger}$										
−LPS	422	427	467	421	410	424	46.2	0.37	0.60	0.77
+LPS	419	425	421	406	387	401	37.2	0.29	0.98	0.89

[1] Methyl group modulator treatments include: Control, Creatine (16.8 g/d supplemental creatine), or GAA (15 g/d supplemental GAA)

[2] Average SEM across all treatments

[3] LPS = lipopolysaccharide

[4] Pairwise means separated as Control > GAA, $P < 0.10$

† Mean fluorescence intensity of oxidative burst

‡ Mean fluorescence intensity of phagocytosis