# Efficacy of guanidinoacetic acid supplementation to growing cattle and relative bioavailability of guanidinoacetic acid delivered ruminally or abomasally

by

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# Abstract

Two experiments were conducted to assess the value of guanidinoacetic acid (GAA) as a feed additive for growing cattle. The first experiment utilized 7 ruminally cannulated Holstein steers (280 ± 14 kg) in a 5 × 5 Latin square design and evaluated relative bioavailability of GAA. Treatments were continuous ruminal or abomasal infusion of 0, 10, or 20 g/d GAA, and blood and urine samples were collected on the final day of each period for analysis of creatine, creatinine, and GAA concentrations. Plasma and urinary creatine concentrations were used as the primary response criteria to calculate ruminal bypass value of GAA by slope-ratio methodology; values were 47% and 49%, respectively. In Exp. 2, effects of GAA supplementation on N retention and methionine (Met) methyl group flux in steers fed corn-based diets were determined utilizing 6 ruminally cannulated Holstein steers (256 ± 14 kg) in a 6 × 6 Latin square design. Factorial treatments were 2 levels of Met (0 or 5 g/d) and 3 levels of GAA (0, 7.5, or 15 g/d) delivered by continuous abomasal infusion. Periods were 10 d in length and included 3 d for total fecal and urine collections; blood samples were collected and Met flux was measured on the final day of each period. Nitrogen retention increased ($P < 0.01$) with Met supplementation and tended to decrease linearly ($P = 0.12$) with increasing amounts of GAA. The response in N retention suggests dietary Met was limiting. Methyl group flux tended to increase linearly ($P = 0.10$) with GAA provision and significantly increased ($P < 0.01$) with Met supplementation. A tendency ($P = 0.10$) for a GAA × Met interaction was also observed, because methyl group flux only increased in response to GAA when Met was supplemented. Plasma and urinary creatine concentrations linearly increased ($P < 0.05$ and $P = 0.06$, respectively) when GAA was supplemented. Urinary GAA concentrations increased ($P < 0.01$) with GAA supplementation. Plasma urea N also increased linearly ($P < 0.05$) with GAA

supplementation, and no differences in plasma total amino acid concentrations were observed ($P \geq 0.29$) across treatments. It can be concluded from these studies that GAA is degraded approximately 50% in the rumen and supplementation of GAA alone or with Met as a methyl donor in a corn-based diet did not improve protein deposition in growing steers.

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"What, then, shall we say in response to these things? If God is for us, who can be against us?"

~ Romans 8:31

# Dedication

*To Dr. Robert J. Keener, who has always provided me endless encouragement and support from the time I set foot in his classroom and continues to be my biggest cheerleader in all my academic pursuits.*

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# Chapter 1 - Literature Review

## *Introduction*

Protein requirements of cattle vary depending on their physiological state and expected level of performance and are met through both diet and microbial sources. The requirements of growing cattle are greater than those of mature animals to support lean tissue growth. Because protein is often the most expensive ingredient in the diet, reducing its inclusion in the diet without adversely affecting animal performance would behoove producers wanting to reduce costs of their cattle operation. In doing this, the efficiency of protein use for gain must be optimized or feed additives designed to improve protein deposition must be incorporated into the diet. Guanidinoacetic acid (GAA), the precursor to creatine, has recently been investigated as supplement for livestock. Creatine is a compound found in muscle tissues that serves to replenish ATP, particularly in those with high energy demands (Wyss and Kaddurah-Daouk, 2000), and is synthesized in the liver by methylation of GAA by *S*-adenosylmethionine (SAM) (Wyss and Kaddurah-Daouk, 2000). Livestock rely heavily on *de novo* synthesis of creatine because their diet is essentially devoid of this nutrient (Snoswell and Xue, 1987). The amount of creatine produced endogenously may not maximize animal performance, so GAA supplementation could improve creatine status of the animal, permitting enough methionine is available to provide methyl groups. This review will provide an overview of the metabolic pathways of creatine and methionine metabolism and how their relationship to one another might affect effectiveness of GAA as a dietary supplement in livestock diets.

## *Creatine Metabolism*

Creatine is a compound in vertebrate muscle tissues that serves as an energy reservoir to help maintain energy homeostasis within the muscle. When phosphorylated by creatine kinase, it is a source of high-energy phosphate bonds with which to replenish ATP in tissues, especially in those where energy demand is high (Wyss and Kaddurah-Daouk, 2000). The final step in creatine metabolism is the nonenzymatic, irreversible degradation of both creatine and phosphocreatine to creatinine, which is subsequently excreted in the urine (Stead et al., 2006). Approximately 1.7% of the total body creatine pool is converted to creatinine daily (Walker, 1979; Wyss and Kaddurah-Daouk, 2000) and must constantly be replaced. Additionally, creatine is required for growing animals to support new tissue growth (Walker, 1979), making their creatine requirement significantly higher than those of adults (Brosnan et al., 2009).

Creatine can be obtained through diet or *de novo* synthesis from 3 amino acids: arginine, glycine, and methionine (Walker, 1979; da Silva et al., 2009). The only dietary sources of creatine are animal-derived, so individuals consuming primarily products of plant origin acquire much of their creatine from *de novo* synthesis (Snoswell and Xue, 1987; Brosnan and Brosnan, 2007). Creatine biosynthesis is a two-step process and is enzymatically regulated to ensure that arginine, glycine, and methionine are not consumed unnecessarily in times of adequate creatine ingestion (Walker, 1979). This regulation occurs in the first step of the pathway, where arginine and glycine are used to form the creatine precursor guanidinoacetic acid.

### *Guanidinoacetic Acid Synthesis*

Guanidinoacetic acid (GAA) is the sole precursor to creatine. It is synthesized from arginine and glycine, a reaction catalyzed by the enzyme L-arginine:glycine amidinotransferase

(AGAT) (Brosnan and Brosnan, 2007). The amidino group of arginine is transferred to glycine, yielding GAA and L-ornithine (Wyss and Kaddurah-Daouk, 2000; Brosnan et al., 2009). Synthesis of GAA is the rate-limiting step of creatine production in animals and is regulated by feedback inhibition to ensure GAA production does not exceed creatine requirements (Wyss and Kaddurah-Daouk, 2000; Stead et al., 2001).

The site of GAA production differs among species due to tissue location of AGAT. It is generally accepted GAA is produced by the kidneys, but both the kidneys and pancreas are known to contain high levels of AGAT in mammals (Wyss and Kaddurah-Daouk, 2000). The pancreas has been found to have a similar amount of activity as the kidneys in piglets (Brosnan et al., 2009), as well as contribute substantially to GAA synthesis in humans and rats (Van Pilsum et al., 1972). Differences in AGAT distribution between humans and rats have been reported by Edison et al. (2007). They discovered GAA production by kidneys of rats fed a creatine-free diet was equal to creatine lost as creatinine, suggesting the kidneys are the primary source of GAA used for *de novo* creatine synthesis and that renal GAA production was adequate to replace endogenous creatine losses in the form of creatinine. Conversely, this was not observed for humans who were not on a creatine-free diet; *in vivo*, only 12% of total creatine loss could be restored by renal GAA production, indicating kidneys do not play a significant role in GAA production (Edison et al., 2007).

### *Creatine Synthesis*

Once GAA has been produced, it is released into the blood and is available for uptake by the liver (Wyss and Kaddurah-Daouk, 2000) by the GAT2 transporter (Tachikawa et al., 2012). In the liver, a methyl group transfer from *S*-adenosylmethionine (SAM) is catalyzed by *N*-

guanidinoacetate methyltransferase (GAMT), an irreversible reaction yielding creatine and *S*-adenosylhomocysteine (SAH) (Stead et al., 2001; Brosnan and Brosnan, 2007; Brosnan et al., 2009). Guanidinoacetate methyltransferase is not regulated by feedback inhibition; rather, it is inhibited by SAH, an end product of methyltransferase reactions (Clarke and Banfield, 2001). The pancreas is also known to also have GAMT activity (Van Pilsum et al., 1972; Walker, 1979; Snoswell and Xue, 1987; Wyss and Kaddurah-Daouk, 2000), but compared to that of the liver is very modest when expressed in terms of body weight (Brosnan et al., 2009). Creatine produced from the GAMT reaction is then transported in the blood for uptake by tissues via the sodium-linked transporter SLC6A8 (Brosnan and Brosnan, 2010). A majority of total body creatine is stored in skeletal muscle (Wyss and Kaddurah-Daouk, 2000; Brosnan and Brosnan, 2007), but it is also found in the heart and nerve tissue (Walker, 1979; Brosnan et al., 2009). Inside the cell, creatine can act as an energy reserve by storing ATP (high energy phosphate bonds) that can be used in periods of high energy demand.

### *Phosphocreatine*

Energy is generated when ATP is hydrolyzed to ADP, inorganic phosphate ($P_i$), and $H^+$ (Brosnan and Brosnan, 2007; Guimarães-Ferreira, 2014). When ATP is being utilized rapidly, phosphocreatine can transfer its phosphate group to ADP and reform ATP to maintain energy homeostasis within the tissue (Walker, 1979; Wyss and Kaddurah-Daouk, 2000). Phosphocreatine formation is simply a reversal of the latter reaction, i.e., a phosphate group is transferred from ATP to creatine. This reversible reaction is catalyzed by creatine kinase, an enzyme present in large quantities in tissues with fluctuating energy demands (Walker, 1979). It

is not surprising, then, that typical creatine content of muscle is comprised of approximately 70% phosphocreatine (Greenhaff, 1997).

Phosphocreatine also acts as a cellular buffer by countering $H^+$ production. Lactic acid produced during anaerobic metabolism of glycogen, in addition to ATP hydrolysis, releases hydrogen ions that reduce intracellular pH (Walker, 1979; Westerblad et al., 2002). Hydrogen ion accumulation is mitigated by phosphocreatine when creatine kinase acts in the direction of ATP regeneration and consumes $H^+$ (Brosnan and Brosnan, 2007). Prior to binding to ADP, release of $P_i$ from the breakdown of phosphocreatine provides additional buffer to attenuate pH changes within the cell (Walker, 1979). Once ATP has been replenished, free creatine then becomes available for phosphorylation again. Even though the creatine kinase reaction maintains equilibrium between creatine and phosphocreatine, both molecules are subject to conversion to creatinine.

### *Creatinine Synthesis*

Irreversible degradation of creatine and phosphocreatine by a spontaneous, nonenzymatic reaction results in the formation of creatinine (Wyss and Kaddurah-Daouk, 2000). Creatinine synthesis occurs predominantly in muscle (Stead et al., 2006), where 90% of the creatine and phosphocreatine is stored (Brosnan and Brosnan, 2007). Approximately 1.1% and 2.6% of body creatine and phosphocreatine, respectively, are transformed to creatinine and excreted in urine daily (Wyss and Kaddurah-Daouk, 2000), which equates to about 1.7% of total body creatine (Walker, 1979; Wyss and Kaddurah-Daouk, 2000). Rate of creatinine excretion by an individual is relatively constant, but between individuals the amount excreted varies due to differences in muscle mass (Stead et al., 2006). Supplemental creatine can also alter creatinine excretion by

way of increasing creatine tissue stores. When subjects in the study of Crim et al. (1975) were supplemented 10 g/d creatine for 10 d, urinary creatinine increased, but the effect was transient and excretion of creatinine returned to basal levels once exogenous creatine was no longer provided. It was noted that individuals with greater creatine retention had greater urinary creatinine excretion following the 10-d period, so they concluded that creatinine excretion was influenced more by the size of the creatine body pool rather than body mass (Crim et al., 1975).

### *Enzymatic Regulation of Creatine Synthesis*

The most important regulatory site, and rate-limiting step, of creatine synthesis is GAA formation, catalyzed by arginine:glycine amidinotransferase (AGAT) (Wyss and Kaddurah-Daouk, 2000; Stead et al., 2001). Feedback repression of AGAT by creatine is the regulatory mechanism, prohibiting excessive creatine formation (Walker, 1979) and consequently allowing conservation of the essential amino acids methionine and arginine (Wyss and Kaddurah-Daouk, 2000; Stead et al., 2001). Creatine exerts its inhibitory effect on AGAT activity by decreasing AGAT mRNA expression (McGuire et al., 1984; Edison et al., 2007). Both *in vitro* and *in vivo* studies provide validation of this feedback repression in the kidney where GAA synthesis is most prominent; however, it should be noted that AGAT suppression will occur in any tissue containing AGAT. An *in vitro* system using isolated renal tubules demonstrated significant suppression of GAA synthesis in a dose-dependent manner when tubules incubated with arginine and glycine had creatine added in concentrations of 100 and 300 mg/L ($P < 0.05$ and $P < 0.01$, respectively) (Takeda et al., 1992). Inclusion of 0.4% creatine in rat diets decreased GAA production 81% by rat kidneys *in vivo* (Edison et al., 2007) and reduced renal AGAT activity 84 to 86% (Edison et al., 2007; da Silva et al., 2009). Similar effects were observed by McGuire et

al. (1984), where rats fed a 0.3% creatine diet experienced 74% less AGAT activity than rats on a creatine-free diet. Furthermore, both GAA and creatine supplementation can depress AGAT activity by 73% and 82%, respectively, when compared to rats who receive neither supplement (Stead et al., 2001). Thus, it can be concluded that increased creatine ingestion or *de novo* creatine synthesis via GAA supplementation results in elevated creatine concentrations in the blood, leading to feedback inhibition of the AGAT enzyme. It has also been proposed that substrate availability, predominantly arginine, provided directly or by way of citrulline, can significantly affect *in vivo* GAA production by the kidneys (Edison et al., 2007). Alternative data in the literature argues dietary arginine does not influence AGAT activity (Funahashi et al., 1981), nor does provision of citrulline and glycine to isolated renal rat tubules result in GAA synthesis (Takeda et al., 1992).

Guanidinoacetate *N*-methyltransferase (GAMT) activity or expression is not controlled by creatine in the same manner as AGAT. As with most methyltransferases, GAMT is inhibited by its product *S*-adenosylhomocysteine (SAH) (Walker, 1979; Clarke and Banfield, 2001) and is dependent on the availability of its substrate (Stead et al., 2001); i.e., GAA provision will stimulate creatine synthesis. da Silva et al. (2009) noted no difference in hepatic GAMT activity or the rate of creatine synthesis between rats receiving creatine supplemented or creatine-free diets; however, elevated plasma creatine concentrations will impair GAA transport into hepatocytes, leading to a reduction in creatine synthesis (Tachikawa et al., 2012). Essentially, GAMT will methylate GAA to produce creatine in the liver, but the quantity of creatine produced will be proportional to the quantity of GAA available.

## *Methionine Metabolism*

Methionine is an essential amino acid in mammals that participates in numerous metabolic pathways beyond protein synthesis. It is the initiating amino acid in the translation step of protein synthesis (Brosnan and Brosnan, 2006), but most importantly is the methyl group donor upon its activation to *S*-adenosylmethionine (SAM) for many transmethylation reactions throughout the body (Lobley, 1992). The end product of these reactions, *S*-adenosylhomocysteine (SAH), is hydrolyzed to homocysteine, which has 2 possible fates. Homocysteine can be recycled back to methionine by 1 of 2 methyltransferases or combined with serine and irreversibly converted to cysteine via transsulfuration. These 3 major pathways – transmethylation, remethylation, and transsulfuration – constitute methionine metabolism.

### *S-adenosylmethionine Synthesis and Use*

The methionine cycle occurs in all tissues and is designed to ensure adequate methionine availability to support protein deposition as well as produce ample amounts of SAM for methyl group donation. Available methionine not being directed towards protein synthesis is instead transformed to SAM by methionine adenosyltransferase (MAT), a catalyst for transferring an adenosyl moiety to methionine after removing all 3 phosphate groups from ATP (Brosnan and Brosnan, 2006). Three different isoforms of MAT (MAT I, MAT II, and MAT III) are found throughout the body; MAT I and MAT III predominate in the liver, and MAT II is the active isoform in extrahepatic tissues (Finkelstein, 2006). Interestingly, each isoform of MAT is regulated by different mechanisms. MAT I and MAT II activity is decreased when dietary methionine levels are high and are feedback inhibited by SAM, whereas MAT III activity increases in response to increased dietary methionine and is feedback activated by SAM

(Finkelstein, 1998). Additionally, MAT III exhibits a low affinity for methionine, thus enabling more hepatic synthesis of SAM in situations of methionine excess (Finkelstein, 2006; Brosnan et al., 2007).

*S*-adenosylmethionine (SAM) can either be decarboxylated for polyamine synthesis or used by many methyltransferases to form products requiring a methyl group. The former reaction does not utilize a significant amount of SAM (Mudd and Poole, 1975), but methionine is still able to be regenerated from 5'-methylthioadenosine in this pathway (Finkelstein, 1998). Therefore, transmethylation reactions consume a majority of SAM to donate methyl groups to synthesize a variety of important molecules such as creatine, choline, neurotransmitters, DNA, and RNA (Walker, 1979). Following methyl group transfer, the resulting *S*-adenosylhomocysteine (SAH) is reversibly hydrolyzed by *S*-adenosylhomocysteine hydrolase to adenosine and homocysteine (Hoffman et al., 1980; Brosnan et al., 2007). Equilibrium of the hydrolase reaction favors the formation of SAH, a potent inhibitor of the methyltransferases responsible for its synthesis (Hoffman et al., 1980; Finkelstein, 1998; Clarke and Banfield, 2001). The reaction will proceed in the direction of hydrolysis when homocysteine is effectively consumed by remethylation or transsulfuration (Hoffman et al., 1980).

### *Remethylation*

Two methyltransferases are capable of recycling homocysteine back to methionine by employing methyl groups originating from the diet or *de novo* synthesis (Brosnan and Brosnan, 2010). Betaine-homocysteine methyltransferase (BHMT) transfers a methyl group from betaine, a product acquired through diet or choline oxidation, to homocysteine, and is most active in the liver. The kidneys and pancreas also exhibit BHMT activity; however, it is negligible compared

to that of the liver (Finkelstein et al., 1971; Brosnan et al., 2009). Although the liver is the primary site of BHMT activity in most species, this is not the case in ruminants. Activity of BHMT in sheep is higher in the pancreas rather than the liver (Xue and Snoswell, 1985a, 1986a), and pancreatic and hepatic activities are relatively similar in cattle (Lambert et al., 2002). The second enzyme, methyltetrahydrofolate-homocysteine methyltransferase, also known as methionine synthase (MS), is present in all tissues (Finkelstein et al., 1971; Lambert et al., 2002) and utilizes 5-methyltetrahydrofolate (MTHF) to remethylate homocysteine. This methyl group donor is synthesized from one-carbon pools throughout the body when 5,10-methylenetetrahydrofolate is reduced by methylenetetrahydrofolate reductase (Finkelstein, 1998; Stead et al., 2001; Brosnan et al., 2007). Remethylation of homocysteine via MS requires several cofactors. When synthesizing MTHF, tetrahydrofolate may obtain its one-carbon unit from serine by way of serine hydroxymethyltransferase, a vitamin $B_6$-dependent enzyme. Methylenetetrahydrofolate reductase contains flavin adenine dinucleotide (FAD) as a cofactor, and MS requires vitamin $B_{12}$ (methylcobalamin) to convert homocysteine to methionine (Finkelstein, 1998; Brosnan and Brosnan, 2006; Brosnan et al., 2007). Thus, a deficiency in any of these vitamins could disrupt folate or methionine metabolism (Brosnan and Brosnan, 2006).

Although hepatic activity of BHMT and MS are rather similar, MS is the more active methyltransferase in extrahepatic tissues (Finkelstein et al., 1971; Lambert et al., 2002; Brosnan et al., 2009). Activities of both enzymes are increased in situations of low methionine intake, and downregulated when dietary intake of methionine is high and methionine does not need to be conserved (Finkelstein et al., 1988; Finkelstein, 2006). Additionally, SAM inhibits BHMT activity and MTHF synthesis by inactivating MTHF reductase (Finkelstein, 2006; Brosnan et al., 2007). Both BHMT and MS share the characteristic of having a high affinity for homocysteine,

which signifies remethylation is preferred when less homocysteine is being produced from transmethylations reactions (Finkelstein, 1998).

### *Utilization of Excess Methionine*

Metabolism of methionine is primarily carried out by the transsulfuration pathway once methionine requirements for protein deposition and transmethylation reactions have been met. In mammals, transsulfuration activity is only present in the liver, kidney, intestine, and pancreas (Finkelstein et al., 1971; Brosnan and Brosnan, 2006; Brosnan et al., 2007). In cattle, transsulfuration is also known to occur in ruminal tissue (Lambert et al., 2002). High concentrations of SAM will activate cystathionine β-synthase (CBS) (Finkelstein et al., 1975; Finkelstein and Martin, 1984; Finkelstein et al., 1988), an enzyme that combines homocysteine with serine in an irreversible reaction to form cystathionine. From here, cystathionine γ-lyase (cystathionase) hydrolyzes cystathionine to cysteine, α-ketobutyrate, and an ammonium ion (Brosnan and Brosnan, 2006; Brosnan et al., 2007). The enzymes CBS and cystathionase are both dependent on Vitamin $B_6$ (pyridoxal phosphate) as a cofactor for their activity (Finkelstein, 1998; Brosnan and Brosnan, 2006). Cysteine may then be diverted to protein synthesis, further metabolized to glutathione or taurine, or oxidized to sulfate; it cannot be converted back to methionine (Finkelstein, 2006).

High concentrations of methionine are also mitigated by glycine *N*-methyltransferase (GNMT). Excess SAM produced by MAT III stimulates GNMT to methylate glycine to sarcosine (Clarke and Banfield, 2001), a metabolite easily converted back to glycine and 5,10-methylenetetrahydrofolate. Unlike the other SAM-dependent methyltransferases, GNMT is relatively unaffected by SAH, a property enabling it to dispose of large amounts of SAM

(Finkelstein, 1998; Brosnan et al., 2007). Just as enzymes of remethylation share similar characteristics, there are notable commonalities between GNMT and enzymes of the transsulfuration pathway. Low affinities for SAM and homocysteine, respectively, and activation by high cellular concentrations of SAM elucidate their involvement in methionine catabolism (Finkelstein, 1998).

### *Methionine Utilization by Growing Cattle*

Methionine is considered the first-limiting amino acid for growth in cattle (Richardson and Hatfield, 1978) because of the low methionine content of microbial protein (Ørskov, 1982). Transmethylation and transsulfuration reactions compete for available methionine, derived from both dietary and microbial protein sources, for SAM and cysteine production; thus, a methionine deficiency could adversely affect multiple biological processes. Because SAM is known to participate in at least 100 methyl group transfer reactions (Lobley, 1992), it may explain why cattle utilize methionine with less efficiency than other amino acids for growth. Therefore, the role of methionine as a methyl group donor may significantly influence the methionine requirement of growing cattle.

Estimates of methionine requirements for growing cattle have been based on breakpoint models of response factors such as plasma methionine concentration (Williams and Smith, 1974; Fenderson and Bergen, 1975; Titgemeyer et al., 1988), nitrogen retention (Titgemeyer and Merchen, 1990; Campbell et al., 1997; Froidmont et al., 2000), and live weight gain (Wilkerson et al., 1993; Klemesrud et al., 2000a). The first technique proposes that plasma concentrations of an amino acid will remain relatively low and constant until the animal has met their requirement, at which point the concentration will rise (Bergen, 1979). Requirements calculated based on

nitrogen retention and live weight gain are more reasonable, accurate estimates because they reflect animal requirements at a certain level of performance. Studies conducted by Titgemeyer and Merchen (1990) and Campbell et al. (1997) demonstrate the disparity between estimates of methionine requirements based on changes in plasma methionine concentration and nitrogen retention in response to supplemental methionine. Breakpoint analysis of nitrogen retention data revealed a methionine requirement of 11 g/d for growing steers; no definitive breakpoint was observed for plasma methionine due to a maximal nitrogen retention response at the lowest level of methionine supplementation (Titgemeyer and Merchen, 1990). Conversely, Campbell et al. (1997) were able estimate a methionine requirement of 4 g/d from plasma methionine data, a value half of the requirement obtained when estimated from nitrogen retention. It is evident, then, that protein deposition of steers is not optimized at the methionine requirement predicted by levels of plasma methionine.

### *Efficiency of Methionine Utilization*

Proper assessment of efficiency of methionine utilization by growing cattle requires use of a research model designed such that increases in nitrogen retention in response to methionine supplementation can be easily measured. Methionine must be the sole-limiting amino acid, and all other amino acids, as well as energy, must be provided in amounts that meet or exceed animal requirements. Assuming retained nitrogen is equal to protein deposition and 2% of protein deposited is methionine (Ainslie et al., 1993), incremental increases in nitrogen retention can be converted to methionine deposition and divided by the corresponding increase in methionine supply to estimate efficiency of methionine use. The NRC (2016) fails to specifically define how efficiently methionine, or any essential amino acid, is utilized for growth. Rather, it

estimates an efficiency of metabolizable protein utilization for growth as 83.4% - (0.114% × empty body weight, kg) for cattle less than 300 kg in body weight, and as 49.2% for cattle weighing more than 300 kg. The NRC (2016) model allocates the same efficiency to each amino acid, an assumption proved by numerous studies to lead to overestimation of efficiencies of each essential amino acid. Furthermore, research indicates that each amino acid has a different efficiency of utilization for growth dependent on many factors other than body weight. Several nitrogen retention experiments have been conducted to estimate efficiency of methionine utilization for growing cattle; reported values are as low as 11% (Schroeder et al., 2006b) and as high as 66% (Lambert et al., 2004). The large variation of these values can be attributed to differences in nutritional conditions generated in each experiment, such as altering supplies of other amino acids (Campbell et al., 1997; Löest et al., 2002; Lambert et al., 2004; Awawdeh et al., 2006), sources and levels of energy (Schroeder et al. 2006a,b) or ruminal ammonia loads (Awawdeh et al., 2004).

### *Improving Methionine Utilization by Sparing Methionine*

As mentioned earlier in this review, methionine acts as a precursor for cysteine formation when homocysteine enters the transsulfuration pathway. Cysteine is considered a conditionally essential amino acid, meaning its synthesis in the body is contingent upon methionine status (Brosnan and Brosnan, 2006). A dietary cysteine deficiency will divert homocysteine away from remethylation and consequently increase an animal's methionine requirement to support protein deposition (Campbell et al., 1997). Cysteine supplementation in such situations can reduce partitioning of homocysteine to transsulfuration and increase remethylation, which may spare methionine and increase protein deposition (Finkelstein et al., 1986, 1988). This methionine-

sparing effect has not been observed in cattle maintained under presumed cysteine-deficient conditions (Campbell et al., 1997; Löest et al., 2002). Campbell et al. (1997) attributed ineffective sparing of methionine by cysteine supplementation to low methyl group availability for homocysteine remethylation; however, Löest et al. (2002) observed no sparing of methionine when betaine and choline were supplemented as methyl group sources. Lack of a regulatory mechanism on CBS activity in cattle, (Lambert et al., 2002), deficiencies of folate or vitamin $B_{12}$ (Lambert et al., 2004), or the modest role of BHMT in remethylation of homocysteine in ruminants (Xue and Snoswell, 1985a) are presumably why cysteine was unable to spare methionine in these studies.

### *Bioavailability of GAA*

Bioavailability refers to a nutrient's capability of being absorbed and utilized by the body. Inferences on the bioavailability of GAA are primarily based on concentrations of GAA as well as creatine in plasma, urine, and tissues. In general, providing an exogenous source of GAA will elevate plasma GAA and creatine concentrations (Ostojic et al., 2013a,b, 2014; Ardalan et al., 2015, 2016; Tossenberger et al., 2016; DeGroot et al., 2018; He et al., 2018), occurring as soon as 1 hr after administration (Ostojic et al., 2014). Increased plasma GAA concentration is indicative of absorption by the gut, whereas increased plasma creatine concentration demonstrates successful methylation of GAA and transport to creatine-requiring tissues. For example, Tossenberger et al. (2016) supplemented a broiler diet with 0.6% GAA and found increased breast meat creatine concentrations to coincide with increases in plasma creatine concentration. Furthermore, liver creatine concentrations were much lower compared to breast meat, which proves effective movement of creatine from its site of formation to the

muscle. Similar responses in muscle creatine with GAA supplementation to broilers have been observed by Lemme et al. (2007a), Ringel et al. (2007), and Michiels et al. (2012).

Urinary concentrations of GAA and creatine follow the same trend as those of plasma in both monogastrics (Ostojic et al., 2013a, 2014; Tossenberger et al., 2016) and ruminants (Ardalan et al., 2015, 2016). Increases in urinary excretion of these compounds with increasing dietary GAA levels suggest that either not all GAA supplied is being utilized for creatine synthesis (Ostojic et al., 2014), or that the body is responding to GAA excess by synthesizing more creatine and subsequently excreting it, as the highest level of GAA supplementation to broilers resulted in the greatest amount of renal GAA and creatine excretion relative to no supplementation (Tossenberger et al., 2016). Ardalan et al. (2015, 2016) observed this effect with postruminal supplementation of GAA to cattle; however, they detected very low concentrations of GAA in urine and plasma, implying that nearly 100% was used for creatine synthesis (Ardalan et al., 2015). Additionally, the rise in urinary GAA and creatine concentration was lessened (Ardalan et al., 2015) or halted (Ardalan et al., 2016) by methionine supplementation. Methionine provides the methyl group necessary for converting GAA to creatine (Walker, 1979); thus, methyl group availability or methionine status could affect how effectively GAA is utilized. Overall, GAA is readily absorbed by the body; however, the extent to which it is used for creatine formation is dependent upon methyl group status.

### *Methyl Group Metabolism*

Considering SAM serves as the methyl group donor for most methylation reactions (Clarke and Banfield, 2001) and regeneration of methionine requires methyl groups, methionine holds a dominant role in methyl group metabolism. Methyl groups are acquired through the diet

as methionine, choline, and betaine, or can be made through irreversible reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahyrdofolate (Stead et al., 2006; Brosnan and Brosnan, 2007). Choline and betaine can also be synthesized by the body; choline oxidation produces betaine (trimethylglycine), the methyl group donor for resynthesis of methionine via BHMT. Either of the two remaining methyl groups can then be transferred to tetrahydrofolate to form (via reduction of 5,10-methylenetetrahydrofolate) the other methyl donor 5-methyltetrahydrofolate, a substrate for MS. Choline is synthesized *de novo* by the liver as phosphatidylcholine when phosphatidylethanolamine *N*-methyltransferase (PEMT) catalyzes 3 methyl group transfers from SAM to phosphatidylethanolamine (Stead et al., 2006). Although GAMT and GNMT consume a significant number of SAM-derived methyl groups for creatine synthesis and excess methionine disposal, respectively, the PEMT reaction is quantitatively the largest consumer of SAM (Stead et al., 2006; Brosnan et al., 2007).

Partitioning of methionine among methylation reactions and protein synthesis depends on methionine supply (Robinson et al., 2016) and the methyl group demand of a specific methyltransferase (McBreairty et al., 2013). For example, Robinson et al. (2016) observed a 30% decrease and a 60% increase in hepatic creatine and phosphatidylcholine synthesis, respectively, in neonatal piglets fed diets deficient in methionine and methyl group sources; protein synthesis and DNA methylation were unaffected by methionine and methyl group restriction. By using a labeled methionine, they were able to determine that 30% of total methionine methyl groups were used for creatine and phosphatidylcholine synthesis and the remaining 70% were incorporated into protein in methyl-deficient pigs (Robinson et al., 2016). These data demonstrate that protein synthesis is favored above transmethylation reactions when methyl group supply is limited. In contrast, McBreairty et al. (2013) observed a 40% reduction

in incorporation of methionine into hepatic protein in piglets administered GAA, indicating methionine was directed away from protein synthesis in order to meet the high methyl group demand for creatine synthesis.

An animal's methyl group status is also the determinant of how homocysteine will be partitioned between remethylation and transsulfuration. A methyl group deficiency results in hyperhomocysteinemia, a condition characterized by elevated homocysteine levels in the blood (Robinson et al., 2016). Here, homocysteine produced from methyltransferase reactions is unable to be effectively utilized by either remethylation or transsulfuration. The condition can be alleviated by supplementing methyl group sources such as choline, betaine, or methionine (Setoue et al., 2008; Ardalan et al., 2015). Serine has also been demonstrated to reduce plasma homocysteine concentration, either by contributing to the one-carbon pool and increasing methyl group status or by providing additional substrate for the CBS enzyme (Stead et al., 2001; Ohuchi et al., 2008).

### *Methyl Group Metabolism in Ruminants*

Enzymatic activity in methyl group metabolism differs slightly in ruminants than in other species due to low dietary intake of methyl groups and the low levels of methionine in microbial protein (Ørskov, 1982; Snoswell and Xue, 1987). Consequently, the major source of methyl groups must come from 5-MTHF generated by the one-carbon pool (Snoswell and Xue, 1987). Ruminants have developed mechanisms that favor methionine conservation rather than catabolism to ensure methionine levels in the body are maintained to support protein synthesis and methylation of biologically important compounds. Dietary choline, for instance, is essentially unavailable to ruminants because of its extensive degradation by microorganisms in

the rumen (Neill et al., 1979), so hepatic BHMT activity along with choline oxidase is strikingly less than in rat liver (Xue and Snoswell, 1986a). It is reasonable to assume this mechanism is why Löest et al. (2002) were unable to improve protein deposition in growing cattle by supplementing betaine and choline despite observed increases of BHMT activity when these compounds were supplemented to rats (Finkelstein et al., 1983). The low activity of BHMT activity in sheep is compensated for by increases in total body MS activity to support remethylation of homocysteine (Xue and Snoswell, 1985a, Snoswell and Xue, 1987). Lastly, hepatic GNMT activity in ruminants is quite low compared to other enzymes involved in transmethylation reactions in order to prevent methionine catabolism and increase its availability for protein deposition and use in other methyltransferase reactions (Xue and Snoswell, 1986a).

### *GAA Supplementation*

Synthesis of GAA by the AGAT enzyme is the control point of the creatine biosynthetic pathway, being regulated by feedback inhibition of creatine to ensure methionine and arginine are not used to produce creatine in amounts exceeding the animal's requirement. Provision of an exogenous source of GAA will bypass this regulatory step and enhance GAMT activity, which continues to synthesize creatine as long as its substrate is available (Stead et al., 2001). Some research has been conducted on GAA supplementation in humans as a replacement for creatine, a supplement well-known to improve muscle performance during exercise; however, it has not been demonstrated to have any use beyond serving as an alternative to creatine (Ostojic et al., 2013a,b; 2014). For this reason, the discussion will be limited to research conducted on GAA supplementation to livestock.

### *Livestock*

Arginine is an essential amino acid for chicks because they lack enzymes for endogenous production and require a significant amount of this amino acid to support rapid rates of protein deposition (Ball et al., 2007). Supplementation of GAA to poultry diets deficient in arginine has been extensively investigated (Dilger et al., 2013; DeGroot et al., 2018) as it has potential to spare arginine, the fifth limiting amino acid for growth in corn-soybean based broiler diets (Waguespack et al., 2009). Exogenous GAA increases creatine production and reduces the need for *de novo* synthesis of GAA, thereby increasing the availability of arginine to be used for protein synthesis (Tossenberger et al., 2016). Creatine supplementation also exerts an arginine-sparing effect in the same manner. Direct comparisons between GAA or creatine-containing diets observed similar improvements in broiler performance (Lemme et al., 2007b; Ringel et al., 2007; Michiels et al., 2012; Dilger et al., 2013), likely as a result of GAA and creatine being equally effective at increasing tissue creatine stores (Stead et al., 2001; Ringel et al., 2007; McBreairty et al., 2015). However, GAA is favored over creatine as a supplement because is a more stable and cost-friendly compound (Baker, 2009).

Further evidence supports enhancement of creatine content in muscle by supplemental GAA. Michiels et al. (2012) and Tossenberger et al. (2016) observed increases in breast muscle creatine concentration in broilers with increasing levels of dietary GAA in a dose-dependent manner. DeGroot et al. (2018) also observed significant increases in total muscle creatine concentration when supplementing GAA to broilers from d 8 to 22 posthatch. Greater muscle creatine concentrations are of benefit to young animals as they may increase the ability of cells to adequately regenerate ATP necessary to support new tissue growth (Walker, 1979; DeGroot et al., 2018). In fact, an increase in breast meat ATP concentrations with GAA supplementation

has been noted in broilers (Lemme et al., 2007a). Conversely, He et al. (2018) noted increases in serum and muscle ATP concentrations can occur independently of increases in muscle creatine concentration in response to supplemental GAA and still improve growth performance of pigs. Creatine synthesized from exogenous GAA was likely stored in muscle tissue as phosphocreatine, the molecule responsible for regenerating ATP; however, this is mere speculation as changes in phosphocreatine concentration were not directly measured in this study (He et al., 2018).

Improvements in weight gain (Lemme et al., 2007b; Ringel et al., 2007; Michiels et al., 2012; Heger et al., 2014), feed conversion (Lemme et al., 2007a,b; Ringel et al., 2007; Michiels et al., 2012; Mousavi et al., 2013; Heger et al., 2014; DeGroot et al., 2018), and breast meat yield (Lemme et al., 2007b; Michiels et al., 2012; Heger et al., 2014) have also been observed in poultry fed corn and soybean meal-based diets. Swine performance data in the literature provides mixed results; Wang et al. (2012) observed no effect of GAA supplementation on average daily gain, average daily feed intake, or feed conversion when pigs were supplemented up to 2.0 g GAA/kg diet, whereas pigs in the study of He et al. (2018) maximized feed conversion at 0.3 g/kg of supplemental GAA in addition to a tendency and a quadratic tendency for hot carcass weight and lean muscle percentages to increase with increasing amounts of GAA up to 1.2 g/kg, respectively.

Only recently has research been conducted on GAA supplementation to cattle, all of which involve postruminal supplementation via continuous infusion in conjunction with supplemental methionine as a methyl donor to examine its effects on creatine status (Ardalan et al., 2015, 2016). In the first study, Ardalan et al. (2015) assigned dairy heifers receiving a corn-based diet to treatments of 0, 10, 20, 30, or 40 g/d of GAA with either 0 or 12 g/d of

supplemental methionine. Improved creatine status was implied by increases in plasma and urinary creatine concentrations at all levels of GAA with provision of 12 g/d methionine and up to 30 g/d GAA in the absence of methionine (Ardalan et al., 2015). Using N retention as a measure of creatine status in a methionine-deficient model, Ardalan et al. (2016) noted tendencies in N retention to increase in growing steers only when GAA was supplemented along with methionine; GAA supplementation alone did not improve N retention. Moreover, the arginine-sparing effect of GAA as described in poultry was demonstrated in dairy heifers by increases in plasma arginine concentrations in response to GAA supplementation (Ardalan et al., 2015). These data demonstrate that GAA supplementation is an effective way to increase creatine synthesis in cattle, providing adequate methyl groups from methionine or other sources are available.

### *Interrelationship between GAA, Creatine, and Methionine*

Methionine and creatine metabolism are linked to one another in that SAM furnishes the methyl group transferred to GAA to synthesize creatine. Creatine synthesis is a large consumer of SAM-derived methyl groups that are permanently lost when creatine is converted to creatinine and excreted in the urine (Walker, 1979). Consequently, this diminishes the size of the body's methionine pool and stimulates MS to resynthesize methionine from homocysteine (Finkelstein et al., 1971; Stead et al., 2001). Methylation of GAA is not a regulated process and will thus proceed at rates corresponding to GAA availability; however, it is limited by methyl group supply (da Silva et al., 2009). In cattle, Ardalan et al. (2015) noted plasma and urinary creatine concentrations increased with provision of supplemental GAA up to 30 g/d but plateaued at 40 g/d in the absence of supplemental methionine; 12 g/d methionine ameliorated this effect.

Moreover, broilers fed methionine-deficient diets supplemented with GAA had lower feed intakes, poor feed conversion, and less weight gain compared to broilers receiving a diet containing GAA and an adequate amount of methionine (Lemme et al., 2010b). Breast meat yield was also lower for broilers when GAA was provided without a methyl group source (Lemme et al., 2010b), indicating that methyl group demand was large enough to divert methionine from protein synthesis (McBreairty et al., 2013).

## *Conclusion*

Methionine is an essential amino acid for mammals and is first-limiting for growing cattle. It can be utilized for protein synthesis or as a methyl donor in the form of SAM for many methylation reactions. Creatine is important for maintaining energy homeostasis within tissues and is synthesized via methyl group transfer from SAM to GAA. Supplementation of GAA to growing animals has resulted in improved performance by increasing protein synthesis or ATP concentration in the body via creatine to support new tissue growth. Preliminary work with postruminal GAA supplementation to growing cattle indicates potential to improve protein deposition providing sufficient methyl groups are available; however, further research of GAA supplementation to growing cattle is required to justify its use as feed additive in a production scenario.

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# Chapter 2 - Relative bioavailability of guanidinoacetic acid delivered ruminally or abomasally to cattle

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## Abstract

This study assessed relative bioavailability of guanidinoacetic acid (GAA) in cattle. Seven ruminally cannulated Holstein steers (initial body weight of 280 ± 14 kg) were used in an experiment with a 5 × 5 Latin square design. Treatments were: control (no GAA, water infusion), ruminal infusion of 10 or 20 g/d GAA, and abomasal infusion of 10 or 20 g/d GAA, with all infusions delivered continuously. Periods were 7 d in length, and on d 7 blood and urine samples were collected to determine concentrations of GAA and its associated metabolites. Plasma creatine concentrations increased ($P < 0.01$) linearly with GAA provision to the abomasum and tended to increase ($P = 0.06$) when GAA was infused ruminally. Urinary creatine concentrations increased linearly with increasing amounts of GAA infused in the abomasum ($P < 0.01$) and the rumen ($P < 0.05$). There were no significant effects of GAA provision to either the abomasum or rumen on plasma or urinary concentrations of GAA. Plasma creatinine concentrations were not affected by GAA supplementation in the abomasum or rumen. Urinary creatinine concentrations decreased when GAA was infused abomasally ($P < 0.05$). Because plasma and urinary creatine concentrations yielded the statistically strongest linear responses, they were selected as the primary response criteria for quantifying ruminal escape of GAA. Estimates for ruminal escape of GAA calculated by slope-ratio methodology based on plasma creatine and urinary creatine concentrations were 47% and 49%, respectively. Ruminally provided GAA was about half as effective as abomasally infused GAA in elevating plasma and urinary concentrations of creatine.

# Introduction

Creatine is a molecule in vertebrate muscle tissues that serves an important role in cell energy metabolism. In its phosphorylated form, creatine can resynthesize ATP in the tissue to replenish energy stores, particularly in those with high energy demands (Wyss and Kaddurah-Daouk, 2000). Approximately 1.7% of total body creatine is lost daily when it undergoes an irreversible, nonenzymatic reaction to form creatinine, which is subsequently excreted in the urine (Walker, 1979; Wyss and Kaddurah-Daouk, 2000). Therefore, there is a need for creatine to constantly be replaced. Significant amounts of creatine may also be lost in the urine, and this will similarly increase the need for creatine synthesis (Crim et al., 1975; Ardalan et al., 2015; Tossenberger et al., 2016).

Creatine can be acquired through diet as well as *de novo* synthesis in the liver by methylation of guanidinoacetic acid (GAA) (Wyss and Kaddurah-Daouk, 2000). Because dietary sources of creatine can only be found in products of animal origin (Stead et al., 2006), livestock consuming plant-based diets rely heavily on *de novo* synthesis of this nutrient (Snoswell and Xue, 1987). Furthermore, growing animals require more creatine to support new tissue growth (Walker, 1979); therefore, growing animals fed creatine-deficient diets may benefit from either creatine or GAA supplementation. Provision of either compound can increase creatine stores in tissue (Ringel et al., 2007; McBreairty et al., 2015) and consequently increase phosphocreatine synthesis in tissues experiencing extreme fluxes in energy demand (i.e. skeletal muscle) (Walker, 1979).

Breast muscle creatine concentrations in broilers have been noted to increase with increasing levels of dietary GAA (Michiels et al., 2012; Tossenberger et al., 2016). DeGroot et al. (2018) observed significant increases in total muscle creatine concentration when

supplementing GAA to broilers from d 8 to 22 posthatch, suggesting GAA may increase the ability of cells to adequately regenerate ATP. In contrast, no effect of GAA supplementation to pigs was observed on muscle creatine concentrations (He et al., 2018); however, the authors did observe an increase in serum and muscle ATP concentrations, suggesting GAA may improve growth performance of pigs by increasing ATP reserves.

Because GAA is a more stable and less expensive compound than creatine (Baker, 2009), GAA supplementation to livestock has been evaluated as an alternative to creatine. Improvements in weight gain (Lemme et al., 2007b; Ringel et al., 2007; Michiels et al., 2012; Heger et al., 2014), feed conversion (Lemme et al., 2007a,b; Ringel et al., 2007; Michiels et al., 2012; Mousavi et al., 2013; Heger et al., 2014; DeGroot et al., 2018), and breast meat yield (Lemme et al., 2007b; Michiels et al., 2012; Heger et al., 2014) have also been observed in poultry fed corn and soybean meal-based diets. Swine performance data in the literature provides mixed results; Wang et al. (2012) observed no effect of GAA supplementation on body weight gain, feed intake, or gain efficiency, whereas pigs in the study of He et al. (2018) showed improvements in feed conversion as well as hot carcass weight and lean muscle percentages.

Some research is available on GAA supplementation to cattle, all of which involved postruminal supplementation via continuous infusion in Met-deficient models (Ardalan et al., 2015, 2016). Increased plasma and urinary creatine concentrations in dairy heifers supplemented with GAA (with or without supplemental Met) implied enhancement of creatine supply (Ardalan et al., 2015). Ardalan et al. (2016) discovered a tendency for an improvement in N retention when GAA was supplemented along with Met to growing steers, but there was no effect of GAA supplementation alone on N retention.

No research has been conducted to assess bioavailability of dietary GAA in cattle, and it is unknown to what extent GAA is degraded in the rumen. Our objective was to determine if, and to what extent, GAA is degraded in the rumen to determine the necessity of utilizing a rumen-protected GAA supplement as means of providing GAA to cattle.

# Materials and Methods

## *Animals and Treatments*

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

Seven ruminally cannulated Holstein steers (average initial body weight of 280 ± 14 kg) were used in an experiment using a 5 × 5 Latin square design composed of 5 periods with 5 treatments. The 2 additional steers received a treatment sequence identical to 2 steers in the Latin square. Treatment sequences in the 5 × 5 Latin square were balanced for carryover effects. Treatments included: control (no GAA, water infusion), ruminal infusion of 10 g/d GAA, ruminal infusion of 20 g/d GAA, abomasal infusion of 10 g/d GAA, and abomasal infusion of 20 g/d GAA. Flexible Tygon polyvinylchloride tubing (2.38 mm i.d.; Fisher Scientific, Pittsburgh, PA) infusion lines were placed in the rumen and abomasum of each steer through the ruminal cannula prior to the study. Rubber flanges (7 cm in diameter) were placed near the end of the abomasal infusion lines to assure they remained fixed in the abomasum. Treatments were infused continuously by a peristaltic pump (Ismatec ISM444A-230V BVP Standard; Cole-Parmer Instrument Company, Vernon Hills, IL) at a rate of 2.77 mL/min, with total solution amounts infused to each location being 4 kg/d.

Periods were 7 d in length, allowing for 6 d of treatment adaptation and 1 d for sample collection. Steers were fitted with neck collars in tie-stalls in a temperature-controlled room (20°C) with ad libitum access to water. Feed was provided in amounts slightly less than ad libitum intake in 2 equal portions at 12-h intervals (0700 and 1900 h). Intakes of individual steers were determined in the diet adaptation period prior to the study. A corn-alfalfa based diet

was fed (16.3% CP, 33.5% NDF, 22.8% ADF; Table 1); chemical composition of dietary ingredients is presented in Table 2.

GAA treatments were prepared by dissolving 91 g GAA in 17,450 g water containing 218.4 g 9.25% (wt/wt) HCl. Once GAA was dissolved, 5% (wt/wt) NaOH was added to raise the solution pH between 6 and 7 to match ruminal pH. Lastly, the solution was brought to a final weight of 18,200 g with water to create a 5 g/kg GAA solution. For steers receiving 10 g/d GAA, 1 kg of solution was added to a bottle and brought to 2 kg with water. For steers receiving 20 g/d GAA, 2 kg of solution was added to a bottle. Bottles were changed every 12 h (0700 and 1900 h), such that each steer received 4 kg of infusate daily.

### *Sample Collection*

Representative samples of the diet were collected from d 5 to 7 of each period and frozen at -20°C. Orts, if present, were removed at 1900 h prior to feeding d 5 to 7 and frozen at -20°C. Feed ingredients were sampled at the time of diet mixing and stored at room temperature (22°C). Blood (10 mL) was collected 5.5 h after the morning feeding on d 7 via jugular venipuncture into vacutainer tubes (Becton, Dickinson and Company; Franklin Lakes, NJ) containing sodium heparin. Samples were promptly placed on ice and centrifuged at 1,000 × *g* at 4°C for 20 min to isolate plasma, which was then frozen at -20°C for later analysis of plasma GAA, creatine, and creatinine concentration. Spot samples of urine were collected during spontaneous urination at 4 time points throughout the day (0500, 1100, 1700, and 2300 h), using a collection cup attached to the end of a wire handle, and immediately frozen at -20°C for later analysis of urinary GAA, creatine, and creatinine concentrations. Subsamples of urine (1 mL from each time point) were composited by steer within period prior to analysis.

### *Laboratory Analyses*

Diet samples and orts were composited within period. Feed and orts subsamples were dried at 55°C for 24 h in a forced-air oven, air-equilibrated for 24 h, and ground in a Wiley Mill (Thomas Scientific USA, Swedesboro, NJ) to pass through a 1-mm screen. All feed and orts samples were dried in a forced-air oven at 105°C overnight to determine DM, then subsequently analyzed for OM by ashing in a muffle oven for 8 h at 450°C. Feed and orts samples were analyzed for N with a TruMac N Analyzer (Leco Corporation, St. Joseph, MI), and CP was calculated by multiplying N × 6.25. NDF and ADF contents of feed samples were determined using an ANKOM Fiber Analyzer (ANKOM Technology, Macedon, NY).

Plasma and urine samples were analyzed by reversed-phase HPLC for creatine, GAA, and creatinine concentrations using a modification of the procedures of Shingfield and Offer (1999). Analyses were performed on a Shimadzu LC-10Ai liquid chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD) outfitted with a quaternary pump (FCV-10AL; Shimadzu Scientific Instruments, Inc., Columbia, MD), Acutect 500 UV/VIS detector (Thermo Fisher Scientific Inc., Waltham, MA) and SpectraSYSTEM AS 1000 autosampler equipped with a 5-µL loop for sample injection (Thermo Fisher Scientific Inc., Waltham, MA). A single channel data system with PeakSimple chromatography software (Model 333; SRI Instruments, Menlo Park, CA) was used for data acquisition. Compounds were separated on a reversed-phase 5-µm Discovery BIO Wide Pore $C_{18}$ column (250 × 4.6 mm i.d., Supelco 568223-U; Sigma-Aldrich, St. Louis, MO) with a 5-µm Discovery BIO Wide Pore $C_{18}$ guard column (20 × 4.6 mm i.d.; Sigma-Aldrich, St. Louis, MO), and the eluted compounds were measured by absorbance at 200 nm.

Plasma was prepared by adding 10% (wt/vol) sulfosalicylic acid (SSA) 1:1 to deproteinize samples, then vortexed and frozen at -20°C overnight. Thawed samples were centrifuged at 13,000 × *g* for 10 min at room temperature (22°C), and the supernatant was filtered through a 0.22-µm membrane syringe filter (Thermo Fisher Scientific Inc., Waltham, MA) into an HPLC vial for injection. Urine samples were diluted 10:1 with a diluent that was prepared by dissolving 0.09 g of monobasic ammonium phosphate and 0.101 g of sodium 1-heptane sulfonic acid in 100 mL of double-deionized $H_2O$ (pH adjusted to 2.2 with 85% phosphoric acid). Samples were then vortexed and filtered through a 0.22-µm membrane syringe filter (Thermo Fisher Scientific Inc., Waltham, MA) into an HPLC vial for injection. The aqueous mobile phase contained 0.09% ammonium monophosphate (wt/vol), 0.101% (wt/vol) sodium 1-heptane sulfonic acid, 70 µL triethylamine (vol/vol) and 3.5% methanol (vol/vol). The final pH of the mobile phase was adjusted to 2.85 with 50% phosphoric acid, and it was filtered through a 0.22-µm membrane filter (Thermo Fisher Scientific Inc., Waltham, MA) and degassed prior to use. The column temperature was maintained at room temperature (22°C). The elution was performed with a flow rate of 0.8 mL/min for 14 min, then increased to 1.2 mL/min for 16 min. Column was then washed with 100% methanol for 10 min at a flow rate of 1.2 mL/min, after which the column was re-equilibrated with the initial eluent before the injection of another sample. Total analysis time per sample was 65 min.

### *Statistical Analysis*

One observation for the control treatment in period 1 was excluded because the steer had erratic feed intake and significant digesta losses through the cannula on sampling day. Data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Inst. Inc., Cary, NC). The

model for plasma and urinary responses included period and treatment as fixed effects and steer as a random effect. LSMEANS were used to calculate treatment means. Polynomial contrasts were used to evaluate linear and quadratic effects of ruminal and abomasal infusions. Linear regression models for evaluating ruminal and abomasal infusion of GAA were also used, and these models included GAA levels nested within infusion site (rumen, abomasum, or none) as regression variables, period as a fixed effect, and steer as a random effect. The intercept was calculated by setting the average effect of period equal to zero. The ESTIMATE statement was used to compare linear regression coefficients of nested terms (abomasal vs. ruminal). Ruminal degradation of GAA was determined by dividing the slope of the linear response to ruminal infusion of GAA by the slope of the linear response to abomasal infusion of GAA.

## Results and Discussion

Dry matter intake (DMI) decreased quadratically ($P < 0.05$) when GAA was infused into the abomasum; however, differences among treatments were small and deemed unlikely to influence responses to the treatments. Plasma and urinary concentrations of GAA, creatine, and creatinine for each route of infusion are presented in Table 3. Plasma creatine concentrations increased ($P < 0.01$) linearly with GAA provision to the abomasum and tended to increase ($P = 0.06$) when GAA was infused ruminally. Similar results have been demonstrated for cattle receiving abomasal infusions of GAA (Ardalan et al., 2015, 2016). Ardalan et al. (2015) observed increases in plasma creatine in a dose-dependent manner when up to 30 g/d GAA was supplied postruminally to dairy heifers, with no increases detected at 40 g/d supplemental GAA. Elevated plasma creatine concentrations indicate GAA was converted to creatine by our steers.

Urinary creatine concentrations increased linearly with increasing amounts of GAA infused in the abomasum ($P < 0.01$) and the rumen ($P < 0.05$), although there was also a quadratic response for the ruminal route because the 10 g/d supplementation amount failed to elevate urinary creatine. Again, these results agree with previous research conducted on GAA supplementation to cattle (Ardalan et al., 2015, 2016) and are similar to those observed in broilers (Tossenberger et al. 2016). Across all treatments, urinary creatine concentrations were lower than urinary creatinine concentrations and ranged from 44 to 83% of creatinine excretion; thus, urinary creatine excretion would represent a significant loss of N to the steers.

There were no significant effects of GAA provision to either the abomasum or rumen on plasma or urinary concentrations of GAA; we attribute this more to variation in measurement than to a lack of biological response. Quantification of GAA in plasma was particularly imprecise due to the low concentrations of GAA present. Plasma GAA concentration, however, was numerically increased by 20 g/d GAA supplementation. Even though we observed no statistically significant increase in plasma GAA concentrations, the magnitude of our increase is similar to the significant increase observed by Ardalan et al. (2016, unpublished data) when considering dosage levels of GAA scaled to BW. In the current study, a 45% increase in plasma GAA concentrations occurred at the highest level of GAA supplementation (0.08 g/kg BW), whereas the study of Ardalan et al. (2016, unpublished data) demonstrated a 40% increase for the same level of GAA supplementation. Our results and those of Ardalan et al. (2016) in growing steers are not in agreement with those of Ardalan et al. (2015) for dairy heifers, as a 150% increase in plasma GAA concentration was observed at a dose of 0.06 g GAA/kg BW. The heifers of Ardalan et al. (2015; 520 kg) were larger than the steers in our study, so it is possible that exponential scaling of BW may be needed to standardize treatment dosages. In response to

GAA supplementation, DeGroot et al. (2018) observed significant increases in serum GAA concentrations in broilers, whereas He et al. (2018) only observed a tendency for serum GAA concentration to increase in pigs. McBreairty et al. (2015) also noted increases in plasma GAA concentration with increasing GAA supplementation to pigs; however, the standard deviation for those values was quite large.

On average, only a 9% increase in urinary GAA concentration occurred when 20 g/d of GAA was provided to our steers. Urinary GAA concentrations were increased by 82% with GAA supplementation up to 30 g/d when no Met was provided to dairy heifers (Ardalan et al., 2015), and urinary excretion of GAA was increased 35% when GAA was supplemented to growing steers in the absence of supplemental Met (Ardalan et al., 2016, unpublished data). Considering heifers in the study of Ardalan et al. (2015) weighed 520 kg and the steers in the study of Ardalan et al. (2016, unpublished data) were of similar weight to ours, it is possible that GAA is utilized more effectively in the earlier stages of growth, or, as noted above, BW may need to be scaled exponentially when considering GAA dosages.

Plasma creatinine concentrations were not affected by GAA supplementation in the abomasum ($P = 0.20$) or rumen ($P = 0.56$). Ardalan et al. (2016) also observed no effect of abomasal GAA administration to cattle on plasma creatinine concentrations, whereas increases in plasma creatinine in broilers have been observed (Tossenberger et al., 2016). Additionally, serum creatinine concentrations were not affected by dietary GAA provision to pigs (He et al., 2018). Urinary creatinine concentrations decreased when GAA was infused abomasally ($P < 0.05$). This was an unexpected result because urinary excretion of creatinine tended to be increased by GAA supplementation previously (Ardalan et al., 2016). It is possible that urinary volume was greater for steers receiving abomasal infusions of GAA, which could account for

changes in creatinine concentrations even if the amount of creatinine excretion was not affected; we have no means to assess this hypothesis. Tossenberger et al. (2016) suggested urinary creatinine excretion increases as a result of excess creatine production, which might suggest the amount of creatine synthesized by our steers was not metabolically excessive. Another possibility is that the body pool size of creatine was not changed enough by the treatments to impact creatinine production, as Crim et al. (1975) noted men who had greater creatine retention following a 10-d period of creatine supplementation had greater levels of urinary creatinine excretion.

Ruminal escape estimates for GAA are presented in Table 4. Supplemental GAA could be available through absorption in the small intestine or across the ruminal wall; therefore, the calculated ruminal escape values would include GAA made available through either route. Ruminal escape of GAA based on plasma creatine concentrations was 47%, whereas the estimate based on urinary creatine concentrations was 49%. Estimates of ruminal escape based on plasma and urinary GAA are also provided, although these values are subject to great uncertainty because the responses were neither significant nor strikingly linear. Urinary concentrations of GAA and creatine divided by urinary creatinine concentrations (to correct for variation in urinary volume) yielded linear responses to GAA infusions, and ruminal escape estimates for GAA were 36% from the urinary GAA/creatinine data and 52% for the urinary creatine/creatinine data. It should be noted that the correction for urine volume using creatinine may be questionable in this experiment because creatinine production might be altered by treatment, although no increases in plasma or urinary creatinine concentrations were detected. The escape value varied with each response variable measured, but because plasma and urinary creatine concentrations yielded the

statistically strongest linear responses, they were selected as the primary response criteria for quantifying ruminal escape of GAA.

## Conclusion

GAA is partially degraded in the rumen. Infusion of GAA into the abomasum led to significantly greater increases in the concentrations of plasma and urinary creatine than when GAA was infused ruminally, suggesting that GAA is more bioavailable when administered postruminally. Ruminal escape estimates for GAA, using plasma and urinary creatine concentrations as response criteria, were 47% and 49%, respectively. If used as a feed additive for ruminants, it could be assumed that levels of GAA in the diet could be approximately doubled to achieve the desired amount the animal is intended to receive, or a rumen-protected GAA could be created. The relative costs of GAA and of creating a rumen protected product, as well as the effectiveness of the protective mechanism, will dictate the benefits of a rumen-protected GAA product.

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**Table 1. Composition of diet fed to steers**

| Item<br>Ingredient | % of dietary DM |
|---|---|
| Alfalfa hay, chopped | 46.5 |
| Corn, dry-rolled | 34.6 |
| Soybean meal, solvent extracted | 8.0 |
| Cottonseed, whole | 4.2 |
| Cane molasses | 4.0 |
| Calcium carbonate | 1.0 |
| Sodium bicarbonate | 0.5 |
| Monobasic calcium phosphate | 0.4 |
| Trace mineral salt[1] | 0.4 |
| Magnesium oxide | 0.2 |
| Vitamin and mineral premix[2] | 0.2 |
| Chemical composition | |
| DM | 86.0 ± 0.34 |
| OM | 91.7 ± 0.47 |
| CP | 16.3 ± 0.55 |
| NDF[3] | 33.5 ± 2.63 |
| ADF[4] | 22.8 ± 2.23 |

[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): 13 ppm Zn, 7 ppm Mn, 4.5 ppm Cu, 0.9 ppm Co (Zn, Mn, Cu, and Co provided as 4-Plex; Zinpro Corporation, Eden Prairie, MN), 3,300 IU vitamin A, 2,100 IU vitamin D, 35 IU vitamin E, and 0.06 mg/kg Se.
[3]Analysis conducted with α-amylase; not corrected for ash.
[4]Sequential ADF analysis; not corrected for ash.

**Table 2. Chemical composition of feed ingredients**

| | Ingredient | | | | | |
|---|---|---|---|---|---|---|
| Chemical composition | Alfalfa | Dry-rolled corn | Soybean meal | Whole cottonseed | Cane molasses | Supplement[1] |
| DM, % | 88.1 | 85.3 | 87.1 | 89.6 | 67.8 | 94.0 |
| OM, % of DM | 90.6 | 98.2 | 91.9 | 95.7 | 81.8 | 9.5 |
| CP, % of DM | 18.6 | 9.6 | 48.8 | 20.3 | 11.2 | --- |
| NDF[2], % of DM | 48.7 | 11.5 | 7.8 | 51.6 | --- | --- |
| ADF[3], % of DM | 36.0 | 2.9 | 4.3 | 35.3 | --- | --- |

[1]Contained calcium carbonate, sodium bicarbonate, monobasic calcium phosphate, trace mineral salt (defined in Table 1), magnesium oxide, and vitamin/mineral premix (defined in Table 1).
[2]Analysis conducted with α-amylase; not corrected for ash.
[3]Sequential ADF analysis; not corrected for ash.

**Table 3. Effects of abomasal or ruminal infusion of guanidinoacetic acid (GAA) on dry matter intake and plasma and urinary concentrations of GAA, creatine, and creatinine**

| | | | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Abomasal GAA | | Ruminal GAA | | | Abomasal | | Ruminal | |
| Item | Control | 10 g/d | 20 g/d | 10 g/d | 20 g/d | SEM[1] | Linear | Quadratic | Linear | Quadratic |
| Dry matter intake, kg/d | 7.44 | 7.10 | 7.39 | 7.19 | 7.15 | 0.17 | 0.77 | 0.04 | 0.12 | 0.47 |
| Plasma, mg/L | | | | | | | | | | |
| GAA | 0.67 | 0.57 | 0.92 | 0.66 | 0.97 | 0.15 | 0.28 | 0.23 | 0.21 | 0.43 |
| Creatine | 24.4 | 26.6 | 27.9 | 24.8 | 26.3 | 0.90 | <0.01 | 0.60 | 0.06 | 0.52 |
| Creatinine | 6.35 | 6.43 | 6.55 | 6.60 | 6.44 | 0.39 | 0.20 | 0.86 | 0.56 | 0.13 |
| Urine, mg/L | | | | | | | | | | |
| GAA | 60 | 69 | 69 | 53 | 62 | 4.4 | 0.18 | 0.47 | 0.80 | 0.14 |
| Creatine | 340 | 514 | 541 | 300 | 486 | 46 | <0.01 | 0.17 | 0.03 | 0.05 |
| Creatinine | 774 | 809 | 649 | 700 | 762 | 73 | 0.02 | 0.03 | 0.82 | 0.11 |
| GAA/creatinine | 0.077 | 0.090 | 0.114 | 0.080 | 0.088 | 0.011 | <0.001 | 0.37 | 0.19 | 0.67 |
| Creatine/creatinine | 0.46 | 0.67 | 0.88 | 0.49 | 0.69 | 0.094 | <0.001 | 0.99 | <0.01 | 0.20 |

[1]SEM for n = 7. n = 6 for Control, n = 7 for all other treatments.

**Table 4. Common intercept linear regression model to estimate ruminal escape of GAA**

| | | Slope (unit change/g treatment GAA) | | | |
|---|---|---|---|---|---|
| | Intercept[1] | Abomasal | Ruminal | *P*-value[2] | Ruminal escape[3] |
| Plasma | | | | | |
| Creatine, mg/L | 24.3 | 0.189±0.044 | 0.089±0.045 | 0.02 | 0.47 |
| GAA[4], mg/L | 0.54 | 0.0156±0.011 | 0.0193±0.011 | 0.70 | 1.24 |
| Urinary | | | | | |
| Creatine, mg/L | 324 | 12.5±3.3 | 6.1±3.4 | 0.05 | 0.49 |
| Creatine/creatinine, mg/g | 425 | 23.1±3.7 | 12.0±3.8 | <0.01 | 0.52 |
| GAA[4], mg/L | 59 | 0.63±0.31 | 0.02±0.32 | 0.04 | 0.03 |
| GAA/creatinine, mg/g | 74 | 1.88±0.36 | 0.68±0.37 | <0.01 | 0.36 |

[1]Calculated with the average effect of period set to 0.
[2]*P*-value for comparing abomasal vs. ruminal slopes.
[3]Calculated as ruminal slope divided by abomasal slope.
[4]Statistics do not suggest this linear model is a good fit; values are provided for completeness.

# Chapter 3 - Effect of guanidinoacetic acid supplementation on nitrogen retention and methionine methyl group flux in growing steers fed corn-based diets

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## Abstract

Six ruminally cannulated Holstein steers (256 ± 14 kg) were used in a 6 × 6 Latin square design to assess effects of guanidinoacetic acid (GAA) supplementation on N retention and methionine (Met) methyl group flux in growing cattle fed corn-based diets. Factorial treatments were 2 levels of Met (0 or 5 g/d) and 3 levels of GAA (0, 7.5, or 15 g/d) delivered by continuous abomasal infusion. Periods were 10 d in length and included 6 d of treatment adaptation, 3 d for total fecal and urine collections, and 1 d for blood samples and flux measures. Urinary N linearly increased ($P < 0.01$) with GAA supplementation and decreased ($P < 0.05$) with Met supplementation. Fecal N excretion was unaffected ($P \geq 0.42$) by treatment. Retained N tended to decrease ($P = 0.12$) with increasing amounts of GAA and increased ($P < 0.01$) with Met supplementation. Flux of Met methyl groups tended to increase linearly ($P = 0.10$) with GAA provision and increased ($P < 0.01$) with Met supplementation. A GAA × Met interaction ($P = 0.10$) was observed, as GAA supplementation linearly increased Met flux when Met was supplemented, but GAA supplementation did not change Met flux when Met was not supplemented. Plasma concentrations and urinary excretion of creatine increased linearly ($P < 0.05$ and $P = 0.06$, respectively) when GAA was supplemented. There was a linear increase ($P < 0.01$) in urinary GAA excretion with GAA supplementation. Plasma and urinary creatinine concentrations were not affected ($P \geq 0.17$) by treatment. No treatment differences ($P \geq 0.13$) were observed for plasma haptoglobin concentrations. Plasma urea N linearly increased ($P < 0.05$) with GAA supplementation. Concentrations of Met and taurine increased ($P < 0.05$) when Met was supplemented. A quadratic increase ($P < 0.05$) in plasma arginine was observed in response to GAA supplementation, with arginine being greatest at the intermediate level of supplemental GAA. No differences in plasma total amino acid concentrations were observed (*P*

≥ 0.29) among treatments. The increase in N retention when supplemental Met was provided suggests Met was limiting in the corn-based diet. Supplementation of GAA alone or with Met as a methyl donor did not increase N retention in growing steers, perhaps because creatine production was favored over protein deposition as a use for Met.

# Introduction

Creatine is an important compound for maintaining energy homeostasis in vertebrate muscle tissues, as it can receive and subsequently donate a phosphate group to resynthesize ATP when energy demand is high (Wyss and Kaddurah-Daouk, 2000). Creatine is also essential to support new tissue growth; thus, the creatine requirement of growing animals is greater than that of adults (Walker, 1979). Dietary creatine is only found in products of animal origin (Stead et al., 2006), so cattle must synthesize most of their own creatine because of the plant-based nature of their diet (Snoswell and Xue, 1987). Creatine is produced in the liver when the methyl group of *S*-adenosylmethionine (SAM), derived from methionine (Met), is transferred to guanidinoacetic acid (GAA) (Wyss and Kaddurah-Daouk, 2000). Methylation of GAA is not a regulated process, so creatine synthesis will occur as GAA becomes available (Stead et al., 2001). This process is a large consumer of methyl groups (Brosnan et al., 2007) and may lead to a methyl group (Met) deficiency in some situations.

Recently GAA has been investigated as a supplement to livestock, primarily in the poultry industry. Several studies conducted with broilers fed corn-soybean meal diets have noted improvements in weight gain (Lemme et al., 2007b; Ringel et al., 2007; Michiels et al., 2012; Heger et al., 2014), feed conversion (Lemme et al., 2007a,b; Ringel et al., 2007; Michiels et al., 2012; Mousavi et al., 2013; Heger et al., 2014; DeGroot et al., 2018), and breast meat yield (Lemme et al., 2007b; Michiels et al., 2012; Heger et al., 2014). Moreover, the response in performance from GAA supplementation has been demonstrated to be greater with provision of Met in the diet. Lemme et al. (2010b) observed increases in weight gain, feed intake, feed conversion, and breast meat yield when broiler diets supplemented with GAA were not limited by Met supply. A study conducted in our lab with growing steers in a Met-deficient model

indicated protein deposition, measured as N retention, tended to increase when GAA was supplemented along with Met but not when GAA was supplemented alone (Ardalan et al., 2016). Those steers were maintained in a Met-deficiency model and not in a scenario representative of normal production conditions (Ardalan et al., 2016). The objective of this study was to evaluate potential benefits of GAA and Met supplementation to growing cattle fed a corn-based diet that might be used in a production scenario. We hypothesized that the corn-based diet would provide adequate amounts of Met and increases in N retention in response to GAA supplementation would occur independent of Met supplementation.

# Materials and Methods

## *Animals and Treatments*

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

Six ruminally cannulated Holstein steers (average initial body weight of 256 ± 14 kg) were used in a 6 × 6 Latin square design with 2 × 3 factorial arrangement of treatments and with treatment sequence balanced for carryover effects. Treatments were all infused abomasally and included 3 levels of GAA (0, 7.5, or 15 g/d) with either 0 or 5 g/d of methionine (Met).

Flexible Tygon polyvinylchloride tubing (2.38 mm i.d.; Fisher Scientific, Pittsburgh, PA) infusion lines were placed in the abomasum of each steer through the ruminal cannula prior to the study. Rubber flanges (7 cm in diameter) were placed near the end of the abomasal infusion lines to assure they remained fixed in the abomasum. Treatments were infused continuously by a peristaltic pump (Ismatec ISM444A-230V BVP Standard; Cole-Parmer Instrument Company, Vernon Hills, IL) at a rate of 2.77 mL/min, with total solution amounts infused being 4 kg/d.

Periods were 10 d in length, allowing for 6 d of treatment adaptation, 3 d for urine and fecal collection, and 1 d for measuring whole body Met methyl group flux. Steers were housed in a temperature-controlled room (20°C) in metabolism crates for total fecal and urine collection. Feed (5.3 kg/d DM) was provided in 2 equal portions at 12-h intervals (0700 and 1900 h), and each steer had ad libitum access to water. Diet adaptation occurred 3 wk prior to the study while steers were housed in tie-stalls. The diet (Table 5) contained 12% CP and represented a diet that might be fed to cattle in a normal production setting. Chemical composition of dietary ingredients is presented in Table 6.

GAA treatment solution was prepared as described in Chapter 2. The Met treatment solution was prepared by dissolving 50 g of methionine into 2 L of deionized $H_2O$. Both treatment solutions were refrigerated until time of use. Infusion bottles were prepared by adding GAA and Met solutions in amounts corresponding to treatment level, and final weights were made to 4 kg with water. For the treatment providing no GAA or Met, water alone was infused. Bottles were changed every 12 h (0700 and 1900 h).

### *Sample Collection*

Representative samples of the diet were collected from d 6 to 8 of each period and frozen at -20°C. Orts, if present, were removed at 1900 h prior to feeding and frozen at -20°C on d 6 to 8. Feed ingredients were sampled at the time of diet mixing and stored at room temperature (20°C). Total fecal and urine collections occurred from d 7 to d 9 of each period, and collections were made daily with weights measured at the time of collection. Urine was collected in buckets containing 900 mL of 10% (wt/wt) $H_2SO_4$ for capture of ammonia. Daily outputs of feces and urine were mixed thoroughly and representative samples were collected and frozen at -20°C. Representative fecal and urine samples were composited by steer within period as a constant percentage of the total weight and frozen at -20°C until time of analysis.

On d 10, whole body Met flux was measured. Blood (20 mL) was collected via jugular venipuncture into vacutainer tubes (Becton, Dickinson and Company; Franklin Lakes, NJ) containing sodium heparin 1 h after the morning feeding and prior to placement of an indwelling jugular catheter (MILACATH #LA1420; MILA International, Inc., Florence, KY) for infusion of methyl-$^2H_3$-L-Met. Samples were promptly put on ice and centrifuged at 1,000 × *g* at 4°C for 20 min to isolate plasma, which was then frozen at -20°C for later analysis of background

enrichment of methyl-$^{2}H_3$-L-Met. Background blood samples were also used for analysis of plasma GAA, creatine, creatinine, amino acid, urea, and haptoglobin concentrations.

The methyl-$^{2}H_3$-L-Met solution was prepared under sterile conditions in a laminar-flow hood by mixing methyl-$^{2}H_3$-L-Met (98%, Cambridge Isotope Laboratories, Inc., Andover, MA) with sterile saline (0.9% NaCl) to make a 0.4% (wt/vol) solution. Solution was filtered through a 0.22-µm membrane syringe filter (Millex-GV; MilliporeSigma, Burlington, MA) into a 500-mL sterile glass bottle with a crimped rubber septum and stored at 4°C until time of use.

Prior to jugular catheter placement 1.5 h after feeding, 2 mL of 2% (wt/vol) lidocaine HCl was provided subcutaneously over the jugular vein for local anesthesia. After lidocaine administration, sterility was maintained for the remainder of the catheterization process. Once the catheter was placed, a 30-in extension (Hospira, Inc., Lake Forest, IL) was attached and filled with heparinized saline to prevent blood clots from forming in the lines. Neck sweats were positioned around the catheter site as a precautionary measure against accidental removal by the animal. Once all steers had catheters in place, a sterile infusion line of flexible Tygon polyvinylchloride tubing (1.5 mm i.d.; Fisher Scientific, Pittsburgh, PA) was attached to the catheter extension. A priming dose of 0.04 g methyl-$^{2}H_3$-L-Met was administered through a 0.22-µm membrane syringe filter (Millex-GV; MilliporeSigma, Burlington, MA). The filter remained connected to the end of the infusion line and was attached to a syringe on a programmable syringe pump (BS-9000-6 Multi-Phaser; Braintree Scientific, Inc., Braintree, MA). Constant infusion of the methyl-$^{2}H_3$-L-Met solution occurred from 1100 h to 1500 h at a rate of 10 mL/h (0.04 g methyl-$^{2}H_3$-L-Met/h). Blood (20 mL) was collected via jugular venipuncture at the end of the infusion period into vacutainer tubes (Becton, Dickinson and Company; Franklin Lakes, NJ) containing sodium heparin. Samples were promptly put on ice

and centrifuged at 1,000 × *g* at 4°C for 20 min to isolate plasma, which was then frozen at -20°C for later analysis of plasma Met enrichment. Blood collected for both background and enrichment measures was collected from the jugular vein contralateral to the catheter.

### *Laboratory Analyses*

Diet samples were composited within period and a subsample was taken from each period. Orts were composited by steer within period and subsampled. Feed and orts subsamples were dried at 55°C for 24 h in a forced-air oven, air-equilibrated for 24 h, and ground in a Wiley Mill (Thomas Scientific USA, Swedesboro, NJ) to pass through a 1-mm screen. All feed, fecal, and orts samples were dried in a forced-air oven at 105°C overnight to determine DM, then subsequently analyzed for OM by ashing in a muffle oven for 8 h at 450°C. Feed, orts, wet fecal, and urine samples were analyzed for N by a TruMac N analyzer (Leco Corporation, St. Joseph, MI); CP was calculated by multiplying N × 6.25. NDF and ADF contents of feed samples were determined using an ANKOM Fiber Analyzer (ANKOM Technology, Macedon, NY).

Plasma and urine samples were prepared and analyzed by reversed-phase HPLC for creatine, GAA, and creatinine concentrations using the same system, chromatographic conditions, and mobile phase described in Chapter 2. Plasma urea nitrogen (PUN) concentrations were measured with an AutoAnalyzer (Bran + Luebbe Analyzer 3, SEAL Analytical, Mequon, WI) according to the methods of Marsh et al. (1965). Plasma haptoglobin concentrations were measured using the procedure of Cooke and Arthington (2013) as a measure of inflammation.

For AA analysis, plasma samples were deproteinized by adding equal parts of 10% (wt/vol) sulfosalicylic acid (SSA) containing 1 mM norleucine as an internal standard. Plasma AA were measured on a lithium cation exchange column (4 mm × 100 mm, Pickering Laboratories Inc., Mountain View, CA) using lithium eluents and post-column derivatization with *o*-phthalaldehyde (OPA). Auto sampler was equipped with a 10-µL loop for sample injection and held at a constant temperature of 4°C, and measurements were made using a fluorescence detector (HP 1046; Hewlett Packard, 46 Alto, CA). The column was held at 32°C for the initial 13 min, then increased to 50°C for 49 min, increased to 71°C for 39 min, and decreased to 32°C for the final 24 min. Total analysis time per sample was 125 min. Eluents were A: 1700-1125 Lithium Eluent (Pickering Laboratories Inc.); B: Li365 Lithium (Pickering Laboratories Inc.); C: Li375 Lithium (Pickering Laboratories Inc.); D: RG003 Lithium (Pickering Laboratories Inc.). A gradient elution was made by the following: 0-15 min, 100% A; 15-27 min, 100% A – 40% A/60% B; 27-45 min, 40% A/60% B – 100% B; 45-60 min, 100% B; 60-60.1 min, 100% B – 100% C; 60.1-85 min, 100% C; 85-85.1 min, 100% C – 70% C/30% D; 85.1-105 min, 70% C/30% D; 105-105.1 min, 70% C/30% D – 100% A; 105.1-125 min, 100% A, with a flow rate of 0.4 mL/min. OPA diluent (Pickering Laboratories Inc.) was mixed with column effluent at a rate of 0.4 mL/min and allowed to react for 10 s at 21°C prior to fluorescence detection with excitation at 330 nm and emission at 465 nm (HP 1046A Fluorescence Detector; Agilent Technologies, Santa Clara, CA).

Plasma samples used for enrichment analysis were deproteinized with 10% (wt/vol) SSA, placed on ice for 30 min, centrifuged at 20,000 × *g* for 15 min at 10°C, and supernatant was collected. Cation exchange columns were prepared with 2 mL of Dowex 50WX8 200 hydrogen form mesh resin (Sigma #217506; Sigma-Aldrich, St. Louis, MO) and washed 4 times; twice

with 10 mL of 1 M HCl followed by 2 washes with 10 mL of deionized water. Supernatant (5 mL) was applied to columns and columns were washed twice with 4 mL of 0.5 M HCl, followed by 2 washes with 4 mL of deionized water. Once columns had been washed with 4 mL of 0.25 M ammonium hydroxide 3 times, samples were eluted with 2 washes (2 mL each) of 2 M ammonium hydroxide and collected into 2-mL microcentrifuge tubes. Sample eluents were then dried under nitrogen at 60°C, and 400 µL of MTBSTFA reagent (Sigma #394882, Sigma-Aldrich, St. Louis, MO) was added to tubes. Tubes were incubated at 80°C for 20 min to create *t*-butyldimethylsilyl derivatives. Enrichments of the methyl-$^2H_3$-L-Met in plasma were measured by an HP 5890 Series II gas chromatograph equipped with an HP 5972 Series mass selective detector for electron impact ionization analysis of the *t*-butyldimethylsilyl derivative separated on a HP-5MS column (60 m × 0.25 mm × 0.25 µm; Agilent Technologies, Santa Clara, CA). Fragment ions were monitored at *m*/*z* of 320 (unlabeled) and 323 (methyl-$^2H_3$-L-Met). Initial oven temperature was 125°C, increased at 2.5°C/min to 175°C, held for 5 min, then increased at 30°C/min to 250°C and held for 10 min. Inlet temperature was 275°C, and the helium flow rate was 1 mL/min. Sample injection volume was 1 µL.

### *Statistical Analysis*

Data were analyzed using the MIXED procedure of SAS (version 9.4, SAS Inst. Inc., Cary, NC). The model included period and treatment (GAA, Met, and GAA × Met) as fixed effects and steer as a random effect. The LSMEANS statement was used to calculate treatment means. Orthogonal contrasts were used to evaluate linear and quadratic effects of GAA and its interactions with Met. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

Data for 1 steer was removed from period 1 and from 2 steers in period 3 due to significant digesta losses through the ruminal cannula on sampling days.

### *Calculations*

Nitrogen retention was calculated as the difference of total N intake (feed and infused N) and total N output (fecal and urinary N). Both dry matter and organic matter digestibility calculations only considered feed as part of intake; i.e., intake via abomasal infusion was excluded. Met flux (Q) was calculated as:

Q = ((infusion rate of label/enrichment in plasma) – infusion rate of label).

Methionine deposition was calculated under the assumptions that protein deposition equals retained N × 6.25 and 2% of deposited protein is Met (Ainslie et al., 1993). Efficiency of supplemental Met utilization was determined by dividing the increase in Met deposition by the increase in Met supply (5 g/d).

## Results and Discussion

### *Nutrient Intake and Digestibility*

Estimated endogenous creatine production of the size of steers used in the current study was 7.5 g/d; therefore, the intermediate level of GAA was designed to meet the animals' need for GAA synthesis and the highest level of GAA was meant to exceed endogenous GAA production. The 15 g/d GAA should therefore have greatly increased creatine production. The 7.5 g/d of GAA was designed to only increase creatine supply to the extent that endogenous production of GAA was not inhibited by the supplement. Total GAA availability would include endogenous

production plus exogenous supply. Using urinary creatine and creatinine excretion values from the control treatment to represent endogenous GAA production, steers excreted 18.1 mmol/d and 63.5 mmol/d, respectively. The 7.5 g/d GAA treatment provided 64.1 mmol/d of GAA, so it matched closely with the requirement of endogenous GAA needed to replace creatinine excreted in the urine.

The effects of GAA and Met supplementation on N retention and digestibility are presented in Table 7. Dry matter and organic matter intake were not affected by GAA supplementation ($P = 0.57$). Apparent DM and OM digestibilities were 73.9 and 74.5%, respectively, and neither were affected by GAA supplementation ($P \geq 0.45$). No interactions between GAA and Met were observed for feed intake or diet digestibilities.

### *Nitrogen Retention*

Total N intakes increased, as expected, due to N being infused as part of the treatment structure. There was no effect of GAA or Met supplementation on fecal N excretion ($P \geq 0.24$). Methionine supplementation decreased ($P < 0.05$) urinary N excretion by an average of 3 g/d, resulting in an increase of 6 g/d of retained N ($P < 0.01$); this indicates a Met deficiency was present for steers fed our corn-based diet. This was unexpected and unintentional because diets high in ground corn are often first-limiting in lysine rather than Met (Burris et al., 1976; Hill et al., 1980; Titgemeyer et al., 1988).

The utilization efficiency of supplemental Met, calculated as the increase in Met deposition divided by the amount of supplemental Met, was 14%, a value much lower than the 54% efficiency calculated by the NASEM (2016) equation. This value is similar to efficiencies reported by Froidmont et al. (2000) and Lambert et al. (2002), but lower than reported values of

24%, 33%, and 43% by Campbell et al. (1997), Titgemeyer and Merchen (1990), and Löest et al. (2002), respectively. The poor efficiency of Met utilization for protein deposition in this study was likely a result of exceeding our steers' Met requirement with 5 g/d of supplemental Met; to what extent it was exceeded is unknown.

Urinary N excretion increased linearly ($P < 0.01$) when GAA was supplemented. Supplemental GAA numerically decreased ($P = 0.12$) retained N regardless of Met supplementation. In contrast, Ardalan et al. (2016) observed no change in N retention when GAA was supplemented to steers extremely deficient in Met, and a tendency ($P = 0.10$) for GAA to increase N retention was observed when 6 g/d Met was provided to steers.

### *Methionine Flux*

In our experiment, Met flux was measured using Met labeled on the methyl group. In this case, flux (Q) is defined as movement of Met methyl groups into or out of the free Met pool in the body. Methionine enters the pool via the diet, protein degradation (PD), and *de novo* synthesis when homocysteine is remethylated by either Met synthase (MS) or betaine-homocysteine methyltransferase (BHMT); methionine leaves the pool when it is utilized for protein synthesis (PS) or for transmethylation reactions with SAM as the methyl group donor. These relationships can be defined as Q = PS + transmethylation reactions = PD + dietary intake + *de novo* synthesis (Storch et al., 1988). Methyl group transfers are the principle part of Met catabolism when measuring flux with a methyl-labeled Met because the methyl group is removed from the labeled Met when SAM serves as the donor in methylation reactions. Although we did not measure protein synthesis and degradation directly, the changes in response to treatment can be estimated from changes in Met deposition. Assuming a 30% incremental

efficiency in the retention of whole-body protein synthesis (Harris et al., 1992), each 0.3 unit increase in Met deposited as protein results in a 1.0 unit increase in Met used for protein synthesis and a 0.7 unit increase in Met released from protein degradation.

***Effects of methionine.*** Methionine supplementation increased ($P < 0.01$) Met flux. When Met was supplemented in the absence of GAA, Met flux increased 1.99 mmol/h. The change in Met deposition between the treatments was 0.27 mmol Met/h (7.7 g retained N/d × 6.25 g protein/g N × 0.02 g methionine/g protein (Ainslie et al., 1993) × 1 mmol Met/0.149 g Met × 1 d/24 h), and the change in Met intake was 5 g/d (1.40 mmol/h). The 0.27 mmol/h increase in Met deposition was calculated to result from increases of 0.90 mmol/h in protein synthesis and 0.63 mmol/h in protein degradation. By using the flux equation described above, a 0.04 mmol/h decrease in *de novo* Met synthesis and 1.09 mmol/h increase in transmethylation reactions were calculated. From these calculations, supplementation of Met increased Met methyl flux as a result of increases in the supplementation itself, increased protein synthesis and degradation (associated with increased protein deposition), and increased transmethylation reactions involving SAM. Notably, the increased use of Met for transmethylation reactions accounted for 55% of the increased Met flux, whereas the increase in protein synthesis accounted for 45% of the increased Met flux. Although more Met was used for transmethylation reactions when Met was supplemented, there was no increase in *de novo* synthesis of Met, suggesting that the increased transmethylation reactions led to increases in Met disposal via transsulfuration.

***Effects of GAA.*** Supplemental GAA tended to increase ($P = 0.10$) Met flux, but the increase was only observed when Met was supplemented (GAA × Met interaction, $P = 0.10$; Table 7). Methylation of GAA is not a regulated process and occurs as long as GAA and adequate methyl groups are available (da Silva et al., 2009; Ardalan et al., 2015). Theoretically,

GAA supplementation would increase methyl group consumption, thereby increasing methyl group flux, and produce more homocysteine that could subsequently be remethylated to Met. Methylation of GAA also might divert Met from being utilized for other transmethylation reactions and protein synthesis. For example, McBreairty et al. (2013, 2015) demonstrated higher rates of Met methyl group incorporation into creatine than phosphatidylcholine or protein when GAA was administered to piglets via venous infusion or diet.

No change in Met flux was observed with supplementation of up to 15 g/d GAA (5.34 mmol/h) in the absence of Met. However, there was a 0.094 mmol/h reduction in Met retention (2.7 g N) that resulted from a 0.31 mmol/h and 0.22 mmol/h decrease in protein synthesis and protein degradation, respectively. Because neither flux nor Met intake changed in response to GAA in the absence of supplemental Met, the increase in transmethylation reactions matched the decrease in protein synthesis, and increases in *de novo* Met synthesis matched the decrease in protein degradation. The changes in transmethylation reactions and in *de novo* Met synthesis were small, but the slightly greater loss of Met through transmethylation reactions than through *de novo* synthesis does account for the minor reduction in Met retention. Additionally, the observed 0.31 mmol/h increase in transmethylation reactions was strikingly less than the 5.34 mmol/h of GAA that was supplemented. Even if GAA supplementation eliminated endogenous GAA production, the increase in transmethylation reactions would still be inadequate to support the additional needs for methylation of the supplemental GAA. Thus, other transmethylation reactions in the body were probably decreased in response to GAA supplementation, and choline synthesis, as the greatest consumer of methyl groups (Stead et al., 2006; Brosnan et al., 2007), was likely the methylation reaction reduced the most.

In the presence of 5 g/d of supplemental Met, 15 g/d GAA led to an increase in methyl group flux of 4.8 mmol/h and a 0.164 mmol/h reduction in Met retention. This reduction in Met retention was calculated to result from respective decreases of 0.55 mmol/h and 0.38 mmol/h in protein synthesis and protein degradation. Within the Met supplemented steers, there was no change in Met intake, and estimated increases of transmethylation reactions and *de novo* Met synthesis were 4.80 and 4.63 mmol/h, respectively, in response to 15 g/d supplemental GAA. The large increase in methyl group flux matched reasonably well with the 5.34 mmol/h of GAA that was provided. Moreover, it appears that, under conditions of Met adequacy, the increase in homocysteine production as a result of increased GAA methylation was largely used for *de novo* synthesis of Met rather than transsulfuration. In previous work with a model using steers starkly deficient in Met, increases in methyl group flux occurred when steers were provided up to 15 g/d of GAA; however, when steers received 6 g/d of Met supplementation (and were still marginally limited by Met supply), Met methyl group flux was unaffected by GAA supplementation (Ardalan et al., 2016, unpublished data). These results are somewhat opposite our observations, although it is possible that GAA can increase Met methyl flux in situations where Met availability is strikingly deficient or when Met is not limiting, with the response being muted in situations where Met is marginally limiting (Met-supplemented steers of Ardalan et al. (2016) and Met-unsupplemented steers in this study).

The similar negative effects of GAA on N retention when Met was or was not supplemented, despite strikingly different responses to GAA in terms of methylation reactions and *de novo* Met synthesis, indicates that effects on Met and/or Met methyl group metabolism may not be the mechanism by which GAA reduced N balance. Responses to GAA in the presence of Met supplementation amounts greater than the 5 g/d used in our experiment might

shed light on the relationship between effects of GAA on methylation reactions and on protein deposition.

### *Plasma and Urinary Metabolites*

***Effects of methionine.*** Supplemental Met had no effect on plasma and urinary concentrations of GAA, creatine, and creatinine ($P \geq 0.18$). Conversely, Ardalan et al. (2016) observed a decrease in plasma creatine concentrations when Met was delivered in conjunction with GAA to the abomasum in growing steers, but that was in a model with steers that were starkly limited by Met availability. Plasma Met and taurine concentrations increased ($P < 0.01$) with Met supplementation. The increase in circulating Met provides further evidence that 5 g/d of supplemental Met exceeded the steers' Met requirement (Bergen, 1979). Taurine concentrations have been observed to increase when Met is provided to steers in amounts above their requirement because of increased production of cysteine from Met (Titgemeyer and Merchen, 1990); however, cysteine production requires serine. A typical response to Met supplementation of Met-deficient cattle, which was not observed in our study, is a decrease in plasma serine concentrations (Titgemeyer and Merchen, 1990; Campbell et al. 1996, 1997; Lambert et al., 2002; Löest et al. 2002; Awawdeh et al., 2004, 2006; Schroeder et al., 2006a,b). Serine is a substrate for cystathionine β-synthase (CBS), an enzyme in the transsulfuration pathway that converts homocysteine to cystathionine. Rats fed diets with excess Met had high levels of CBS activity (Finkelstein and Martin, 1984; Finkelstein et al., 1988), so a decrease in serine levels in plasma might indicate more homocysteine being diverted towards transsulfuration. However, Lambert et al. (2002) did not observe a change in hepatic CBS activity in steers supplemented with 10 g/d of Met. The fact that plasma serine concentrations

were relatively unchanged by Met supplementation in our study suggests remethylation of homocysteine was favored over cysteine formation via the transsulfuration pathway and fits well with the increase in Met flux that was observed in response to Met supplementation (Table 7). Increases in plasma taurine concentrations and decreases in N retention could also imply that some cysteine was not utilized for protein deposition and instead further metabolized to taurine. Another consideration related to the plasma serine data is that our steers were likely not as deficient in Met as were the steers in other studies where Met supplementation reduced plasma serine.

***Effects of GAA.*** With the exception of plasma ornithine concentrations, no interactions were observed between GAA and Met for plasma concentrations ($P \geq 0.13$) or urinary excretions ($P \geq 0.17$) of any of the measured metabolites (Tables 8 and 9). Plasma urea concentrations increased ($P < 0.05$) in steers supplemented with GAA, perhaps reflecting the increased N intake as well as the numerically decreased protein deposition (Table 9). No differences in plasma haptoglobin concentrations were detected in response to GAA supplementation (Table 9). As expected, plasma creatine concentrations increased ($P < 0.05$) and urinary creatine excretion tended to increase ($P = 0.06$) with GAA supplementation. Averaged across all of the treatments, renal reabsorption of creatine averaged 90% with no differences among treatments (data not shown). Urinary GAA excretion increased ($P < 0.01$) when GAA was supplemented, but no significant increase in plasma GAA concentrations were observed.

Previous research with postruminal supplementation of GAA to cattle has also noted increases in plasma concentrations and urinary excretions of creatine (Ardalan et al., 2015, 2016); however, in growing steers urinary creatine excretion only increased in response to GAA supplementation when Met was not provided (Ardalan et al., 2016). Additionally, GAA

supplementation to livestock has resulted in elevated plasma creatine concentrations in broilers (Tossenberger et al., 2016; DeGroot et al., 2018) and piglets (McBreairty et al., 2015) and urinary creatine excretion in broilers (Tossenberger et al., 2016). Our response in urinary GAA agrees with the response observed by Ardalan et al. (2015) in dairy heifers and by Tossenberger et al. (2016) in broilers supplemented with GAA. Plasma GAA concentrations we observed differ from those observed in growing steers in the study of Ardalan et al. (2016), where plasma GAA concentrations significantly increased with postruminal infusion of GAA. Responses in plasma GAA concentrations seem to differ among species, as DeGroot et al. (2018) observed significant increases in serum GAA concentrations in broilers when GAA was incorporated at 0.12% of the diet, and He et al. (2018) only observed a tendency for serum GAA concentrations to increase in pigs at 0.09% dietary GAA. McBreairty et al. (2015) also noted increases in plasma GAA concentrations with increasing GAA supplementation to pigs; however, standard deviation for these values was quite large.

Plasma and urinary concentrations of creatinine were not affected by GAA supplementation, with the average amount of creatinine excreted across treatments being about 7.2 g/d. Creatinine is formed from creatine in an irreversible nonenzymatic reaction and is excreted in urine at a constant rate of 1.7% of the body's creatine pool per day (Wyss and Kaddurah-Daouk, 2000), so even though *de novo* creatine production seemed to increase in response to GAA supplementation in our steers, it appears that total muscle mass (i.e., creatine pool size) has more influence over creatinine production than short-term changes in creatine production. Ardalan et al. (2016) also observed no effect of abomasal GAA administration to cattle on plasma creatinine concentrations, and serum creatinine concentrations in growing pigs are also not altered by dietary GAA provision (He et al., 2018). In contrast, increases in plasma

creatinine levels in broilers fed GAA have been observed (Tossenberger et al., 2016). Numeric increases in urinary excretion of creatinine by GAA supplementation to cattle have previously been observed (Ardalan et al., 2016, unpublished data); this effect has been demonstrated in poultry as well (Tossenberger et al., 2016). Tossenberger et al. (2016) suggested urinary creatinine excretion increases as a result of excess creatine production, so it is possible that metabolically excessive amounts of creatine were not synthesized by steers in this experiment.

Quadratic responses in plasma citrulline ($P = 0.10$) and arginine ($P < 0.05$) concentrations were observed and were greatest at 7.5 g/d of GAA supplementation. A linear GAA × Met interaction ($P = 0.10$) was detected for plasma ornithine concentrations because of its tendency to increase in response to increasing doses of GAA when Met was provided and tendency to decrease without Met supplementation. Arginine is a metabolic precursor to GAA, and Ardalan et al. (2015) observed an increase in plasma arginine concentrations when GAA was supplemented to dairy heifers, indicating an exogenous source of GAA has an arginine-sparing effect. The urea cycle contains arginine, citrulline, and ornithine as metabolic intermediates, and it is interesting that their plasma response patterns were similar. Ornithine is also a by-product of the arginine:glycine amidinotransferase (AGAT) reaction (Brosnan and Brosnan, 2010). Therefore, arginine utilization for *de novo* GAA synthesis appears to have been suppressed to some extent by GAA supplementation.

## Conclusion

Previous work from our lab suggested that GAA may improve N retention when steers were not starkly deficient in Met. We expected that our corn-based diet would provide an adequate amount of Met to the steers; however, the increase in N retention in response to Met

supplementation illustrates that a deficiency was present. Postruminal supplementation of GAA did not improve N retention under our experimental conditions whether Met was deficient or adequate, and actually led to numeric decreases in N retention. When Met was not supplemented, Met use for total methylation reactions was not increased by GAA supplementation, suggesting that methylation of GAA to creatine occurred at the expense of other methylation reactions, most likely choline synthesis. In contrast, in the presence of adequate Met supply, GAA supplementation increased Met methyl group flux such that GAA methylation to creatine did not come at the expense of other methylation reactions. Our data suggest that supplementation of GAA with or without 5 g/d Met did not improve N retention in growing steers fed a corn-based diet.

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**Table 5. Composition of diet fed to steers**

| Item | % of dietary DM |
|---|---|
| Ingredient | |
| Corn, dry-rolled | 75.6 |
| Alfalfa hay, chopped | 12.7 |
| Soybean meal, solvent extracted | 6.2 |
| Cane molasses | 4.2 |
| Limestone | 1.0 |
| Trace mineral salt[1] | 0.3 |
| Vitamin and mineral premix[2] | 0.1 |
| Chemical composition | |
| DM | 88.1 ± 0.59 |
| OM | 94.5 ± 0.76 |
| CP | 12.0 ± 0.33 |
| NDF[3] | 16.9 ± 1.15 |
| ADF[4] | 8.2 ± 0.54 |

[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; not corrected for ash.
[4]Sequential ADF analysis; not corrected for ash.

**Table 6. Chemical composition of feed ingredients**

| | Ingredient | | | | |
|---|---|---|---|---|---|
| Chemical composition | Corn, dry-rolled | Alfalfa, chopped | Soybean meal | Cane molasses | Supplement[1] |
| DM, % | 87.3 | 92.5 | 89.8 | 66.6 | 99.9 |
| OM, % of DM | 97.9 | 93.3 | 92.0 | 80.6 | 6.8 |
| CP, % of DM | 9.1 | 12.1 | 48.8 | 9.0 | --- |
| NDF[2], % of DM | 10.0 | 58.6 | 8.5 | --- | --- |
| ADF[3], % of DM | 2.3 | 41.0 | 5.0 | --- | --- |

[1]Contained limestone, trace mineral salt (defined in Table 1), and vitamin/mineral premix (defined in Table 1).
[2]Analysis conducted with α-amylase; not corrected for ash.
[3]Sequential ADF analysis; not corrected for ash.

**Table 7. Effect of postruminal GAA and Met supplementation on diet digestibility, nitrogen balance, and methionine flux of growing steers**

| | Met, g/d | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 5 | | | | | | | | |
| | GAA, g/d | | | | | | | *P*-value | | | | |
| Item | 0 | 7.5 | 15 | 0 | 7.5 | 15 | SEM[1] | Met | Linear GAA | Quadratic GAA | Linear GAA × Met | Quadratic GAA × Met |
| *n* | 6 | 5 | 6 | 5 | 5 | 6 | | | | | | |
| Intake, kg/d[2] | | | | | | | | | | | | |
| DM | 5.26 | 5.28 | 5.29 | 5.29 | 5.29 | 5.29 | 0.014 | 0.27 | 0.31 | 0.76 | 0.27 | 0.76 |
| OM | 4.97 | 4.99 | 5.00 | 5.00 | 5.00 | 5.00 | 0.013 | 0.27 | 0.31 | 0.75 | 0.27 | 0.75 |
| Apparent digestibility, % | | | | | | | | | | | | |
| DM | 72.2 | 74.7 | 75.7 | 73.3 | 72.9 | 74.7 | 2.42 | 0.75 | 0.23 | 0.92 | 0.61 | 0.64 |
| OM | 72.6 | 75.4 | 76.4 | 73.6 | 73.6 | 75.1 | 2.48 | 0.70 | 0.21 | 0.96 | 0.59 | 0.66 |
| N, g/d | | | | | | | | | | | | |
| Feed | 100.9 | 101.4 | 101.5 | 101.5 | 101.5 | 101.5 | 0.28 | 0.26 | 0.31 | 0.79 | 0.27 | 0.79 |
| Infused | 0.0 | 2.7 | 5.4 | 0.5 | 3.2 | 5.8 | | | | | | |
| Intake[3] | 100.9 | 104.0 | 106.9 | 102.0 | 104.7 | 107.4 | 0.28 | <0.01 | <0.01 | 0.80 | 0.29 | 0.79 |
| Urinary | 25.5 | 29.4 | 34.5 | 22.3 | 27.2 | 29.9 | 2.20 | <0.05 | <0.01 | 0.89 | 0.64 | 0.56 |
| Fecal | 36.9 | 37.6 | 36.6 | 33.6 | 36.4 | 36.0 | 1.78 | 0.24 | 0.56 | 0.43 | 0.42 | 0.80 |
| Retained | 38.5 | 37.1 | 35.8 | 46.2 | 41.0 | 41.5 | 2.60 | <0.01 | 0.12 | 0.49 | 0.66 | 0.52 |
| Met flux, mmol/h[3] | 15.6 | 15.4 | 15.6 | 17.6 | 19.1 | 21.9 | 1.26 | <0.01 | 0.10 | 0.70 | 0.10 | 0.86 |

[1]Average SEM across all treatments.
[2]Excludes abomasal infusions.
[3]Feed N + Infused N.

**Table 8. Effect of postruminal GAA and Met supplementation on plasma concentrations and urinary excretions of GAA, creatine, and creatinine**

| | Met, g/d | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 5 | | | | | | | | |
| | GAA, g/d | | | | | | | *P*-value | | | | |
| Item | 0 | 7.5 | 15 | 0 | 7.5 | 15 | SEM[1] | Met | Linear GAA | Quadratic GAA | Linear GAA × Met | Quadratic GAA × Met |
| *n* | 6 | 5 | 6 | 5 | 5 | 6 | | | | | | |
| Plasma, mg/L | | | | | | | | | | | | |
| Creatine | 22.1 | 24.8 | 23.7 | 19.4 | 22.5 | 26.3 | 1.88 | 0.59 | 0.03 | 0.63 | 0.16 | 0.50 |
| Creatinine | 6.12 | 7.05 | 6.20 | 6.25 | 6.63 | 7.26 | 0.57 | 0.52 | 0.27 | 0.40 | 0.34 | 0.27 |
| GAA | 0.82 | 0.99 | 0.99 | 0.85 | 0.81 | 0.88 | 0.09 | 0.21 | 0.25 | 0.82 | 0.43 | 0.38 |
| Urine, g/d | | | | | | | | | | | | |
| Creatine | 2.37 | 3.12 | 2.95 | 2.01 | 2.17 | 2.97 | 0.54 | 0.18 | 0.06 | 0.85 | 0.63 | 0.28 |
| Creatinine | 7.18 | 7.58 | 6.92 | 6.89 | 6.70 | 7.99 | 0.52 | 0.93 | 0.39 | 0.82 | 0.17 | 0.17 |
| GAA | 0.20 | 0.44 | 0.67 | 0.23 | 0.26 | 0.51 | 0.11 | 0.19 | <0.01 | 0.56 | 0.32 | 0.55 |

[1]Average SEM across all treatments.

**Table 9. Effect of postruminal GAA and Met supplementation on plasma haptoglobin, urea, and amino acid concentrations of growing steers**

| | Met, g/d | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | 5 | | | | | | | | |
| | GAA, g/d | | | | | | | *P*-value | | | | |
| Item | 0 | 7.5 | 15 | 0 | 7.5 | 15 | SEM[1] | Met | Linear GAA | Quadratic GAA | Linear GAA × Met | Quadratic GAA × Met |
| *n* | 6 | 5 | 6 | 5 | 5 | 6 | | | | | | |
| Haptoglobin, µg/mL | 886 | 683 | 671 | 681 | 861 | 686 | 94 | 0.96 | 0.27 | 0.63 | 0.25 | 0.13 |
| Urea, m*M* | 1.34 | 1.56 | 1.67 | 1.07 | 1.55 | 1.34 | 0.16 | 0.08 | 0.04 | 0.12 | 0.81 | 0.25 |
| AA, µ*M* | | | | | | | | | | | | |
| Met | 27.9 | 33.1 | 29.3 | 37.1 | 38.9 | 35.9 | 3.02 | <0.01 | 0.96 | 0.14 | 0.60 | 0.62 |
| Tau | 49.3 | 41.9 | 44.4 | 59.0 | 56.9 | 55.6 | 3.97 | <0.01 | 0.29 | 0.44 | 0.85 | 0.53 |
| Ser | 93.2 | 91.0 | 87.0 | 96.5 | 91.3 | 98.8 | 7.50 | 0.33 | 0.76 | 0.64 | 0.50 | 0.53 |
| Gly | 247.1 | 259.7 | 268.7 | 261.4 | 251.5 | 275.6 | 18.38 | 0.76 | 0.30 | 0.63 | 0.83 | 0.55 |
| Arg | 121.6 | 144.9 | 121.4 | 118.1 | 135.6 | 127.9 | 9.87 | 0.75 | 0.55 | 0.02 | 0.53 | 0.47 |
| Thr | 108.2 | 110.0 | 97.1 | 107.6 | 117.2 | 110.3 | 11.93 | 0.37 | 0.64 | 0.35 | 0.44 | 0.96 |
| Ile | 59.5 | 62.0 | 50.3 | 52.4 | 56.8 | 49.8 | 4.58 | 0.21 | 0.15 | 0.10 | 0.42 | 0.86 |
| Asn | 43.8 | 44.4 | 41.2 | 44.4 | 44.6 | 44.8 | 3.97 | 0.62 | 0.76 | 0.77 | 0.66 | 0.78 |
| Glu | 133.1 | 156.7 | 136.6 | 135.1 | 136.1 | 131.0 | 9.83 | 0.21 | 0.97 | 0.09 | 0.62 | 0.19 |
| Gln | 287.5 | 310.5 | 288.4 | 301.7 | 286.7 | 295.3 | 23.38 | 0.94 | 0.84 | 0.68 | 0.79 | 0.20 |
| Val | 223.3 | 222.7 | 187.5 | 204.4 | 227.4 | 198.2 | 14.23 | 0.91 | 0.11 | 0.08 | 0.25 | 0.71 |
| Ala | 237.7 | 231.2 | 207.7 | 239.3 | 225.1 | 222.7 | 15.56 | 0.75 | 0.09 | 0.92 | 0.61 | 0.55 |
| Cit | 63.7 | 69.8 | 62.6 | 68.2 | 75.4 | 70.8 | 4.03 | 0.08 | 0.87 | 0.10 | 0.64 | 0.92 |
| Lys | 113.5 | 117.1 | 95.0 | 99.3 | 109.7 | 102.4 | 10.43 | 0.53 | 0.40 | 0.21 | 0.25 | 0.82 |
| Tyr | 140.8 | 140.2 | 132.8 | 148.0 | 150.5 | 133.6 | 10.72 | 0.37 | 0.18 | 0.40 | 0.69 | 0.68 |
| Phe | 60.2 | 60.2 | 54.4 | 60.2 | 59.8 | 57.6 | 4.51 | 0.78 | 0.30 | 0.61 | 0.68 | 0.78 |
| Trp | 214.6 | 222.0 | 231.5 | 218.9 | 213.7 | 229.2 | 14.61 | 0.86 | 0.35 | 0.66 | 0.82 | 0.73 |
| Orn | 65.7 | 67.2 | 59.7 | 58.9 | 68.1 | 66.8 | 5.42 | 0.91 | 0.82 | 0.21 | 0.10 | 0.91 |
| His | 85.0 | 88.5 | 75.0 | 82.3 | 82.1 | 81.8 | 7.03 | 0.81 | 0.20 | 0.26 | 0.24 | 0.26 |
| Leu | 78.9 | 76.6 | 67.8 | 74.4 | 74.7 | 71.4 | 6.23 | 0.84 | 0.20 | 0.62 | 0.45 | 0.89 |
| Asp | 13.4 | 13.1 | 13.4 | 12.4 | 12.8 | 10.7 | 0.75 | 0.01 | 0.17 | 0.40 | 0.19 | 0.16 |
| α-aminoadipic acid | 5.3 | 5.9 | 5.3 | 4.9 | 6.3 | 5.5 | 0.79 | 0.79 | 0.59 | 0.10 | 0.59 | 0.57 |
| Total AA | 2473 | 2573 | 2357 | 2483 | 2526 | 2476 | 116 | 0.75 | 0.55 | 0.29 | 0.60 | 0.56 |

[1]Average SEM across all treatments.