CREATINE SYNTHESIS AND AMINO ACID SPARING IN NEONATAL PIGLETS

By

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Abstract

Arginine and methionine are indispensable amino acids in neonates and have a metabolic role in creatine synthesis as well as protein synthesis. Arginine transfers its amidino group to glycine to form guanidinoacetic acid (GAA) which is then transmethylated to creatine. Methionine is the primary methyl donor for transmethylation reactions via S-adenosylmethionine (SAM). SAM is demethylated to S-adenosylhomocysteine and transfers its methyl group to synthesize creatine, phosphatidylcholine (PC) and methylated DNA. In rapidly growing animals, the balance between requirements for growth versus those for maintenance becomes more critical as growth demands more amino acids for expansion of body protein mass as well as for the increased need for other critical metabolites. Therefore, our hypothesis is that dietary creatine and its precursor GAA can spare methionine and arginine. L-[methyl-^{3}H] methionine was infused to measure the sparing effect of dietary creatine and GAA and to quantify the partitioning of transmethylation reactions. The fractional synthetic rate of creatine, PC, DNA and protein were measured using isotope kinetics. Creatine supplementation increased the availability of methyl groups for other transmethylation reactions by reducing the labile methyl groups needed for creatine synthesis. GAA supplementation increased hepatic creatine levels 5-fold, but only when methionine was not limited. Excess dietary methionine enhanced PC synthesis by double but not hepatic protein synthesis. Sparing methionine with creatine or supplementing methionine did not increase hepatic protein synthesis, suggesting that hepatic protein synthesis is conserved in neonates when methionine is limiting. Therefore, creatine levels in neonatal piglets can be maintained only when the supply of methyl groups is expanded in the diet.

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Disclosure

All of the data in this thesis were generated by the auther except for some data from the same pigs generously shared by O. Chandani Dinesh (Table 3.2: Hepatic GAA, Table 3.4: Kidney GAA and creatine and Figure 3.9: AGAT activity).

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Abbreviations

ADP	Adenosine diphosphate
AGAT	Arginine:glycine amidinotransferase
ANOVA	Analysis of variance
ANTS	Amino-naphthalene-1,3,6-trisulfonic acid disodium salt
Arg	Arginine
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthase
ATP	Adenosine triphosphate
BHMT	Betaine-homocysteine methyltransferase
CBS	Cystathionine β-synthase
CGL	Cystathionine γ-lyase
CPS-1	Carbamoyl phosphate synthetase-1
CRE	Creatine
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DMG	Dimethylglycine
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
FSR	Fractional synthetic rate
GAA	Guanidinoacetic acid
GAMT	Guanidinoacetate methyltransferase
GNMT	Glycine N-methyltransferase
HPLC	High-performance liquid chromatography
IG	Intragastric

IV	Intravenous
Km	Michaelis-Menten constant
MAT	Methionine adenosyltransferase
MeOH	Methanol
Met	Methionine
MS	Methionine synthase
MTHFR	Methylenetetrahydrofolate reductase
NRC	National Research Council
OAT	Ornithine aminotransferase
OTC	Ornithine transcarbamoylase
P5C	Pyrroline-5-carboxylate
P5CDH	Pyrroline-5-carboxylate dehydrogenase
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PITC	Phenylisothiocyanate
PTFE	Polytetrafluoroethylene
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
SAM	S-adenosylmethionine
SBD-F	7-fluorobenzo-2, 1, 3-oxadiazole-sulfonic acid ammonium salt
SDS	Sodium dodecyl sulfate
SHMT	Serine hydroxymethyltransferase
SRA	Specific radioactivity
TBASH	Tetrabutylammonium hydrogen sulfate

TCA	Tricarboxylic acid
TCEP	Tris(2-carboxyethyl)phosphine
TEA	Triethylamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofolate
TLC	Thin layer chromatography
VLDL	Very low density lipoprotein
WHC	Water holding capacity

1. Introduction

Many amino acids play an important role in protein synthesis, as well as in other metabolic reactions. Indeed, a significant portion of the total amino acid pool ends up in non-protein roles. In particular, indispensable amino acids used in non-protein roles must be carefully considered when establishing dietary requirements for these amino acids. Methionine is a sulfur-containing indispensable amino acid which is not only important for protein synthesis but also necessary for synthesis of cysteine, taurine, glutathione (Finkelstein, 1990), and also provides methyl groups for the synthesis of creatine, phosphatidylcholine and methylation of DNA (McBreairty *et al.*, 2013). Three-quarters of the creatine accretion in neonatal piglets is via de novo creatine synthesis (Brosnan *et al.*, 2009). As a result, in growing piglets, there is a high demand for methionine for expansion of body protein and creatine synthesis. In addition, arginine is a conditionally essential amino acid in piglets that is necessary to maintain the urea cycle, as well as for the synthesis of creatine, nitric oxide and polyamines (Cynober *et al*., 1995). Arginine and methionine are involved in the process of creatine synthesis. When more than one amino acid is involved in a synthetic pathway, up- or down-regulation of this pathway can spare amino acids for synthesis of protein or other metabolites.

1.1. Creatine biosynthesis and regulation

Creatine biosynthesis is a simple two-step process. In the kidney, arginine transfers its amidino group to glycine to synthesize guanidinoacetate (GAA) and ornithine via arginine:glycine amidinotransferase (AGAT) (Bloch & Schoenheimer, 1941; Wyss & Kaddurah-Daouk, 2000). GAA is transferred to the liver, where SAM

gives its methyl group to GAA to synthesize creatine and SAH via GAMT (Cantoni & Vignos, 1954) (Figure 1.1). In neonatal piglets, a high activity of AGAT has been reported only in kidney and pancreas, whereas GAMT activity is high in liver and intermediate in the pancreas, suggesting most of creatine synthesis is by the renal hepatic axis; but the role of the pancreas in creatine synthesis remains unknown (Brosnan *et al.*, 2009).

Creatine supplementation down-regulates AGAT activity as well as lowers the expression of mRNA of AGAT enzyme (McGuire *et al.*, 1984), demonstrating that creatine synthesis is thought to be regulated via GAA production. On the other hand, GAMT activity is not regulated by creatine but it is competitively inhibited by SAH (Clarke & Banfield, 2001).

1.1.1. Amino acids involved in creatine synthesis

1.1.1.1. Methionine

In piglets, up to ~35% of the dietary methionine end up in creatine synthesis (Brosnan *et al.*, 2009). The rate of transmethylation flux in 27 day old piglets is 33-40 $\mu mol.kg^{-1}.h^{-1}$ (Riedijk *et al.*, 2007) and the rate of creatine synthesis in growing piglets was estimated approximately 25 $\mu mol.kg^{-1}.h^{-1}$, suggesting that creatine synthesis may consumes 63-77% of the labile methyl groups used by piglets (Brosnan *et al.*, 2009). Therefore, creatine synthesis can potentially be limited by methionine availability.

a. Methionine requirement

Methionine is an essential amino acid important for protein synthesis, as well as for synthesis of taurine and cysteine and for supplying methyl groups to form methylated products. Therefore, it is important to estimate the methionine requirement

considering these many end products. Parenteral and enteral methionine requirements are 0.26 g/kg/d and 0.44 g/kg/d, respectively, in piglets and were estimated using the indicator phenylalanine oxidation technique (Shoveller *et al.*, 2003b). The above requirements were estimated with no cysteine in the diet. The authors suggested that first-pass splanchnic metabolism consumes 30% of the dietary methionine in healthy, enterally-fed piglets, based on the difference in requirement relative to the route of feeding. Parenteral and enteral methionine requirements were also estimated using the above technique in the presence of excess cysteine. Parenteral and enteral methionine requirements are 0.18 g/kg/d and 0.25 g/kg/d, respectively, with cysteine in the diet.

b. Transmethylation

Methionine can be adenylated to form S-adenosylmethionine (SAM) via methionine adenosyltransferase (MAT) (Finkelstein, 1990). SAM is a universal methyl donor for more than a hundred transmethylation reactions (Brosnan and Brosnan, 2006). SAM transfers methyl groups to various substrates for the synthesis of transmethylated products and S-adenosylhomocysteine (SAH) via various methyltransferases. SAM level is regulated by glycine N-methyltransferase (GNMT) (Mato & Lu, 2007). SAM:SAH ratio can be used as a transmethylation index whereby a lower ratio suggests higher total transmethylation. SAH is an inhibitor of transmethylation reactions and is further hydrolyzed to homocysteine and adenosine via S-adenosylhomocysteine hydrolase (Hoffman *et al.*, 1980). The most quantitatively important transmethylation pathways are phosphatidylcholine (PC) and creatine syntheses (see1.1) via phosphatidylethanolamine N-methyltransferase (PEMT) and guanidinoacetate methyltransferase (GAMT),

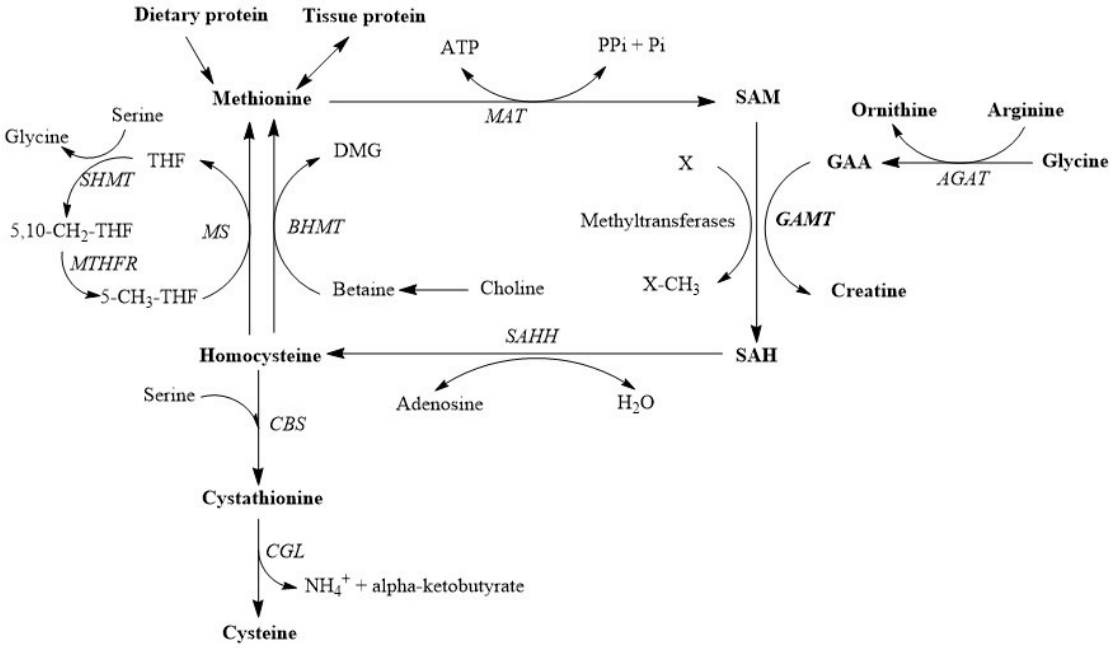

Figure 1.1: Methionine cycle and creatine synthesis (Source: MacKay *et al*., 2012 and Stead *et al*., 2001)

AGAT, L-arginine:glycine amidinotransferase; ATP, Adenosine triphosphate; BHMT, betaine:homocysteine methyltransferase; CBS, cystathionine β-synthase; CGL, cystathionine ϒ-lyase; DMG, dimethylglycine; GAA, guanidinoacetate; GAMT, guanidinoacetate methyltransferase; Pi, inorganic phosphate; PPi, inorganic diphosphate; MAT, methionine adenosyltransferase; MS, methionine synthase, MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAHH, S-adenosylhomocysteine hydrolase; SAM, S-adenosylmethionine; SHMT, serine hydroxymethyltransferase; THF, tetrahydrofolate

respectively (Mudd *et al.*, 2007; McBreairty *et al.*, 2013). Less quantitatively important transmethylation reactions are sarcosine synthesis and DNA methylation via GNMT and DNA methyltransferase (DNMT), respectively (Mudd *et al.*, 2007).

i. PC synthesis

Approximately 70% of PC synthesis occurs via the cytidine-diphosphate-choline pathway, also known as the Kennedy pathway, which is not SAM dependent; the remaining 30% of PC is synthesized via the PEMT pathway (Reo *et al.*, 2002). PC synthesis catalyzed by choline kinase, CTP:phosphocholine cytidylyltransferase and choline-phosphatetransferase to form PC from choline, where CTP:phosphocholine cytidylyltransferase is the rate-limiting enzyme (DeLong *et al.*, 1999). SAM provides three methyl groups to phosphatidylethanolamine to produce PC via the PEMT pathway, which demonstrates the quantitative importance of methyl consumption for PC synthesis, compared to creatine synthesis which only consumes one methyl group (Stead *et al.*, 2006). Plasma homocysteine was reduced by 50% in $PEMT^{-/-}$ mice compared to wild type mice suggesting the PEMT pathway significantly contributes to homocysteine production in the liver (Stead *et al.*, 2006). There are two forms of PEMT enzyme that are specific to the liver: PEMT 1 is localized on the endoplasmic reticulum and methylates phosphatidylethanolamine (PE) to phosphatidyl-N-monomethylethanolamine in the rate limiting step followed by two subsequent methylation reactions via PEMT 2, which is associated with mitochondria (Vance *et al.*, 1997). The up-regulation of the PEMT pathway has been observed in piglets fed a choline-free diet with methionine restriction, suggesting PEMT flux is regulated by PC demand (Robinson *et al.*, 2013). Moreover, a minimal effect on hepatic PC and PE has been observed in $PEMT^{-/-}$ mice demonstrating the coordinated regulation of PC

synthesis (Walkey *et al.*, 1998). A large amount of PC found in the liver is secreted as lipoproteins. In particular, PC is important for secretion of very low density lipoproteins (VLDL) (Vance *et al.*, 2007).

ii. DNA methylation

SAM transfers its methyl group to the 5' carbon on cytosine residues in the CpG site of DNA to form methylated DNA via DNMT enzyme. This modification leads to alteration of the expression of certain genes (Hermann *et al.*, 2004). These epigenetic changes are tissue-specific and nutritionally influenced by dietary methyl donors such as methionine, choline and folate. For example, excess methionine induces changes in DNA methylation and expression of several genes (Waterland, 2006). However, it has not been firmly established whether excess methionine induces hyper- or hypomethylation (Waterland, 2006). With multiple methyl donors, diets deficient in methionine, choline and folic acid (Wainfan *et al.*, 1989), or choline (Locker *et al.*, 1986; Tsujiuchi *et al.*, 1999) led to hypomethylation as observed in rat liver. Moreover, DNA hypomethylation and patterns of the gene expression were restored by folate therapy in humans (Ingrosso *et al.*, 2003). Although the relationship between dietary methyl donors and epigenetic modifications is not well described, it is rather consistent that these modifications are alterable by manipulating the diet.

iii. N-methyl glycine (sarcosine) synthesis

SAM transfers a methyl group to glycine to form N-methyl glycine, also known as sarcosine, and SAH via glycine-N-methyl transferase enzyme (GNMT). This pathway is considered an overflow to maintain hepatic methionine, SAM and homocysteine at normal levels when methionine is excess (Mudd *et al.*, 2007). In humans, absence of GNMT causes a rise in SAM and methionine, demonstrating the

lack of SAM utilization via GNMT and suggesting its importance in SAM homeostasis (Mudd *et al.*, 2001).

c. Remethylation and transsulfuration

SAH, a product of transmethylation, is hydrolyzed to homocysteine and adenosine via SAH hydrolase. The homocysteine can either remethylate to form methionine by BHMT or MS, convert to cysteine via transsulfuration, or be exported into blood (Brosnan *et al.*, 2004). In neonatal piglets, it has been shown BHMT and MS activities are similar (i.e., 65.3 and 54.5 $\mu mol.kg^{-1}.h^{-1}$, respectively) in the liver (Brosnan *et al.*, 2009). Also, Riedijk et al. estimated the rate of total remethylation is 8-14 $\mu mol.kg^{-1}.h^{-1}$ in 27 day old piglets, suggesting piglets have sufficient capacity to refill the liver methionine pool (Riedijk *et al.*, 2007).

Irreversible oxidation of homocysteine forms cystathionine via cystathionine-β-synthase (CBS) using condensation of serine and vitamin B_6 as a cofactor (Finkelstein, 1990). Cystathionine-γ-lyase further oxidizes cystathionine to form cysteine and α- ketoglutarate, also using vitamin B_6 as a cofactor. Cysteine can be further metabolized to taurine and glutathione (Finkelstein, 1990). CBS is the rate-limiting enzyme for transsulfuration and its activity is limited to liver, kidney, intestine and pancreas (Bauchart-Thevret *et al.*, 2009) and is allosterically activated by SAM (Prudova *et al.*, 2006).

1.1.1.2. Arginine

Arginine is a conditionally essential amino acid in neonatal piglets, and plays an important role in protein synthesis and other metabolic pathways such as synthesis of creatine, nitric oxide and polyamines. Furthermore, arginine plays an important role

in ammonia detoxification via the urea cycle. In the liver, arginine is broken down into urea and ornithine by arginase. Ammonia and bicarbonate are converted into carbamoyl phosphate by carbamoyl phosphate synthetase-1 (CPS-1), which is the substrate for ornithine transcarbamoylase (OTC). Jones demonstrated that conversion of glutamate to L-pyrroline-5-carboxylate (P5C) is limited to the small intestine and thymus because P5C synthase is found exclusively in these tissues (Jones, 1985). The liver is the primary site for urea synthesis due to very high arginase activity. The kidney is the primary site for arginine synthesis from citrulline and is also involved in ammonia removal via the glutamine nitrogen shuttle (Bertolo & Burrin, 2008). Excess dietary arginine down-regulates CPS-1 and OTC, but low arginine in the diet up-regulates CPS-1 and OTC to maintain citrulline synthesis thereby maintaining arginine metabolism in the kidney (Bertolo & Burrin, 2008).

In piglets, up to ~20% of the arginine end up in creatine synthesis (Brosnan *et al.*, 2009). In one week old pigs, sow's milk provides ≤ 40% of arginine requirements. In sow-fed piglets, approximately 17-20% of milk arginine may be used for creatine synthesis (Wu *et al.*, 2004). In piglets, endogenous arginine synthesis is inadequate to meet their requirement when fed arginine free diet (Brunton *et al.*, 1999). Therefore, creatine synthesis can potentially be limited arginine availability.

a. Arginine de novo synthesis in adults

Glutamate, glutamine and proline are the dietary precursors for arginine synthesis in adults (Van De Poll *et al.*, 2007). In de novo synthesis of arginine, glutamate and proline are converted to P5C by P5C synthase and proline oxidase, respectively. P5C is converted to either proline by P5C reductase or ornithine by ornithine aminotransferase (Brunton *et al.*, 1999). In the gut, low argininosuccinate

synthase (ASS) and argininosuccinate lyase (ASL) activities and higher arginase activity lead to net synthesis of citrulline and export into the portal vein (Cynober, 2002). In the kidney, higher ASS and ASL activities with low renal arginase lead to net arginine synthesis and export into circulation (Bertolo & Burrin, 2008). Therefore, de novo arginine synthesis in adults occurs through this small intestine-renal axis (Ligthart-Melis *et al.*, 2008).

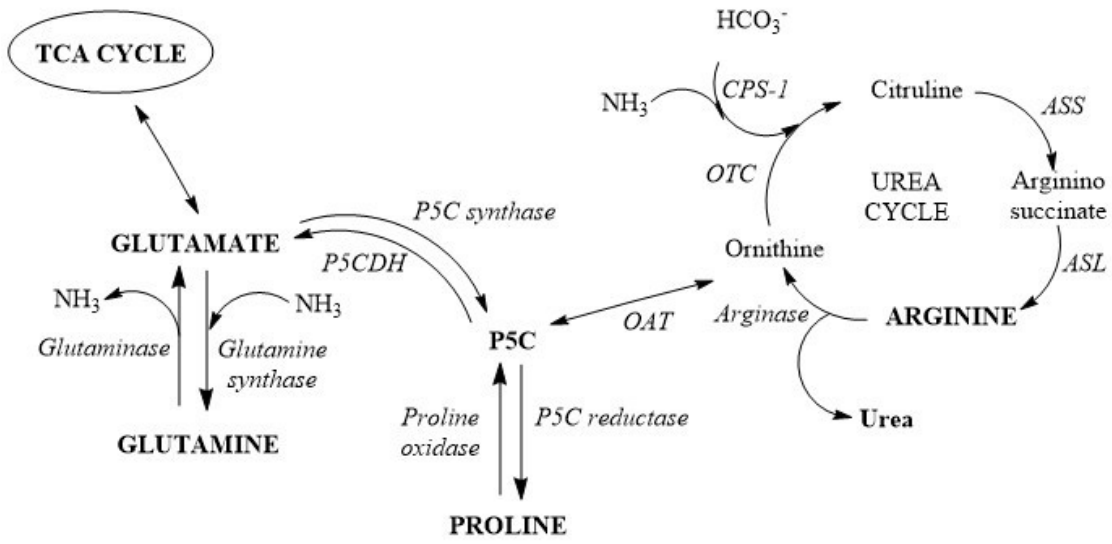

Figure 1.2: Amino acids involved in arginine metabolism (Source: Bertolo and Burrin, 2008)

ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CPS-1,carbamoyl phosphate synthetase; OAT, ornithine aminotransferase; OTC, ornithine transcarbamoylase; P5C, pyrroline-5-carboxylate; P5CDH, pyrroline-5-carboxylate dehydrogenase

b. Arginine de novo synthesis in neonates

In contrast to adult rats, de novo arginine synthesis does not follow the intestinal-renal axis in neonates. Several studies in neonatal piglets have demonstrated that arginine is synthesized in and released by the small intestine (Brunton *et al.*, 1999; Bertolo *et al.*, 2003b; Wilkinson *et al.*, 2004). Bertolo et al. demonstrated that 50% of the arginine requirement is synthesized in the neonatal intestine suggesting that a significant fraction of the requirement should enter from the diet (Bertolo *et al.*, 2003a). Wu and co-workers demonstrated that sow's milk is deficient in arginine and only ~40% of the arginine requirement is derived from sow's milk in 1 week old piglets. Therefore, they suggested that piglets need to synthesize substantial arginine to compensate for deficient arginine in sow's milk (Wu *et al.*, 2004). In neonatal piglets, feeding arginine-free diets via the intravenous (IV) route leads to onset of severe hyperammonemia, regardless of proline levels in the diet (Brunton *et al.*, 1999). The same diet when delivered via the gastric route led to development of moderate hyperammonemia after 8 h of feeding, which suggests that de novo arginine synthesis from proline only takes place when diet is delivered into the gut. Proline is the only precursor for arginine synthesis in neonates due to lack of P5C synthase activity in the neonatal intestine (Brunton *et al.*, 1999).

c. Arginine requirement

Based on the National Research Council (NRC) recommendation, which is an extrpolation from older pigs, the dietary requirement of arginine is estimated at 0.38 g/kg/d for neonatal piglets (National Research Council, 1988). Suckling piglets gain arginine (0.47 g/kg/d) via sow milk (Moughan *et al.*, 1991) which contains more than the requirement recommended by NRC. However, there is question as to whether the

NRC requirement is adequate given data by Wu and co-workers (Wu *et al.*, 2004). Brunton et al. demonstrated that >1.2 g arginine/kg/d is necessary to maximize muscle protein synthesis in enterally fed piglets (Brunton *et al.*, 2003), demonstrating that the NRC requirement is likely underestimated.

1.1.1.3. Glycine

Brosnan et al. (2009) also estimated that up to 12% of dietary glycine ends up in creatine synthesis compared to 2.7% of the net glycine used for protein synthesis in the body. However, pigs can synthesize a considerable amount of glycine (Wang *et al.,* 2013) and glycine is also readily available from protein. Therefore, creatine synthesis would not likely place a metabolic burden on the availability of glycine as it does for arginine and methionine.

1.2. Creatine transport and excretion

The resulting creatine is exported from liver and transported into high energy demand tissues via the sodium dependent creatine transporter (Fitch & Shields, 1966). Creatine is reversibly phosphorylated to phosphocreatine via creatine kinase enzyme. The larger pools of creatine and phosphocreatine are stored in high energy demand tissues such as skeletal muscle, cardiac muscle and brain tissue (Wyss & Kaddurah-Daouk, 2000). During high energy demands, phosphocreatine acts as an energy buffer by synthesizing ATP (Wallimann *et al.*, 1992).

About 1.7% of the total creatine pool is irreversibly and spontaneously converted into creatinine each day which is excreted via urine in humans (Brosnan *et al.*, 2009). Therefore, there is a need for continual replacement of this creatine lost as

creatinine. Creatine can accrue in the body both by de novo synthesis and from dietary sources rich in creatine such as red meat and fish (Wyss & Kaddurah-Daouk, 2000).

1.3. Sources of creatine in neonates

In neonatal piglets, ~25% of creatine accretion comes from sow milk and the rest is synthesized endogenously. A previous study in neonatal piglets reported that creatine synthesis may consume two thirds of the labile methyl groups, demonstrating the high demand for creatine during early development (Brosnan *et al.*, 2009). Edison and colleagues (2013) have shown that breast-fed infants and infants receiving cow milk-based formula receive 9% and 36% of their creatine needs from their respective diets. In contrast, a soy-based formula has been shown to have a negligible amount of creatine (Edison *et al.*, 2013). Therefore, the entire creatine requirement needs to be synthesized endogenously in infants receiving soy-based formulas. The variability in dietary creatine intake potentially leads to a considerable metabolic burden on the precursor amino acids for creatine synthesis.

1.4. Applications of creatine

1.4.1. Feed additives

Creatine is commonly used as a feed additive in the poultry industry to increase muscle creatine and growth rate. Several studies showed that adding creatine to arginine deficient diets elicits a growth response in chicks (Fisher *et al.*, 1956; Austic & Nesheim, 1972). Creatine supplementation has also been shown to increase the weight gain in finishing pigs (Maddock *et al.*, 2002; Young *et al.*, 2005). It has been suggested that creatine monohydrate increases the water holding capacity (WHC) of pork (Berg & Allee, 2001; Young *et al.*, 2005). Thus, it remains unknown

whether increased weight gain from creatine supplementation is due to WHC. It has also been shown that creatine supplementation decreases plasma homocysteine in rats (Stead *et al.*, 2001; Deminice *et al.*, 2009) and in humans (Korzun, 2003) suggesting a lower production of GAA. Although in contrast, Steenge and colleagues have reported no effect of creatine supplementation on levels of plasma homocysteine in humans (Steenge *et al.*, 2001). Creatine synthesis has been shown to be proportional to GAA availability in rats (Da Silva *et al.*, 2009) and in piglets (McBreairty *et al.*, 2013). Moreover, in humans, a single dose of GAA ingestion has been shown to increase plasma GAA, creatine and homocysteine (Ostojic *et al.*, 2014). In piglets, portal vein infusion of GAA increases methyl incorporation to creatine. This methyl incorporation into creatine leads to lower methyl incorporation into PC as well as lower methionine incorporation into protein (McBreairty *et al.*, 2013), suggesting GAA increases the methyl demand and limits methyl and methionine availability. Therefore, further studies are required to assess the effective use of creatine as feed additives.

1.4.2. Sport nutraceutical

Creatine supplementation has been shown to increase total muscle creatine in humans (Harris *et al.*, 1992). Further, Harris et al. demonstrated that supplementation of creatine increases the total creatine in exercised legs more so than in control legs. Creatine accretion is also higher when creatine is supplemented with a high carbohydrate diet (Green *et al.*, 1996). In other studies, it has been demonstrated that ingestion of creatine at a rate of 20 g/day for six days rapidly increases the creatine content of skeletal muscle and this accrued creatine can be maintained by supplementing creatine at a rate of 2 g/day (Hultman *et al.*, 1996). An alternative

regime is supplementing creatine at a dose of 3 g/day for 4 weeks which leads to a creatine load in skeletal muscle similar to the load of supplementing creatine at a dose of 20 g/day for six days (Hultman *et al.*, 1996). Therefore, creatine supplementation is effective in athletics by enhancing the creatine content of muscle and accommodating the high energy demand during exercise.

1.5. Amino acids sparing

In the animal feed industry, a major component of the total feed costs are amino acids and protein. As a result, synthetic amino acids are used to replace protein ingredients to minimize the feed cost. Moreover, most of the amino acids in the diet could be partially spared by a precursor or related metabolite. For example, creatine and GAA can spare methionine and arginine for protein synthesis and growth. Hegsted et al. suggested many years ago that creatine can spare arginine and glycine in poultry (Hegsted *et al.*, 1941). Several other studies have shown that adding creatine to casein-based diets improved growth rate in chicks, since casein was observed to be lacking in arginine (Fisher *et al.*, 1956; Austic & Nesheim, 1972).

GAA or creatine supplementations have been shown to increase weight gain and muscle creatine in poultry suggesting GAA can replace creatine (Almquist *et al.*, 1941). Further, Almquist et al. first mentioned in 1941 that GAA can spare arginine and glycine in the same manner that creatine could in poultry. In growing piglets, creatine supplementation did not seem to induce growth, whether added to the diet (Miller *et al.*, 1962; Guzik *et al.*, 2000) or injected intraperitoneally (Baker *et al.*, 1961). Also, Baker et al. and Miller et al. reported that creatine supplementation did not increase muscle creatine (Baker *et al.*, 1961; Miller *et al.*, 1962). It is important that dietary arginine and methionine should be marginal in order to observe sparing

effects of these amino acids. In growing-finishing pigs, large doses of creatine monohydrate (25 g/d) for a short period (10 days) did not influence growth performance (O'Quinn *et al.*, 2000).

Recently it was shown that GAA can spare arginine in poultry (Ringel *et al.*, 2008). Further studies showed that GAA can only spare arginine when arginine is deficient in diets in broiler chicks (Dilger *et al.*, 2013). McBreairty et al. were the first to demonstrate in neonatal piglets that acute portal infusion of GAA resulted in an 80-120% increase in methyl incorporation into creatine with a concomitant decrease by 75–85% and 40% in methyl incorporation into PC and protein, respectively, suggesting methyl groups were limited for PC and protein synthesis (McBreairty *et al.*, 2013). With respect to methionine, it has been shown that cysteine can spare methionine in animals and humans. Shoveller et al. estimated that ~40% of the methionine requirement can be spared by excess cysteine in growing piglets (Shoveller *et al.*, 2003a). Moreover, folate and betaine can spare methionine (Fukagawa, 2006) and betaine has been shown to spare methionine in chickens (Dilger *et al.*, 2007) but not in finishing pigs (Matthews *et al.*, 2001). Supplementing creatine with or without glycine has been shown not to have an effect on the growth response suggesting that creatine does not spare glycine for growth in chicks (Waterhouse & Scott, 1961).

1.6. Tissue protein synthesis in neonatal piglets

Protein synthesis and breakdown are together called protein turnover (Yin *et al.*, 2013). In rapidly growing animals, there is a high demand for amino acids for growth and other metabolic pathways. Of note, methionine and arginine are essential amino acids in piglets and both are involved in protein synthesis as well as in creatine

synthesis (Brosnan *et al.*, 2009). In piglets, 75% of the creatine must be synthesized via de novo synthesis (Brosnan *et al.*, 2009). Therefore in rapidly growing animals the requirement of these amino acids is high as the body protein mass expands. Liver and gut fractional protein synthesis is much higher than in other tissues and organs and together both of these tissues account for 25% of the whole-body protein synthesis (Stoll *et al.*, 1998). Bauchart-Thevret and coworkers demonstrated that the fractional synthetic rate of hepatic protein synthesis was reduced in piglets receiving sulfur amino acids free diets, as determined using an (^{15}N)-cysteine tracer method (Bauchart-Thevret *et al.*, 2009). Recently, Robinson et al. demonstrated that methyl and methionine deficient diets led to ~70% reduction in fractional protein synthesis in muscle, suggesting that methionine can be re-synthesized via the remethylation pathway when methyl donors are available (Robinson *et al.*, 2014). It has also been demonstrated that adding arginine to milk-replacement diets (deficient in arginine) increased the growth rate in piglets (Kim *et al.*, 2004). Enteral arginine supplementation increases the hepatic protein synthesis compared to parenteral arginine supply as demonstrated using [^{3}H] phenylalanine isotope infusion technique in piglets (Dinesh *et al.*, 2013). Therefore, tissue protein synthesis in piglets is sensitive to methionine and arginine intakes.

1.7. Piglets as a model for infants

The pig is an excellent model to examine questions related to amino acid metabolism (Miller & Ullrey, 1987). The piglet's digestive anatomy and physiology and amino acid metabolism resemble that of humans during early development. Moreover, the miniature piglet's small size is beneficial for biomedical research (Miller & Ullrey, 1987). Studies of amino acid metabolism and requirements are well

established in piglets, and these requirements are proportionately very similar to human requirements. Indeed, Chapman et al. demonstrated that piglet amino acid requirement data are transferrable to the human infant when adjustments are made for rapid growth of the piglets (Chapman *et al.*, 2009). In particular, the methionine requirement has been studied in enterally fed and parenterally fed neonatal piglets with or without excess levels of cysteine (Shoveller *et al.*, 2003a; Shoveller *et al.*, 2003b). Similarly, the arginine requirement has been estimated in neonatal piglets in terms of muscle protein synthesis as a functional determinant of adequate arginine (Brunton *et al.*, 2003). Therefore, piglets are a clinically relevant model to study amino acid metabolism as it relates to human infants.

1.8. Rationale, hypothesis and objectives

Creatine synthesis requires glycine, arginine and methionine. It has been postulated that creatine can spare arginine and glycine for growth (Hegsted *et al.*, 1941). However, creatine synthesis likely does not present a burden on glycine in pigs as glycine is rapidly synthesized. In contrast, Wietlake et al. proposed that creatine and arginine are interchangeable in poultry since arginine is essential. Even though creatine can spare arginine, this only occurs when arginine is limiting; no improvement has been demonstrated when creatine is supplemented to an arginine adequate diet (Wietlake *et al.*, 1954). In studies in pigs, creatine supplementation did not induce growth in weanling or growing-finishing pigs (O'Quinn et al 2000). However, this may be due to arginine and methionine not being marginal in the diet so the sparing effect on these amino acids would not be expected. Indeed, Robinson et al. has demonstrated that creatine synthesis is more sensitive to methyl and methionine in the diet when their supply is limited (Robinson *et al.*, 2013). Overall, creatine

synthesis potentially consumes approximately 35% of dietary methionine and 20% of dietary arginine in suckling neonatal piglets (Brosnan *et al.*, 2009). However, this consumption is an upper estimate as methionine and arginine can be re-synthesized via remethylation and the urea cycle, respectively, in the liver. Therefore, evaluating the burden of creatine synthesis on methionine and arginine metabolism is more complex.

Creatine synthesis is proportional to GAA availability. Almquist et al. first mentioned that GAA can spare arginine and glycine, similar to creatine, when it was demonstrated that the creatine load in muscle can be achieved either by creatine or GAA supplementation (Almquist *et al.*, 1941). GAA can spare arginine for growth when GAA is supplemented to an arginine deficient diet which has been demonstrated in broiler chicks (Dilger *et al.*, 2013). In piglets, arginine may also limit growth due to limiting arginine in sow milk (Wu *et al.*, 2004). Therefore, supplementation of GAA could also be beneficial in suckling piglets when piglets are incapable of synthesizing sufficient amount of arginine for growth.

We hypothesized that dietary creatine can spare arginine and methionine for protein synthesis and growth in piglets, and that dietary GAA could similarly spare arginine.

Objectives

01. To quantify the amino acid sparing effects of dietary creatine and GAA for protein synthesis and growth when arginine and methionine are marginally deficient in diet.

02. To quantify the partitioning of methionine-derived methyl groups into the quantitatively important transmethylation reactions in response to dietary creatine and GAA supplementation when arginine and methionine are deficient or excess in diet.

2. Materials and methods

2.1. Animals and surgical procedures

Our hypothesis was tested in a Yucatan miniature pig model. All animal procedures were reviewed and approved by the Institutional Animal Care Committee at Memorial University of Newfoundland and conformed to the guidelines of the Canadian Council on Animal Care. Thirty four, 7-10 day-old Yucatan miniature piglets were obtained from a breeding colony at Memorial University of Newfoundland, St. John's, NL, Canada. A sample size of six per group was calculated with significance level of 0.05 and power of 0.80 to detect a 10% difference using previous variance estimates from data for transmethylation partitioning in piglets; however, I used a sample size of seven, except for the Base+GAA group (n=6). The study planned to block by litter and use five animals from each litter for each of the five treatments; however, this goal could not be achieved in one set of piglets because of an unexpected mortality.

Piglets were transported from the breeding colony via Animal Care Services staff to the Biotechnology building, Memorial University of Newfoundland just before the surgery. Upon arrival, piglets were weighed and anaesthetized with an intramuscular injection of ketamine hydrochloride (Bimeda Canada, Cambridge, ON, Canada) and acepromazine (Vetoquinol, Quebec, Canada) at doses of 22 mg/kg and 0.5 mg/kg, respectively. Piglets were then given an atropine sulfate (Rafter and Products, Canada) injection of 0.05 mg/kg via subcutaneous route to reduce air-way secretions. Afterwards, piglets were intubated and anaesthesia was maintained with 1.5% isoflurane (Abbott Laboratories Inc., Canada) mixed with oxygen (1.5 l/min).

The animal was cleaned with soap and Proviodine prior to the surgery. Just before surgery, piglets were also given a dose of buprenorphine analgesic (Temgesic, Reckitt Benckiser Healthcare, UK) providing 0.03 mg/kg intramuscularly; analgesic was repeated every 12 h for 2 days. In each piglet, two venous catheters and a gastric catheter were surgically implanted. One silastic catheter was introduced into the left external jugular vein and advanced to the cranial vena cava immediately cranial to the heart. The other catheter was introduced into the left femoral vein and advanced to the caudal vena cava immediately caudal to the heart. The jugular catheter was used for diet infusion during the initial 2 days of the study period, and the femoral catheter was used to collect blood samples throughout the protocol. A mid ventral incision was made to open the abdominal cavity for implantation of a gastric catheter (Rombeau & Caldwell, 1984). The body temperature, respiratory rate, heart rate and oxygen saturation were monitored throughout the surgical procedure.

Immediately following surgery, piglets were transferred to the animal housing room, with a 12-h light and 12-h dark cycle where the temperature was maintained at 28 °C with supplemental heating lamps. Piglets were housed individually in metabolic cages which allowed visual and aural contact with other piglets. The IV and intragastric (IG) lines were connected via a dual-port swivel and tether system (Lomir Biomedical, Montreal, Quebec, Canada) to facilitate continuous IG diet infusion and to allow the piglets to move freely around the cage. Piglets received an IV injection of 0.5 ml of the anti-bacterial Borgal (Trimethoprim 40 mg/ml and sulfadoxine 200 mg/ml; Intervet Canada Ltd, Canada) which was diluted to 10 ml with saline. Anti-bacterial veterinary ointment was applied on the incision sites until they healed.

2.2. Experimental design and diet

Complete elemental diets (Appendix I) was prepared in the laboratory using crystalline L-amino acids (Evonik Industries AG, Hanau-Wolfgang, Germany or Sigma, St. Louis, MO, USA). The amino acid profile was based on a commercially available parenteral nutrition solution for infants (Vaminolact; Fresenius Kabi, Germany) with slight modifications for piglets (Wykes *et al.,* 1993). Dry amino acids were thoroughly mixed together and then dissolved in water at 55-65 °C under a cover of nitrogen gas to protect from oxidation. D-glucose and major minerals (Appendix II) were added and the solution was brought up to the desired volume using pyrogen-free water. The diets were sterilized by filtering through a 0.22 μm filter (ACROPAK, Pall Corporation, Switzerland) into sterile IV bags (Baxter Corporation, Mississauga, ON, Canada) in a laminar flow hood and kept refrigerated and protected from light until needed. Similar to the above procedure, five experimental diets (Appendix I) were prepared to test our hypothesis. Alanine and aspartate were adjusted to make the diets isonitrogenous.

2.2.1. Diet groups

Base diet (Base): Base diet was designed to marginally control transmethylation reactions and protein synthesis. In this diet, methionine was added to provide 0.2 g/kg/day; this represents 80% of the methionine requirement determined by Shoveller et al. with excess cysteine (Shoveller *et al.*, 2003b). Arginine was fed at 0.3 g/kg/day, which corresponded to 79% of NRC requirement (i.e., 0.38 g/kg/day); this chosen intake also corresponded to 50% of the intake value at the breakpoint in the plasma arginine concentration when piglets were fed increasing levels of arginine (Brunton *et al.,* 2003). These marginal levels of methionine and arginine allowed us to detect both

decreases and increases in amino acid utilization due to supplement treatments. In other words, if a supplement spared methionine for protein synthesis, this increase in protein synthesis can only be detected if methionine was limiting in the first place.

Base diet with GAA (Base+GAA): The purpose of this diet was to determine whether or not GAA can spare arginine. Base diet was supplemented with GAA at a rate of 0.09 g/kg/day. This amount of GAA, if entirely converted into creatine, would fulfill the total creatine accretion rate of the piglet at this age (Brosnan *et al.*, 2009).

Base diet with creatine (Base+CRE): The purpose of this diet was to determine whether or not creatine can spare arginine and methionine for protein synthesis. Base diet was supplemented with creatine (as creatine monohydrate) at a rate of 0.12 g creatine/kg/day, which is equal to the total creatine accretion rate of the piglet (Brosnan *et al.*, 2009).

Excess arginine and methionine diet (Excess Arg/Met): Excess dietary arginine (1.8 g/kg/day) and methionine (0.5 g/kg/day) levels were chosen so each are in excess of the respective whole body requirements (i.e., at least twice the estimated requirements).

GAA with Excess methionine (GAA/Excess Met): The purpose of this diet was to determine if methionine can limit creatine synthesis from supplemented GAA. If methionine is limited in the Base+GAA diet, then utilization of GAA (ie conversion to creatine) might be limited by marginal methionine. So this group was supplemented with GAA plus excess methionine to ensure GAA is methylated to creatine.

2.2.2. Trace element mixture

All trace elements were dissolved in pyrogen-free water and filtered through a 0.22 μm syringe filter (Millipore Ireland Ltd., Ireland) into a sterile intravenous bag and kept in a refrigerator and protected from light until needed (Appendix III).

2.2.3. Vitamin mixture

The vitamin mixture was calculated based on the Multi-12/K1 pediatric multivitamin commercial solution dose. All water soluble vitamins and the water soluble form of vitamin K were prepared separately and neutralized with sodium citrate (Appendix IV). The solutions were filtered through a 0.22 μm syringe filter into a sterile intravenous bag and kept in refrigerator and protected from light until needed. Fat soluble vitamins A, D and E were mixed in vegetable oil and stored in a sterile bottle under refrigeration and protected from light until needed.

Three ml of Multi-12/K1 pediatric vitamins, 1 ml of iron dextran (Ventoquinol Canada Inc., Canada) that provides 2 mg of iron per kg of body weight, 3 ml of trace element mix and 145 ml of 20% Intralipid (Fresenius Kabi, Uppsala, Sweden) were mixed with 750 ml of diet just before feeding. Commercially available multivitamins (Multi-12/K1 Pediatric multivitamins, Baxter, Canada) were used during the adaptation period (which included IV feeding) and then laboratory-made multivitamins were used to mix with the experimental diets. Fat soluble vitamins were directly injected into the stomach via the gastric catheter. The complete diet provided 15 g amino acids/kg/day and 1.1 MJ of metabolizable energy/kg/day with lipids supplying 50% of non-protein energy. Vitamins were provided at more than 100% and trace minerals were provided at a maximum of 200% of requirements (National Research Council, 1998).

On the day of surgery, complete elemental diet (adaptation diet) was continuously infused IV at 50% of the maximal rate (13.5 ml/kg/h) by dual channel infusion pumps (Baxter Healthcare Corporation, Deerfield, USA). On the morning of the following day, 50% of diet was infused IV and 50% was infused via the gastric catheter; IG infusion was increased to 75% and IV infusion was reduced to 25% of the maximal rate that evening. By the next morning, 100% of diet was infused IG. After 2 days of adaptation to the complete elemental diet, piglets were switched to one of the above test diets which was infused via the gastric catheter at 13.5 ml/kg/h for 5 days. During this period, the body weight of each animal was measured every day in the morning and the diet infusion rate was adjusted to the body weight.

2.3. Isotope infusion protocol and necropsy procedure

On study day 7, all piglets received a primed dose of 30 μCi/kg L-[methyl-^{3}H] methionine (30-80 Ci/mmol; American Radiolabeled Chemicals) infused via the gastric catheter. At the same time a constant infusion of 30 μCi/kg/h of L-[methyl-^{3}H] methionine was initiated and continued for 6 h. The constant infusion was provided as half of the hourly dose infused intragastrically every 30 min (Figure 2.1). During the infusion, 1 ml of blood was obtained from the femoral catheter at baseline and every 30 min. The blood samples were immediately transferred to a heparinized vacutainer and centrifuged at 1300g (VWR Clinical 200, Hermle Labortechnik, Wehingen, Germany) for 5 min to separate plasma. Plasma samples were stored at -80°C until used for analyses.

At the end of the 6 h infusion, piglets were anaesthetized with 1.5% isoflurane mixed with oxygen (1.5 l/mim) by mask. The abdomen was opened and tissue samples were obtained from the pancreas, liver and kidney, which were quickly

freeze-clamped in liquid nitrogen. Samples of other tissues (biceps femoris muscle) were then removed and freeze-clamped. A 30 cm long section of proximal jejunum was removed, flushed with ice-cold saline, and the mucosa was scraped from the muscularis using a glass microscope slide and freeze-clamped. All samples freeze-clamped time was recored and stored at -80°C until further analysis.

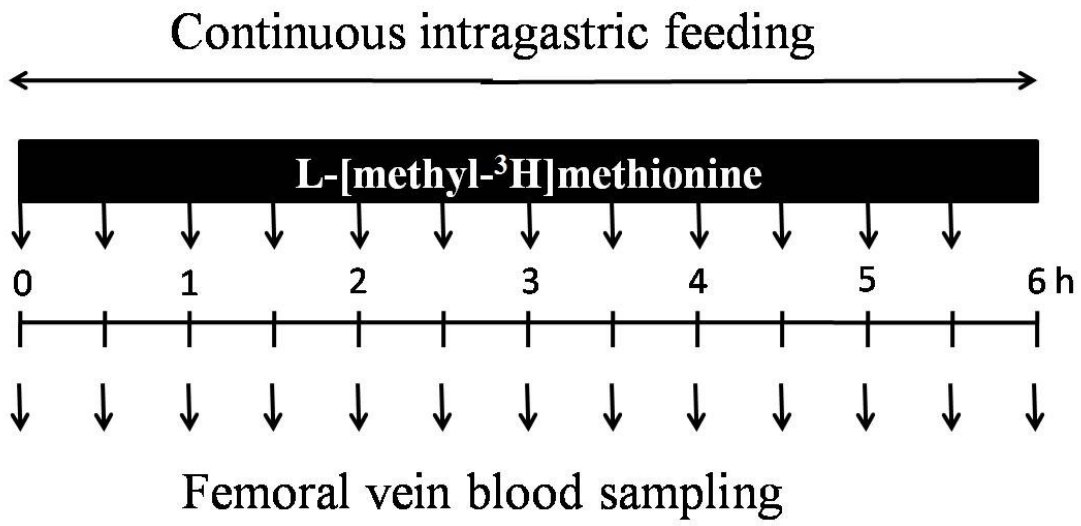

Figure 2. 1: L-[methyl-^{3}H] methionine infusion and blood sampling protocol

2.4. Analytical procedures

2.4.1. Plasma and tissue amino acid concentrations and radioactivity associated with methionine

2.4.1.1. Plasma amino acids analysis

Plasma amino acid concentrations were measured by reverse-phase high-performance liquid chromatography (HPLC) following derivatization with phenylisothiocyanate (PITC) (Thermo Scientific, USA) (Bidlingmeyer *et al.*, 1984). Briefly, 100 µl of plasma was mixed with 20 µl of 2.5 µmol/ml norleucine internal

standard (Sigma Aldrich, Oakville, Canada) and 1 ml of 0.5% trifluoroacetic acid (TFA) (Sigma Aldrich, Oakville, Canada) in methanol (MeOH) (Fisher Scientific, Whitby, Canada) in a micro centrifuge tube, to precipitate proteins. The samples were vortexed and centrifuged at 2655 x g for 5 min. Then the supernatant was poured into a plastic tube and flash frozen in liquid nitrogen and lyophilized overnight in a freeze dryer (Thermo Savant, Canada). One-hundred microliters of 2:2:6 triethylamine (TEA) (Sigma Aldrich, Oakville, Canada): MeOH: water was added to each sample followed by freeze drying for ~1 h. Samples were then incubated for 35 min at room temperature with 50 µl of 1:1:7:1 solution of water: TEA: MeOH: phenylisothiocyanate (PITC). The labelling with PITC was stopped by flash freezing with liquid nitrogen and then placed on a freeze dryer overnight. Samples were re-suspended in 200 µl of sample diluent (710 mg of Na_2HPO_4 (Sigma Aldrich, Oakville, Canada) in 1 liter of water, pH 7.4 with 10% H_3PO_4 acid; 5% of the volume was replaced with acetonitrile (Fisher Scientific, Whitby, Canada)).

Forty microliters of each sample was injected into a reverse-phase C18 Pico-Tag column (Waters, 60Å, 4 µm 3.9 X 300 mm) connected to a HPLC system. The HPLC system consisted of a Waters 1525 Binary HPLC pump, Waters 2487 Dual λ absorbance detector and Waters 717 plus Auto sampler (Waters Corporation, Milford, MA, USA). The column temperature was maintained at 46 ^{o}C to facilitate the separation of amino acids. Mobile phase A consisted of 70 mM sodium acetate (Fisher Scientific, Whitby, Canada) and 2.5% acetonitrile (Fisher Scientific, Whitby, Canada) at pH 6.55 and mobile phase B consisted of 45% acetonitrile 15% MeOH and 40% water. Mobile phases were filtered through a 0.45 µm MAGNA nylon filter (Canadian Life Science, Peterborough, ON, Canada) and degassed using a Waters In Line-

Degasser AF. The column was allowed to equilibrate with mobile phase A at a rate of 1 ml/min. The phenylthiocarbamyl amino acids were detected at 254 nm. Peaks were integrated using Breeze Software (Waters, version 3.3, 2002, Waters Corporation, Woburn MA, USA) and the amino acid concentrations were determined by comparing to the area produced by the internal standard. A fraction collector (Waters Fraction Collector III) was used to collect the eluent that was associated with methionine peak. The radioactivity associated with methionine was determined by liquid scintillation counter (Perkin Elmer Tri-Carb 2810, Woodbridge, ON, Canada) after adding 10 ml of ScintiVerse (TM) (Fisher Scientific, Whitby, Canada). Unfortunately, sarcosine and assiociated radioactivity could not be successfully separated using this method and so were not included in this thesis.

2.4.1.2. Tissue amino acids analysis

a. Tissue preparation

Liver and muscle tissue were homogenized (1:3 w/v) with 2% cold perchloric acid at 50% speed using a mechanical homogenizer for ~45 s and centrifuged at 3000 x g for 15 min to separate tissue-free amino acids from tissue-bound amino acids. The supernatant was collected into a scintillation vial. This step was repeated 3 times for the complete separation of tissue free amino acids. Norleucine (25 μmol/ml) was added to the scintillation vial as an internal standard and stored at -80 ^{o}C until needed. The acid-insoluble protein pellet in the tube was used for tissue-bound amino acid analysis.

b. Tissue-free amino acids

The supernatant was neutralized with 2.0 M K_2CO_3 (12.5% v/v) in a plastic tube and centrifuged at 5000 x g for 3 min to separate the supernatant from the precipitate. One milliliter of supernatant was added to another plastic tube which was flash frozen in liquid nitrogen and placed on a freeze dryer overnight. Then sample was derivatized as described in 2.4.1.1.

c. Tissue-bound amino acids

The pellet was re-suspended in 1.0 M NaOH (1:8 w/v) and mixed. The tubes were placed in a water bath for ~1.5 h at 37 °C to solubilize the protein. Fifty microliters of solubilized protein solution was transferred to a micro centrifuge tube and stored at -80 °C to analyze the protein concentration. Protein was re-precipitated by adding half of the volume of solubilized protein to cold 20% perchloric acid and samples were placed on ice for 20 min. Then samples were centrifuged at 3000 x g for 15 min and the supernatant was discarded. The internal standard, norleucine (25 µmol/ml) was added. Then 6.0 M HCl (10 ml/g tissue wt) was added to the tube and a glass rod was used to disrupt the pellet, followed by transfer of all contents to a Pyrex tube. Then the solution was placed in a 110 °C oven for 24 h to hydrolyze. The resulting hydrolysate solution was left to cool overnight. Hydrolysates were brought to a final volume of 25 ml with HPLC water. One milliliter was then transferred into a plastic tube using a 0.45 µm PTFE syringe filter (Canadian Life Sciences, Canada) and tubes were placed in a vacuum oven overnight, and were subsequently derivatized as described above (see 2.4.1.1). Muscle samples were directly hydrolyzed with HCl after adding the internal standard to the insoluble protein pellet; a solubilized protein

sample was not needed for this tissue. The radioactivity associated with methionine was determined by HPLC as described above (see 2.4.1.1).

2.4.2. Analyses of transmethylation products

2.4.2.1. Determination of creatine concentration and specific radioactivity

Tissue creatine concentration was determined using a modified method by Lamarre (Lamarre *et al.*, 2010). Tissue was homogenized for 45 s in 50 mM Tris buffer (pH 7.4) (0.2/0.9 w/v) and left at room temperature for 20 min to allow for complete conversion of phosphocreatine to creatine. Homogenates were transferred to a 1.5 ml micro centrifuge tube and TFA (97% minimum) was added to the samples which were set on ice. After 10 min, samples were centrifuged at 3825 x g at 4 °C for 10 min. Samples were then filtered using a Bond-Elut C18 solid phase extraction cartridge and were separated via HPLC using porous graphitized carbon column (Hypercarb, 7 μm, 100 X 4.6 mm, Thermo Scientific, Canada). The peak was detected using a Waters 2487 Dual λ absorbance detector at an absorbance of 210 nm. An isocratic mobile phase of 0.1% TFA and 3% MeOH was used with a flow rate of 1 ml/min and run time of 20 min. A standard curve was used for quantification and peaks were determined using Breeze Software (Waters, version 3.3, 2002, Waters Corporation, Woburn, MA, USA). The creatine fraction was collected and radioactivity of creatine was determined using a liquid scintillation counter as described above (2.4.1.1)

2.4.2.2. Determination of phosphatidylcholine concentration and radioactivity

Lipids were extracted from liver by using Folch method (Folch *et al.*, 1957). One-hundred milligram of liver was homogenized with 300 μl of 50 mM NaCl in a

glass culture tube and transferred to a Pyrex tube. One and a half milliliters of chloroform and methanol (2:1) was added and samples were extensively vortexed and left at 4 ^{o}C overnight. After overnight extraction, samples were centrifuged at 2000 x g for 10 min and the lower organic layer was removed and evaporated under nitrogen gas for 15-20 min. The residue was re-dissolved in 100 µl isopropanol (99.9%) (Fisher Scientific, Whitby, Canada). Ten microlilers of samples were applied onto a thin layer chromatography (TLC) silica gel 60 plate (EMD, Millipore Corporation, Billerica, MA, USA). The plate was inserted in a saturated TLC chamber (with chloroform/methanol/acetic acid (17.4 M)/water, 25:15:4:2) and left until the mobile phase migrated to the top of the plate. Iodine was used for visualization of the corresponding bands to the PC and PE standards. The PC and PE bands were scraped into an acid-washed Pyrex tube containing 300 µl of perchloric acid. The tubes were heated at 180 ^{o}C for ~2 h until no dark colour was visible. Samples were cooled to room temperature.

PC and PE were quantified via measuring total phosphate using a modified Bartlett method (Bartlett, 1959). Phosphate standards were made from stock solutions containing KH_2PO_4 in H_2SO_4 (1 M) solution. Fifty microliters of 5% ammonium molybdate and 50 µl of a 1-amino-2-naphthol-4-sulfonic acid solution (1.0 g 1-amino-2-naphthol-4-sulfonic acid in 390 ml of 15% sodium metabisulfite plus 10 ml of 20% sodium sulfite) were added to samples and standards. Then samples and standards were extensively vortexed and placed in a boiling water bath for 12 min to allow colour development. A Thermo Spectronic Scientific Bio Mate 3 spectrophotometer was used to read the absorbence at 815 nm and a standard curve was used for quantification of total phosphorus. A portion of the colour developed sample was used

for scintillation counting to determine the radioactive PC using scintillation counter as described above (2.4.1.1)

2.4.2.3. Determination of DNA concentration and radioactive DNA

DNA was extracted using the phenol extraction method. Samples were homogenized for 45 s in a buffer (50 mM Tris (pH 8), 1% SDS, 100 mM EDTA, and 100 mM NaCl) and Proteinase K (50 mg/5 ml) (0.4/0.5/0.4 w/v/v). Samples were incubated overnight in a water bath at 56 ^{o}C. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each sample. The samples were vigorously shaken and centrifuged for 15 min at 4500 x g. The top aqueous phase was transferred to a new tube. This extraction process was repeated several times until no protein interface was visible. Finally, chloroform: isoamyl alcohol (24:1) was added to the aqueous layer and centrifuged at 4500 x g for 10 min after vigorously shaking. The DNA was precipitated using an equal volume of isopropanol (99.9%) and 1/5 volume of 1 M sodium acetate and centrifuged at 4500 x g for 5 min. DNA pellets were washed twice in 70% ethanol and once in 95% ethanol followed by centrifugation for 10 min at 6700 x g. The pellet was dried and re-suspended in 10 mM Tris buffer (pH 8) with 0.1 mM EDTA. The DNA concentration was determined using Thermo Scientific Nano Drop 2000 spectrophotometer and DPM were determined with liquid scintillation counter as described above (2.4.1.1).

2.4.3. Determination of SAM/SAH concentration and radioactive SAM

Hepatic SAM and SAH were determined using a method established by Ratnam et al. (Ratnam *et al.*, 2006). Tissue was homogenized in cold 8% trichloroacetic acid for 45 s and cold centrifuged at 12,000 rpm for 5 min to precipitate protein. Then samples were filtered using a 45 µm polytetrafluoroethylene

(PTFE) syringe filter (Canadian Life Science, Peterborough, Canada) and analyzed using reverse-phase HPLC.

Samples were injected into a Vydac C18 reverse phase column (250 X 4.5 mm, 5 µm Cat#218TP54). A standard curve was used for quantification and peaks were determined using Breeze Software (Waters, version 3.3, 2002, Waters Corporation, Woburn, MA, USA). Mobile phase was made fresh and filtered with a 0.45 µm MAGNA nylon filter (Canadian Life Science, Peterborough, Canada). Mobile phase A consisted of 50 mM NaH_2PO_4 and 10 mM heptanesulfonic acid at pH 3.2 and mobile phase B consisted of 4% acetonitrile. Samples were detected by Waters 2487 Dual λ absorbance detector at an absorbance of 258 nm with a flow rate of 1 ml/min and run time of 30 min. The SAM fraction was collected and radioactivity of SAM was determined via liquid scintillation counter as described above (2.4.1.1).

2.4.4. Determination of plasma and hepatic homocysteine, cysteine and glutathione concentration

Plasma and hepatic homocysteine, cysteine and glutathione were determined using a modified method by Vester and Rasmussen and Pfeiffer et al. (Vester & Rasmussen, 1991; Pfeiffer *et al.*, 1999).

2.4.4.1. Plasma

One-hundred and fifty microliters of plasma was combined with 50 µl of 0.2 mM 8-amino-naphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) (dissolved in 0.1 mM borate buffer with 2 mM EDTA at pH 9.5) and 30 µl of tris-(2-carboxyethyl) phosphine (TCEP) (100 mg/ml of 1 X PBS (137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4) which was vortexed and left at room temperature

for 30 min. After 30 min, 125 µl of 0.6 M perchloric acid was added to samples and the samples were vortexed and left at room temperature for 10 min followed by centrifugation at 4 °C at 4000 x g for 5 min. one-hundred microliters of supernatant was then transferred to a flat-top micro centrifuge tube (amber colour) and 200 µl of 2 M borate buffer (pH 10.5) was added. Samples were derivatized by adding 100 µl of 7-flurobenzo-2,1,3-oxadiazole-sulfonic acid ammonium salt (SBD-F) solution (1 mg/ml SBDF in 0.1 M borate buffer with 2 mM EDTA at pH 9.5). Samples were covered with aluminum foil and vortexed. Then samples were incubated at 60 °C in a water bath for 60 min. Samples were then removed and placed on ice for 5 min and subsequently filtered using a 45 µm PTFE syringe filter (Canadian Life Science, Peterborough, Canada) and analyzed using reverse-phase HPLC. Samples were injected into a YMC-Pack Pro C18 column (150 X 4.6 mm I.D, 3 µm; YMC America, Inc, Allentown, PA, USA) and homocysteine, cysteine and glutathione were separated via reverse phase HPLC using a dual mobile phase system (Mobile phase A: 980 ml 33.33 mM sodium acetate trihydrate in 66.66 mM glacial acetic acid (pH 4) mixed with 20 ml of 100% methanol and mobile phase B: 800 ml 33.33 mM sodium acetate trihydrate in 66.66 mM glacial acetic acid (pH 4) mixed with 200 ml of 100% methanol). Samples were detected by Waters 474 Scanning fluorescence detector at 515 nm emission and 385 nm excitation. The run time for each sample was 20 min with a flow rate of 1 ml/min. The ANTS was used as internal standard for quantification. Data were obtained using Empower 2 software (Waters, Milford, MA, USA).

2.4.4.2. Liver

One-hundred milligrams of tissue was combined with 500 µl of 0.1 M potassium borate buffer (pH 9.5) in 2 mM EDTA and 50 µl of internal standard (2-mercaptopropionyl-glycine) followed by homogenization for 30 s. Then 50 µl of TCEP (32 µg/ml in 0.1 M potassium borate) was added and left at room temperature for 30 min after vortexing. After 30 min, 150 µl of 70% perchloric acid was added and mixed followed by centrifugation at 4000 x g for 6 min. Five-hundred microliters of supernatant was neutralized with 35 µl of 2 M KOH followed by derivatization with SBD-F and analyzed using reverse-phase HPLC, as described above (see 2.4.4.1).

2.4.5. Determination of plasma creatine and creatinine

Plasma creatine concentration was determined using a modified method by Lamarre (Lamarre *et al.*, 2010). Plasma, 50 mM Tris buffer (pH 7.4) and TFA (3:2:0.5) were mixed together in a micro centrifuge tube and set on ice for 10 min. After 10 min, samples were centrifuged at 4000 x g for 10 min at 4^{o}C. Creatine and creatinine concentration, and specific radioactivity of creatine were determined as described above (see 2.4.2.1).

2.4.6. Determination of plasma and tissue GAA concentration

Tissue and plasma GAA was determined according to the method of Buchberger and Ferdig (Buchberger & Ferdig, 2004).

2.4.6.1. Plasma GAA

Two-hundred microliters of plasma was mixed with 14 µl of 30% perchloric acid and placed on ice for 15 min followed by centrifugation at 15,000 x g for 8 min at 4 ^{o}C. One-hundred and fifty microliters of supernatant was transferred to a micro

centrifuge tube and 22.4 µl of 20% KOH was added. Then samples were placed on ice for 15 min followed by centrifugation at 10,000 x g for 5 min. After centrifugation, 150 µl of supernatant was used for derivatization with ninhydrin (see section 2.4.6.3).

2.4.6.2. Tissue GAA

Tissue was homogenized with cold 1 M perchloric acid (1:4 w/v) followed by centrifugation at 4 ^{o}C for 20 min at 15,250 x g. Supernatant was transferred to a tube quantitatively followed by adding of 50% K_2CO_3 and 20% KOH to neutralize (pH 6.5-7) the samples. Samples were centrifuged again for 10 min at 10,600 x g at 4 ^{o}C. Two-hundred microliters of supernatant was used for derivatization with ninhydrin (see section 2.4.6.3).

2.4.6.3. Derivatization with ninhydrin

Samples, 1.3 M KOH and 0.9% ninhydrin (4:3:1.5) were mixed together and left at room temperature for 15 min. Five percent ascorbic acid and 5 M phosphoric acid (1:1) were added to samples and mixed followed by incubation in a 90 ^{o}C water bath for 30 min. After derivatization, samples were cooled and filtered using a 0.45 µm PTFE syringe filter (Canadian Life Science, Peterborough, Canada). Samples were injected into a YMC-Pack Pro C18 column (150 X 4.6 mm I.D, 3 µm; YMC America, Inc, Allentown, PA, USA) and derivatized GAA was separated via reverse-phase HPLC using a dual buffer system (Mobile phase A: 50 mM formic acid and mobile phase B: 100% methanol). Samples were detected by Waters 474 scanning fluorescence detector at 470 nm emission and 390 nm excitation. The run time for each sample was 32 min with a flow rate of 1 ml/min. A standard curve was used for quantification and peaks were determined using Breeze Software (Waters, version 3.3, 2002, Waters Corporation, Woburn, MA, USA).

2.4.7. Determination of hepatic GAMT activity

GAMT activity was measured using fresh liver samples using the method of Ogawa et al. (Ogawa *et al.*, 1983). Liver tissue was homogenized with a buffer solution (1:5 w/v) consisting of 0.25 M sucrose, 1 mM EDTA and 10 mM HEPES (pH 7.2), Homogenates were centrifuged at 100,000 x g at 4 ºC for 1 h and the supernatant was used to determine GAMT activity. Briefly, 10 µl supernatant was combined with 350 µl of tris-β-mercaptoethanol solution (100 mM Tris buffer (pH 7.4), 20 mM 2-mercaptoethanol) and 50 µl S-adenosylmethionine solution (8.8 mg SAM in 1 ml of water and 1 ml of tris-β-mercaptoethanol buffer) followed by incubation at room temperature for 10 min. The assay was started by addition of 10 µl of GAA (4.68 mg of GAA in 1 ml of water and 1 ml of tris-β-mercaptoethanol buffer) followed by incubation at 37 ^{o}C for 20 min. Blanks for the assay did not contain supernatant. At the end of 20 min of incubation, the assay was stopped by addition of 750 µl of 15% (w/v) trichloroacetic acid and samples were kept on ice for 10 min. Then 720 µl of 1 M Tris (pH 7.4) was added for neutralization followed by centrifugation at 10,000 x g for 5 min. Four-hundred microliters of supernatant was derivatized as described above (2.4.6.3) to measure creatine production in order to determine GAMT activity.

2.4.8. Determination of muscle metabolites

Muscle metabolites (phosphocreatine, ATP and ADP) were determined using a modified method by Volonte et al. (Volonté *et al.*, 2004). Briefly 250 mg of the biceps femoris muscle was homogenized with 1 ml of 0.4 M perchloric acid followed by the addition 375 µl of 1 M KOH for neutralization. Samples were centrifuged at 3000 x g for 5 min. Then samples were filtered using a 45 µm PTFE syringe filter (Canadian

Life Science, Peterborough, Canada) and injected into a Waters Symmetry C18 column (100Å, 5µm, 4.6 X 150 mm). The peak was detected using Waters 2487 Dual λ absorbance detector at an absorbance of 220 nm. An isocratic mobile phase of 215 mM potassium dihydrogen phosphate, 2.3 mM tetrabutylammonium hydrogen sulfate (TBASH), 4% acetonitrile and 0.4% potassium hydroxide (1 M) was used with a flow rate of 1 ml/min and run time of 20 min. Standard curves were used for quantification and peaks were determined using Breeze Software (Waters, version 3.3, 2002, Waters Corporation, Woburn, MA, USA).

2.5. Calculations

Specific radioactivity (SRA) of the transmethylation products, methionine and SAM were expressed as DPM/nmol. The mean of the plasma SRA of methionine was calculated for data between 4 to 6 h of the infusion. All mean SRAs included ≥ 4 times points. Methionine plateaus were confirmed by regression analysis by determining that the slope was not different from zero. Methionine flux was calculated using following equation.

Flux ($\mu mol.kg^{-1}.h^{-1}$) = Methionine dose rate ($DPM.kg^{-1}.h^{-1}$)/Mean SRA of methionine at plateau (DPM/µmol)

Fractional synthetic rate (FSR) of the transmethylation products and protein synthesis were calculated using following equation.

Fractional Synthetic Rate (%/h) = $SRA_{\text{of product}}/SRA_{\text{of precursor}}*100/t$

SRA of hepatic SAM was used as the precursor for calculation of fractional synthetic rate of transmethylation products, but SRA of the tissue-free methionine was used to calculate tissue specific protein synthesis, with "t" being the duration of labelling in hours.

Proprotion of the ^{3}H-methyl products remaining in the liver at the end of study was calculated using following equation.

$$\% = (DPM_{product}/g \text{ of tissue}) / (DPM_{\text{sum of all measured products}}/g \text{ of tissue}) * 100$$

2.5. Statistical analysis

Data were analyzed by one-way ANOVA with Newman-Keuls multiple comparisons post-hoc test using GraphPad Prism 5 (Graph Pad Software, San Diego, CA, USA). The alpha level was set at 0.05. All data were reported as mean ± standard deviation.

3. Results

This study was designed for two major outcomes: whole body stable isotope analysis (completed by another graduate student) and tissue specific transmethylation (subject of this thesis). Based on the sample size calculation, it was decided to have a sample size of 6 per group. However, steady state kinetics were not achieved for the stable isotope infusions in the first set of pigs, so we added another set of pigs. This allowed a sample size of 7 per group for transmethylation outcomes, but one of the piglets from Base-GAA group did not complete the experiment because it had a volvulus and died during the adaptation period. All other piglets were healthy during the study period.

3.1. Growth and liver to body weight ratio

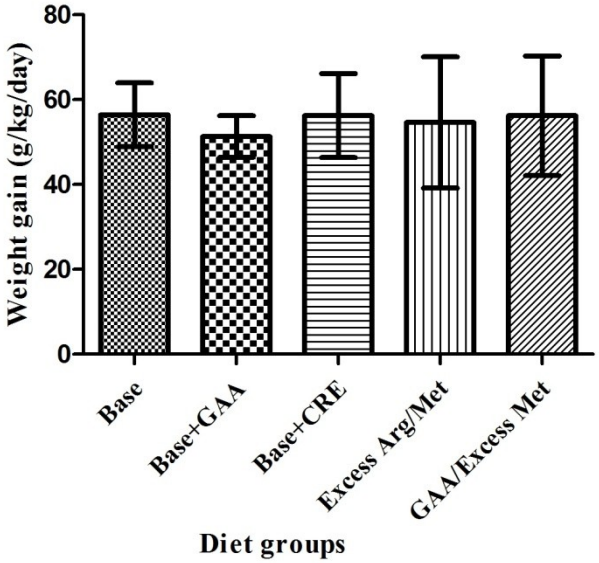

Figure 3.1: Effects of different test diets on weight gain in neonatal piglets.

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine

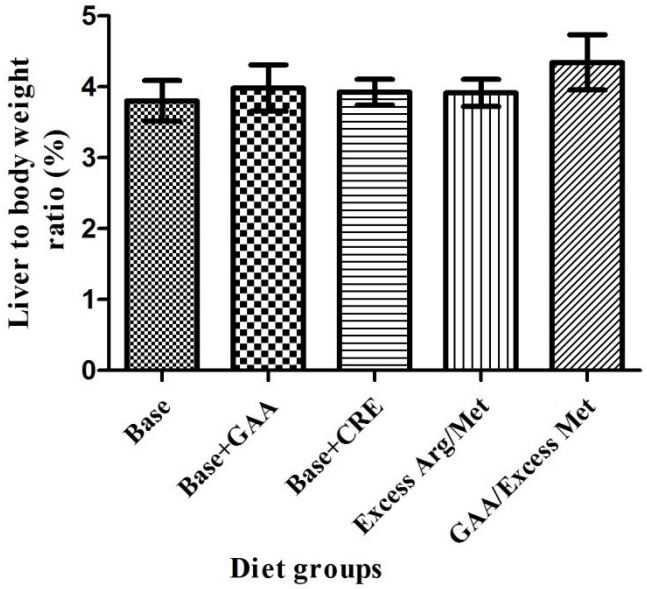

Figure 3.2: Effects of different test diets on liver to body weight ratio in neonatal piglets.

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine

There were no differences in animal body weight gain among groups after five days of test diets (Figure 3.1). The ratio of liver weight to body weight at the time of necropsy also did not differ significantly among groups (Figure 3.2).

3.2. Plasma amino acids

Plasma creatine was higher when creatine was added to the base diet of piglets (Table3.1). Supplementing GAA led to higher plasma creatine concentration only when methionine was excess in the diet compared to the Base-GAA diet. An excess methionine and arginine level in the diet led to higher plasma creatine concentrations compared to the Base and Base-GAA groups.

Table 3.1: Plasma concentration of metabolites after 5 days of treatment

	Diet Treatment				
	Base	**Base-+GAA**	**Base+ CRE**	**Excess Arg/Met**	**GAA /Excess Met**
Sulfur Amino Acid Metabolites					
Methionine	150±53[a]	121±35[a]	152±31[a]	226±56[b]	233±59[b]
Homocysteine	20±7.9[a]	18±7.4[ab]	11±2.0[b]	10±3.0[b]	14±4.4[ab]
Cysteine	168±20[a]	178±45[a]	154±23[a]	130±37[b]	158±32[a]
Taurine	170±28[a]	165±25[a]	167±29[a]	240±49[b]	253±27[b]
Glutathione	2.22±1.34	2.09±2.01	1.96±0.97	0.85±0.26	1.61±0.55
Glycine	1679±615[a]	1636±425[a]	1636±249[a]	1004±166[b]	1584±201[a]
Serine	1098±323[a]	1003±247[a]	1029±220[a]	574±125[b]	874±238[ab]
Indispensable Amino Acids					
Arginine	71±19[a]	71±23[a]	83±21[a]	427±97[b]	71±16[a]
Histidine	90±29	86±20	116±33	80±14	105±15
Isoleucine	251±50	238±27	257±30	230±47	240±74
Leucine	605±189	531±103	584±96	521±87	560±138
Lysine	484±98	464±126	438±129	343±95	472±145
Phenylalanine	119±26	125±15	124±18	151±20	129±32
Threonine	535±71	515±124	597±140	451±130	480±118
Tryptophan	84±20	80±20	81±19	87±23	80±34
Valine	517±91	486±41	503±78	489±80	469±75
Dispensable Amino Acids					
Alanine	1789±596[a]	1423±332[a]	1528±420[a]	607±112[b]	1538±392[a]
Aspartate	22±9.8	19±10	22±10	18±9.9	28±6.5
Hydroxyproline	107±28	90±23	89±33	80±25	88±15
Proline	811±214	749±128	837±202	892±257	840±186
Tyrosine	160±88	135±52	118±26	158±45	114±26
Glutamate	121±21	120±49	152±39	127±39	165±40
Glutamine	455±111[a]	338±102[a]	426±93[a]	168±93[b]	367±115[a]
Citrulline	177±48	149±62	128±45	97±39	141±62
Ornithine	80±16[a]	83±14[a]	84±35[a]	191±60[b]	73±22[a]
Other Metabolites					
Creatine	130±19[a]	129±44[a]	364±81[b]	284±141[b]	325±63[b]
Creatinine	30±4[a]	32±4[a]	56±14[b]	36±6[a]	38±7[a]
GAA	5.9±1.8[a]	6.1±2.1[a]	8.0±4.6[a]	12±4.3[b]	6.0±1.8[a]

Columns represent means (μM) (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Means not sharing a superscript letter were different.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine

A deficient dietary methionine level led to decreased plasma methionine and taurine concentrations compared to piglets on diets with excess methionine (Table 3.1). Higher plasma taurine concentration with excess dietary methionine diet could suggest that there is lower synthesis of taurine in piglets on methionine-deficient diets. Arginine deficiency in the diet led to a decrease in plasma arginine, ornithine and GAA concentrations. Piglets with excess dietary arginine showed a reduction of plasma glycine concentration compared to piglets in arginine-deficient groups. Piglets on an arginine-deficient diet had higher plasma glutamine concentrations when compared to the group with excess arginine. Interestingly, GAA supplementation did not increase plasma GAA concentration in Base-GAA and GAA/Excess Met groups. However, the plasma GAA concentration was higher in the group that received Excess Arg/Met. The plasma homocysteine concentration was lower in the Base-CRE and Excess Arg/Met groups compared to the Base group. There were no differences in the plasma glutathione concentration among groups. The group that received Excess Arg/Met had lower plasma cysteine compared to other groups. The plasma serine was lower in Excess Arg/Met group compared to Base, Base-GAA and Base-CRE groups but not different from the GAA/Excess Met group.

3.3. Hepatic free amino acids and metabolites

Unlike plasma concentrations, the hepatic methionine concentration did not change in piglets with different levels of dietary methionine (Table 3.2). However, hepatic SAM and taurine concentrations were reduced in methionine-deficient groups when compared to groups with excess methionine.

Table 3.2: Hepatic concentration of free amino acids at the end of study

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	**GAA /Excess Met**
Sulfur Amino Acid Metabolites					
Methionine	109±24	106±25	124±33	125±40	133±38
SAM	34±12[a]	35±11[a]	36±11[a]	119±9[b]	76±16[c]
SAH	21.9±2.7[a]	22.5±3.9[ab]	20.0±3.7[a]	26.8±4.1[b]	24.5±1.3[ab]
SAM/SAH	1.6±0.6[a]	1.6±0.6[a]	1.8±0.6[a]	4.5±0.8[b]	3.1±0.7[c]
Homocysteine	438±112	497±60	488±76	409±101	437±77
Cysteine	9354±2172	10470±1635	10049±2543	9041±2990	7487±1545
Taurine	7676±1006[a]	7720±2177[a]	7361±1058[a]	9812±2159[b]	10132±1116[b]
Glutathione	18806±5392	17436±4252	16332±5070	22061±5138	21887±3373
Glycine	1446±247[a]	1239±55[a]	1256±235[a]	573±210[b]	1181±380[a]
Serine	4233±1085[a]	4205±942[a]	3892±1073[a]	3083±651[ab]	2638±536[b]
Indispensable Amino Acids					
Arginine	218±28	211±90	230±49	251±58	238±83
Histidine	370±31	358±46	375±83	395±54	400±71
Isoleucine	246±29	235±36	256±36	236±35	246±53
Leucine	521±62	489±90	532±102	505±81	503±118
Lysine	788±90	669±85	750±151	677±172	655±206
Phenylalanine	135±13	138±18	142±23	153±24	138±31
Threonine	537±81	546±98	591±122	595±128	455±100
Valine	453±49	442±44	451±58	455±64	433±79
Indispensable Amino Acids					
Alanine	2489±457	2061±713	2213±587	1564±512	2417±666
Aspartate	1042±195	1042±207	1090±250	915±260	993±327
Hydroxyproline	137±47	127±33	125±37	118±23	115±16
Proline	985±257	1028±322	1011±300	1481±633	1082±219
Tyrosine	193±42	164±39	153±25	180±73	148±51
Glutamate	1795±398	2065±430	2002±403	1503±461	1503±415
Glutamine	1446±247[a]	1239±55[a]	1256±235[a]	597±177[b]	1181±380[a]
Citrulline	159±51	138±30	159±52	123±37	117±22
Ornithine	358±89[a]	348±98[a]	379±158[a]	1256±304[b]	318±128[a]
Other Metabolites					
Creatine	918±317[a]	978±855[a]	2694±1177[ab]	3767±2012[bc]	5733±2781[c]
GAA*	18±2	18±5	12±2	16±4	19±7
PE	9057±3104	8465±2653	8299±2319	7327±1617	6962±943
PC	14409±3736	15758±3684	14767±3410	17079±4067	16848±3611
PE/PC	0.71±0.34[a]	0.52±0.12[ab]	0.57±0.17[ab]	0.44±0.12[ab]	0.40±0.10[b]

Columns represent means (nmol/g) (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Means not sharing a superscript letter were different.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine

***** Hepatic GAA data were analyzed by O. Chandani Dinesh for her thesis studies.

Hepatic SAM concentrations were reduced when piglets on excess methionine were supplemented with GAA compared to piglets with excess dietary Arg/Met. There was a reduction in SAM:SAH ratio in methionine-deficient groups compared to groups with excess methionine. A reduction in the SAM:SAH ratio was observed when the excess methionine diet was supplemented with GAA compared to the group with Excess Arg/Met. Hepatic SAH concentration was higher in Excess Arg/Met group compared to Base and Base-CRE groups.

Supplementing GAA led to higher hepatic creatine concentration only when methionine was excess in the diet when compared to the Base-GAA diet. Also, excess arginine and methionine diet led to higher hepatic creatine concentration compared to the base diet. Supplementation of the base diet with creatine resulted in a ~3-fold increase in hepatic creatine but there was also a 4-5-fold increase in hepatic creatine in Excess Arg/Met and GAA/Excess Met groups compared to the base group.

The hepatic concentration of arginine did not change with dietary arginine levels. However, hepatic ornithine concentration was lower with arginine-deficient diets. Also, there was a higher level of hepatic glutamine in arginine-deficient groups compared to the group with excess arginine. Excess arginine in the diet led to a reduction in hepatic glycine concentration when compared to other groups. Serine concentrations were lower in GAA/Excess Met groups compared to Base, Base-GAA and Base-CRE groups. There were no differences in hepatic PC and PE concentration among groups but there was a reduction of PE: PC ratio in GAA/Excess Met group compared to the Base group. Similar to findings in plasma, GAA supplementation did not increase hepatic GAA concentration in Base-GAA and GAA/Excess Met groups.

3.4. Skeletal muscle free amino acids and energy metabolites

Table 3.3: Muscle concentration of metabolites

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	**GAA/ Excess Met**
Methionine	106±35[a]	116±23[a]	122±19[a]	188±36[b]	179±28[b]
Creatine	25375±5394	26132±917	25977±2389	28439±2953	27127±5885
Taurine	5285±1045	5155±790	4947±743	5221±743	5192±869
Glycine	2219±484[a]	1927±487[a]	2190±654[a]	610±325[b]	1735±596[a]
Phosphocreatine	5535±1820	5537±2403	6501±2615	5653±2994	5539±2691
ADP	680±116	731±104	653±116	776±238	659±158
ATP	3052±370	3544±255	3128±421	3150±462	3344±414

Columns represent means (nmol/g) (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Means not sharing a superscript letter were different.

ADP, Adenosine 5′-diphosphate; ATP, Adenosine 5′-triphosphate; Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine

A deficient level of dietary methionine led to lower muscle methionine concentrations compared to diets with excess methionine (Table 3.3). In contrast to plasma and liver concentrations, there were no differences in muscle taurine and creatine among groups. Also, there were no differences in muscle phosphocreatine, ATP and ADP among groups. Excess arginine in the diet led to a reduction in muscle glycine concentration compared to other groups, perhaps suggesting that glycine was consumed during creatine synthesis.

3.5. Kidney creatine and GAA concentrations

Table 3.4 Kidney concentration of metabolites

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	**GAA/Excess Met**
Creatine*	363±105[a]	443±291[a]	1009±427[b]	952±437[b]	1135±461[b]
GAA*	345±113[a]	388±187[a]	259±129[a]	645±344[b]	274±82[a]

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Means not sharing a superscript letter were different.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine; GAA; Guanidinoacetic acid

* Kidney GAA and creatine concentrations were analyzed by O. Chandani Dinesh for her thesis studies.

A deficient level of dietary arginine led to lower kidney GAA concentrations compared to diets with excess arginine. Supplementing GAA led to higher renal creatine concentration only when methionine was excess in the diet when compared to the Base-GAA diet. Also, excess arginine and methionine in diet led to higher renal creatine concentration compared to the base diet. Supplementation of the base diet with creatine resulted in a ~3-fold increase in renal creatine.

3.6. Methionine kinetics

3.6.1. Plasma methionine kinetics and methionine flux

3.6.1.1. Methionine enrichment

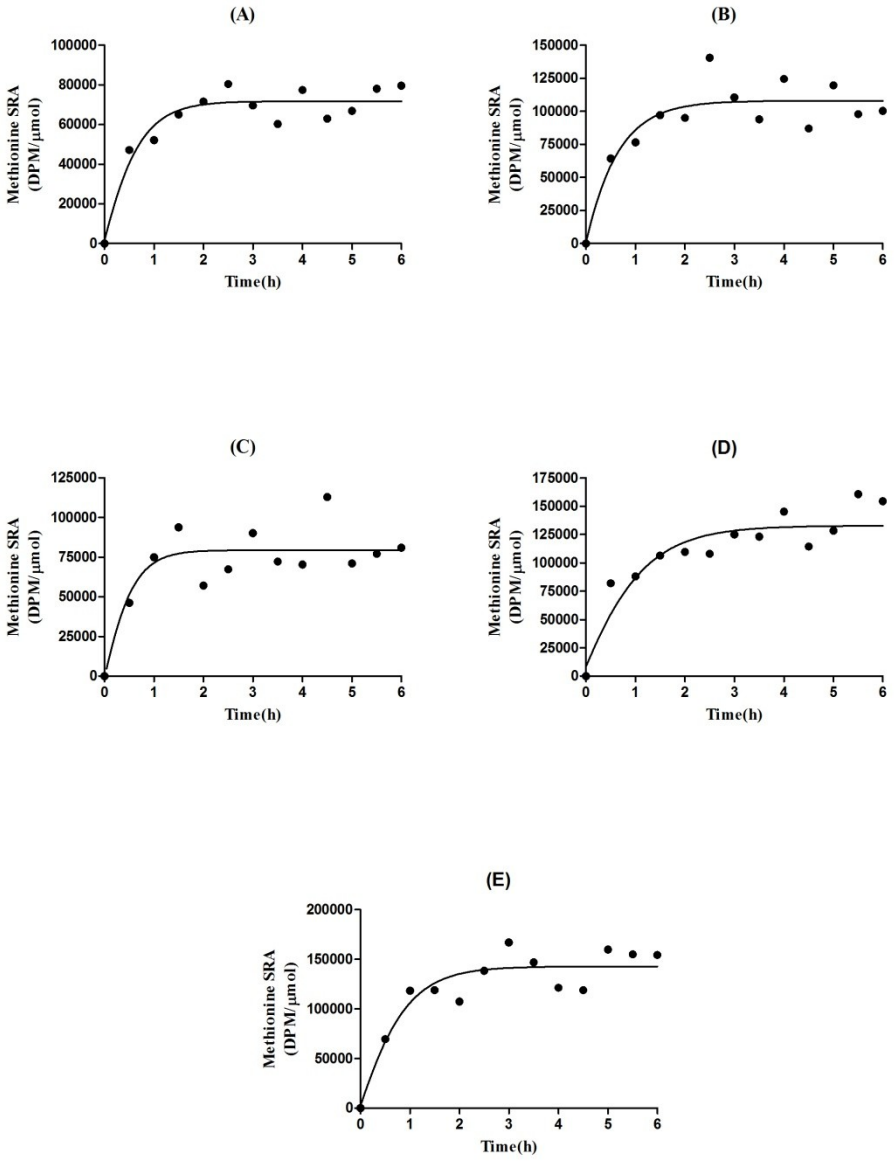

Figure 3.3: Plasma methionine SRA over time in representative pigs from each test group that received gastric infusions of L-[methyl-^{3}H] methionine at a constant rate of 30 µCi/kg/h.

(A) Base, (B) Base+GAA, (C) Base+CRE, (D) Excess Arg/Met, (E) GAA/Excess Met

Arg, Arginine; DPM, Disintegrations per minute; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine; SRA, Specific radioactivity

As shown in Figure 3.3, a steady-state of plasma isotopic enrichment of L-[methyl-^{3}H] methionine was achieved during 4 h of constant infusion. Figure 3.4 shows the average methionine SRA in plasma for each group. The mean plasma enrichment between 4 to 6 h was used to calculate the methionine SRA in plasma and methionine flux. There were no differences in either mean methionine SRA or methionine flux between methionine deficient and methionine excess diets.

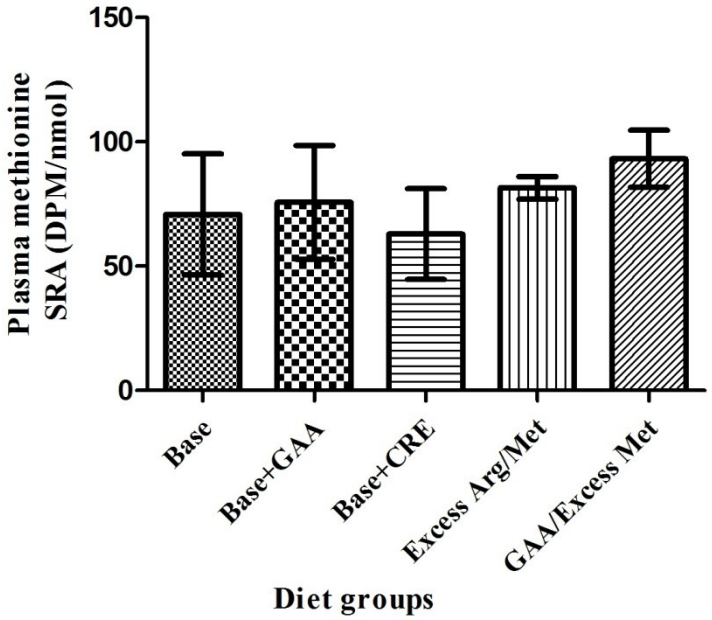

Figure 3.4: The mean plasma SRA of methionine between 4 and 6 h of infusion of L-[methyl-^{3}H] methionine (ie at steady state).

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets.

Arg, Arginine; DPM, Disintegrations per minute CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine; SRA, Specific radioactivity

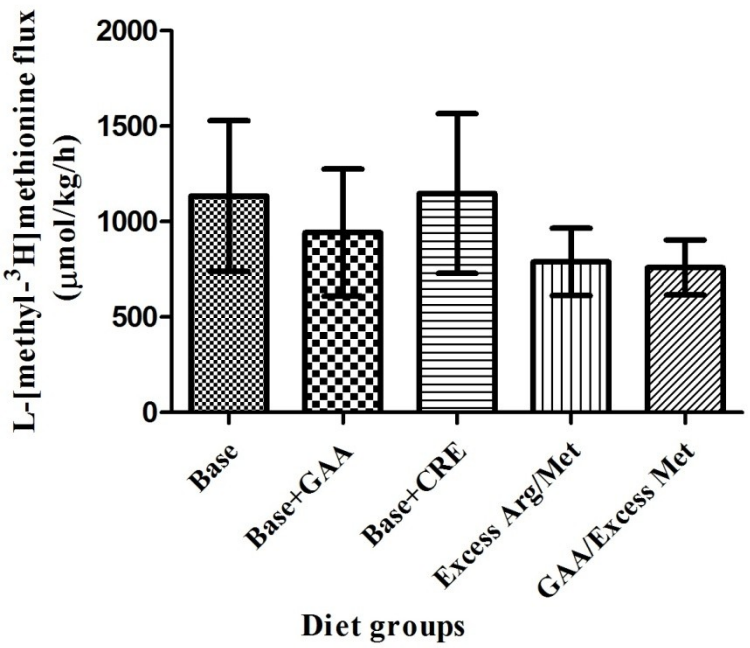

Figure 3.5: Whole body methionine flux of different diet groups.

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine

3.6.1.2. Plasma creatine specific radioactivity

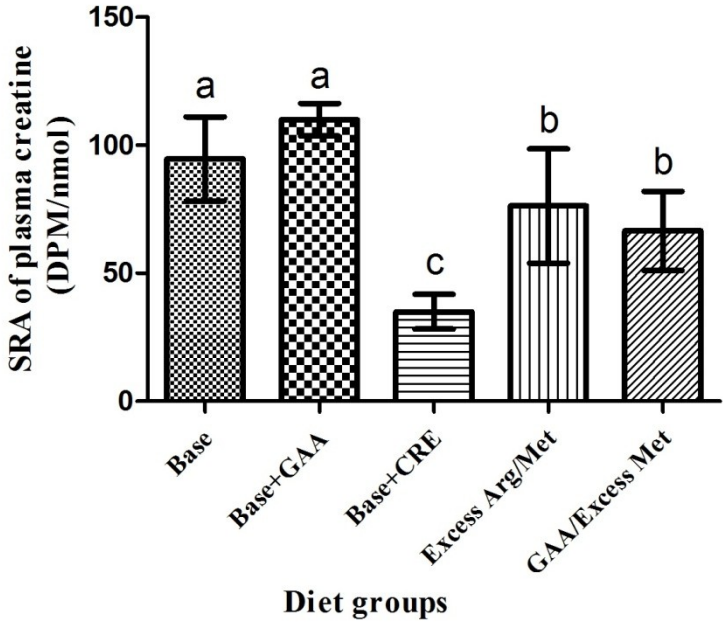

Figure 3.6: SRA of plasma creatine in different diet groups at the end of L-[methyl-^{3}H] methionine infusion for 6 h.

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons (α = 0.05) were used to test for differences between diets. Significant differences between groups are shown by the different letters above each column.

Arg, Arginine; DPM, Disintegrations per minute CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine; SRA, Specific radioactivity

The SRA of the plasma creatine was higher in Base and Base-GAA groups compared to Excess Arg/Met and GAA/Excess Met groups. Supplementing creatine led to lower plasma creatine SRA compared to other groups (Figure 3.6).

3.6.2. Hepatic methionine kinetics

3.6.2.1. Specific radioactivity

Table 3.5: Specific radioactivity (SRA) of hepatic metabolites

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	**GAA /Excess Met**
Free Met	81±8	70±37	76±32	93±16	95±16
Protein-bound Met	11.0±1.9	10.9±2.8	13.9±1.7	10.7±2.5	10.2±2.2
Creatine	78±14[a]	65±13[a]	47±6[b]	73±12[a]	76±14[a]
PC	10±3[a]	12±5[a]	19±6[b]	25±7[b]	22±5[b]
SAM	119±28	123±25	145±48	127±16	150±21
DNA	0.80±0.08[a]	0.93±0.08[a]	0.91±0.07[a]	1.15±0.25[b]	1.21±0.19[b]

Columns represent means (DPM/nmol) (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Means not sharing a superscript letter were different.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine; SAM, S-adenosylmethionine

There were no differences in the SRA of hepatic tissue free methionine, hydrolyzed (ie protein-bound) methionine or SAM among groups. In creatine supplemented pigs, the SRA of hepatic creatine was low compared to other groups. The SRA of the hepatic PC was higher in Base-CRE, Excess Arg/Met and GAA/ Excess Met groups compared to other groups. Groups with excess methionine had a higher SRA of hepatic DNA compared to methionine-deficient groups.

3.6.2.2. Hepatic fractional synthetic rate

Table 3.6: Fractional synthetic rate of transmethylation products and protein in liver

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	GAA/Excess Met
Creatine	11.4±3.0[a]	9.3±2.8[a]	5.7±1.1[b]	9.8±2.7[a]	8.7±2.5[a]
PC	1.52±0.63[a]	1.75±0.61[a]	2.22±0.90[a]	3.32±1.01[b]	2.46±0.64[a]
Protein	2.3±0.5	2.7±1.3	2.7±1.5	2.0±0.6	1.8±0.5
DNA	0.045±0.010	0.050±0.011	0.048±0.012	0.059±0.014	0.052±0.009

Columns represent means (%/h) (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Means not sharing a superscript letter were different.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine

The fractional synthetic rate of creatine was lower in piglets supplemented with creatine when compared to other groups. The fractional synthetic rate of hepatic PC was significantly higher in Excess Arg/Met group compared to other groups. There were no differences in the fractional synthetic rate of hepatic protein and DNA among groups.

3.6.2.3. ^{3}H-Methyl products remaining in the liver at necropsy

Table 3.7: ^{3}H-Methyl products remaining in the liver after 6 h constant infusion with L-[methyl-^{3}H] methionine

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	**GAA / Excess Met**
Creatine	74±35^{a}	76±51^{a}	114±57^{a}	202±148^{a}	372±122^{b}
PC	137±43^{a}	174±85^{a}	223±80^{ac}	343±58^{bd}	287±78^{cd}
DNA	7.8±0.7^{a}	9.0±1.4^{ab}	9.1±1.3^{ab}	11.0±3.2^{b}	11.0±2.4^{b}
Free Met	8.8±2.2	8.5±2.6	8.7±1.7	11.5±4.1	12.4±3.4
Protein-bound Met	66±33	70±32	90±23	67±35	75±34
SAM	4.0±1.2^{a}	4.4±2.0^{a}	5.6±3.3^{a}	15.0±2.2^{b}	11.0±2.2^{c}

Columns represent means (x1000 DPM/g) (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Means not sharing a superscript letter were different.

Arg, Arginine; CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine; SAM, S-adenosylmethionine

After 6 h, newly synthesized hepatic creatine, derived from L-[methyl-^{3}H] methionine, was higher in GAA/Excess Met group compared to other groups. Newly synthesized hepatic PC, via transmethylation from L-[methyl-^{3}H] methionine, was higher in Excess Arg/Met and GAA/Excess Met groups compared to the Base and Base-GAA groups. In pigs on methionine-deficient diets, newly synthesized L-[methyl-^{3}H] SAM was lower compared to pigs with excess dietary methionine. There was a reduction in newly synthesized SAM, derived from L-[methyl-^{3}H] methionine in GAA/Excess Met group compared to the Excess Arg/Met group. There was higher newly methylated DNA, methylated by L-[methyl-^{3}H] methionine, in the Excess Arg/Met and GAA/Excess Met groups compared to the Base group. There were no

differences in the newly incorporated tissue free and protein-bound L-[methyl-^{3}H] methionine among groups.

Table 3.8: Proportion of the ^{3}H-methyl products remaining in the liver after 6 h constant infusion with L-[methyl-^{3}H] methionine

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	**GAA/Excess Met**
Creatine	22±4	27±18	25±11	27±13	48±8
PC	48±6	46±15	49±10	56±13	38±7
DNA	3.0±1.4	2.7±1.2	2.1±0.5	1.8±0.4	1.5±0.6
Free Met	3.3±1.2	2.7±2.1	2.0±0.5	1.9±0.8	1.7±0.5
Protein-bound Met	22±6	20±10	21±6	10±3	10±4
SAM	1.4±0.3	1.2±0.3	1.2±0.5	2.6±0.9	1.6±0.6
Total measured ^{3}H-products (DPMx1000/g)	**299±100**	**385±150**	**450±116**	**650±226**	**770±171**

Columns represent means (% of total measured ^{3}H-products) (± standard deviations).

Arg, Arginine; CRE, Creatine; DPM, Disintegrations per minute; GAA, Guanidinoacetic acid; Met, Methionine; PC, Phosphatidylcholine; SAM, S-adenosylmethionine

In pigs on methionine-deficient diets, ~20% of the dietary methionine was used for protein synthesis and ~75% for transmethylation products (Table 3.8). Also notable was that only 40-60% of dietary methionine remained in liver when methionine was deficient. When dietary methionine was excess, ~10% of the dietary methionine was used for protein synthesis and ~85% for transmethylation products. Overall, these data suggest that the vast majority of dietary methionine ends up in transmethylation products.

Supplementation with GAA doubled the proportion of dietary methionine ending up in creatine, but only when methyl groups were not limited by low methionine. Approximately half of the ^{3}H-methyl groups were diverted to PC synthesis in all groups, but the proportion in PC was somewhat lower in GAA/Excess Met group when creatine synthesis by GAA methylation was facilitated.

3.6.3. Skeletal muscle methionine kinetics

3.6.3.1. Specific radioactivity

A deficient level of dietary methionine led to a decreased SRA of the tissue free methionine but increased the SRA of the protein-bound methionine compared to the excess methionine groups. A similar trend was observed in newly incorporated methionine from L-[methyl-^{3}H] methionine in muscle tissue free and tissue bound methionine (Table 3.10).

Table 3.9: Specific radioactivity (SRA) of muscle metabolites

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	**GAA /Excess Met**
Free Met	54±15^{a}	57±20^{a}	56±31^{a}	133±41^{b}	143±19^{b}
Protein-bound Met	6.6±1.3^{a}	7.2±1.7^{a}	7.1±0.9^{a}	3.7±1.5^{b}	4.5±0.9^{b}
Creatine	1.8±0.6	1.6±0.5	0.9±0.3	1.4±0.6	1.3±0.4

Columns represent means (DPM/nmol) (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Means not sharing a superscript letter were different.

Arg, Arginine; CRE, Creatine; GAA; Guanidinoacetic acid; Met, Methionine

Table 3.10 ^{3}H-Methyl products found in the muscle after 6 h constant infusion with L-[methyl-^{3}H] methionine

	Diet Treatment				
	Base	**Base+GAA**	**Base+CRE**	**Excess Arg/Met**	**GAA /Excess Met**
Free Met	5.4±1.3^{a}	6.2±1.2^{a}	6.6±3.1^{a}	23.8±4.3^{b}	25.6±5.3^{b}
Protein-bound Met	122±22^{a}	131±12^{a}	122±24^{a}	65±17^{b}	88±19^{b}
Creatine	48±12^{a}	46±14^{a}	22±6^{b}	37±15^{ab}	33±11^{ab}

Columns represent means (x1000 DPM/g) (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons (α = 0.05) were used to test for differences between diets. Means not sharing a superscript letter were different.

Arg, Arginine; CRE, Creatine; GAA; Guanidinoacetic acid; Met, Methionine

3.5.3.2. Fractional synthetic rate of muscle protein

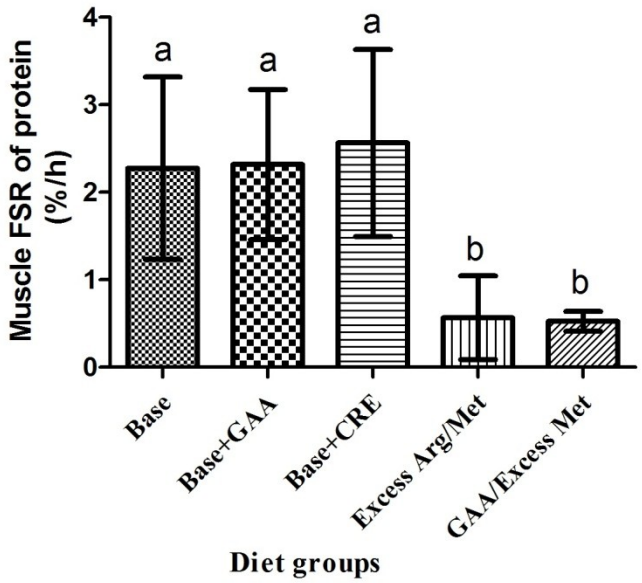

Figure 3.7: Effect of deficient methionine on protein synthesis.

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons (α = 0.05) were used to test for differences between diets. Significant differences between groups are shown by the different letters above each column.

Arg, Arginine; CRE, Creatine; FSR, Fractional synthetic rate; GAA, Guanidinoacetic acid; Met, Methionine

There were no differences in the SRA of muscle creatine among groups. In methionine-deficient pigs, the fractional synthetic rate of protein was ~4-fold higher than in pigs with excess methionine.

3.6. Enzyme activities

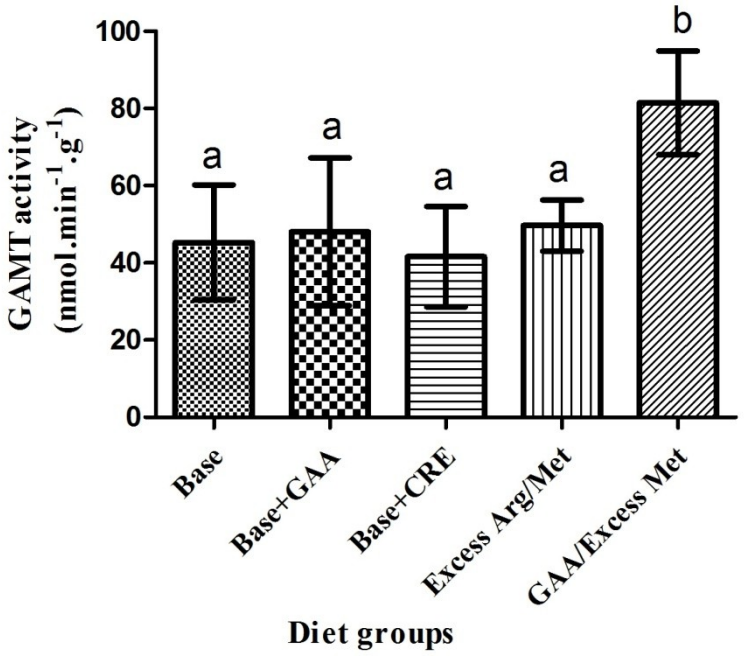

Figure 3.8: Effect of GAA and creatine feeding on liver GAMT activity

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Significant differences between groups are shown by the different letters above each column.

Arg, Arginine; CRE, Creatine; GAMT, Guanidinoacetate methyltransferase; GAA, Guanidinoacetic acid; Met, Methionine

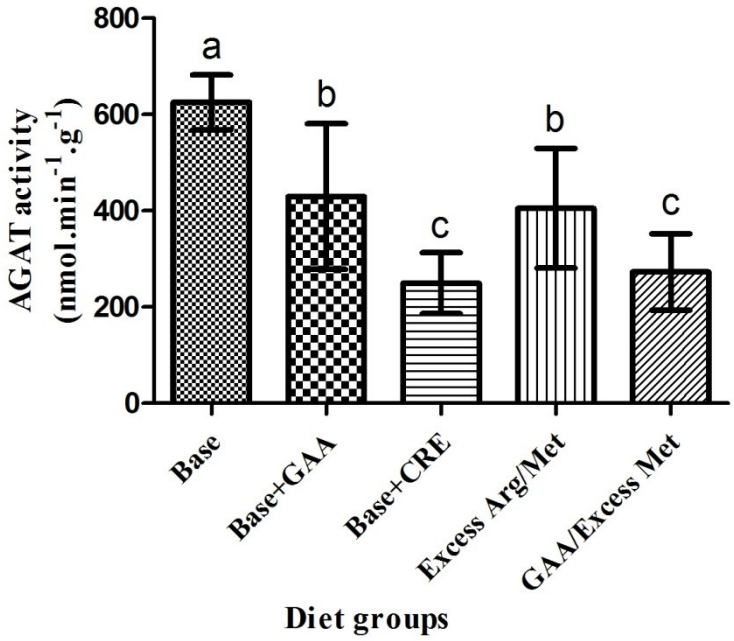

Figure 3.9: Effect of GAA and creatine feeding on kidney AGAT activity.

Columns represent means (± standard deviations). One-way ANOVA followed by Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to test for differences between diets. Significant differences between groups are shown by the different letters above each column.

Arg, Arginine; AGAT, Arginine:glycine amidinotransferase CRE, Creatine; GAA, Guanidinoacetic acid; Met, Methionine

Remark: Kidney AGAT activity was analyzed by O. Chandani Dinesh for her thesis studies.

Supplementation with GAA and excess methionine resulted in a significant increase of hepatic GAMT enzyme activity (Figure 3.8). Supplementing creatine or GAA with excess methionine led to lower AGAT activity compared to Base, Base+GAA and Excess arginine and methionine diet (Figure 3.9).

4. Discussion

When creatine was supplemented to the diet (Miller *et al.*, 1962; Guzik *et al.*, 2000) or injected intraperitoneally (Baker *et al.*, 1961), it did not induce growth in weaning piglets, likely because arginine and methionine, precursors for creatine, were not limiting in the first place. In order to observe the sparing effect of creatine or GAA, the diets should be marginally deficient in arginine and/or methionine. Therefore, in this study using arginine- and methionine-deficient diets, we investigated the effects of dietary creatine on sparing methionine and arginine for protein synthesis, and of its precursor GAA on sparing arginine for protein synthesis. We hypothesized that creatine and GAA supplementation can change the flux of transmethylation and protein synthesis in the liver by sparing the methyl groups needed from methionine for creatine synthesis. The main objectives of this study were to quantify the partitioning of transmethylation reactions in response to dietary GAA and creatine supplementation and to quantify the sparing effects of dietary GAA and creatine on protein synthesis.

4.1. Rationale of diet groups

The methionine requirement for gastrically-fed piglets is 0.25 g/kg/day (Shoveller *et al.*, 2003a). We chose to feed methionine at a rate of 0.20 g/kg/day, which is 80% of the piglets' requirement, to marginally impair protein synthesis and transmethylation reactions. The effects of this low methionine are evaluated with supplementation of GAA, creatine and excess methionine. In suckling piglets, the methionine intake from milk is estimated at 27.8 mmol or 4.1 g between 4 to 11 days of age (Brosnan *et al.*, 2009). Assuming an average body weight of 2.25 kg at this age,

this translates to a whole body methionine requirement of 0.26 g/kg/day, which is similar to the empirically determined estimate by Shoveller *et al.,* 2003a)

Suckling piglets gain 12.5 mmol of total creatine between 4 to 11 days of age (Brosnan *et al.*, 2009). Based on that finding, the total body creatine accretion rate at this age is 0.103 g/kg/day; so we infused 0.118 g creatine monohydrate/kg/day to account for this creatine accretion rate. Also, creatine down-regulates AGAT activity which would increase the arginine available for protein synthesis, provided arginine is deficient. Based on this creatine accretion rate, we calculated the amount of GAA that is enough to satisfy the amount of creatine that would be consumed and synthesized in piglets at this age; the molar equivalent of GAA to satisfy this creatine accretion rate is 0.093 g GAA/kg/day. GAA supplementation would also reduce the conversion of arginine into GAA, thus leading to further arginine availability for protein synthesis. In addition, the amount of methionine needed to convert GAA to creatine in suckling piglets is 0.12 g/kg/day (Brosnan *et al.*, 2009). Thus, if creatine is not provided in the diet, then the piglet requires approximately 0.38 g methionine/kg/day (0.26 plus 0.12 g/kg/day) to convert GAA to creatine in the GAA/excess methionine diet. Therefore, this 'excess' methionine was provided at a rate of 0.5 g/kg/day, which fulfils the requirement for the whole body, as well as the methyl groups needed for the conversion of GAA to creatine at a normal creatine accrual rate.

The arginine requirement for piglets is 0.38 g/kg/day (National Research Council, 1998), but in enterally-fed piglets receiving a graded dietary concentration of arginine, the breakpoint in the plasma concentration was ~0.6 g/kg/day (Brunton; unpublished work). Brunton and co-workers also demonstrated that ~0.6 g arginine/kg/day is required to maximize muscle protein synthesis in enterally-fed

neonatal piglets. Other studies showed that at least 0.2 g/kg/day was needed to avoid acute hyperammonemia (Wilkinson *et al.*, 2004; Urschel *et al.*, 2007). Therefore, we chose a 'marginally deficient' arginine level at a rate of 0.3 g/kg/day, which is 79% of NRC requirement.

The effects of low arginine were tested through comparisons with diets supplemented with GAA, creatine or excess arginine. Excess arginine was provided at a rate of 1.8 g/kg/day, which was metabolically sufficient for the whole body, and also accommodates whole body GAA synthesis, as calculated from creatine accretion during this age (Brosnan *et al.*, 2009). The purpose of excess arginine and methionine diet was to provide sufficient substrates to synthesize creatine at a maximum level without limiting other metabolic pathways. This treatment could be considered a 'positive' control situation with excess nutrients for all metabolic products, while the Base treatment can be considered the 'negative' control. GAA provided to the liver is rapidly converted to creatine with no feedback regulation at GAMT (Da Silva *et al.*, 2009; McBreairty *et al.*, 2013), which requires excess methyl groups. If methionine is limited in the Base-GAA diet, then GAA conversion to creatine could be limited by lack of methyl supply; so we supplemented GAA with excess methionine to enable creatine synthesis and ensure methionine is available for protein synthesis and for other methylated products.

4.2. Effects of creatine supplementation and methyl demand in neonatal piglets

A previous study in piglets reported that creatine synthesis may consume 66 to 77% of all of the labile methyl groups (Brosnan *et al.*, 2009). Therefore, creatine supplementation could increase the availability of methyl groups for other transmethylation reactions by reducing the labile methyl groups needed for creatine

synthesis. There was no increase in hepatic SAM in creatine supplemented pigs, suggesting creatine is not sparing labile methyl groups and expanding the SAM pool. We observed a 50% decrease in the fractional rate of de novo creatine synthesis in the liver when creatine was fed with the base diet. To support this finding, we also observed a similar trend in plasma creatine SRA in the creatine supplemented group compared to our base group. Creatine bio-synthesis is regulated by the enzyme AGAT. In rats, creatine supplementation down-regulates AGAT activity (McGuire *et al.*, 1984; Guthmiller *et al.*, 1994; Edison *et al.*, 2007; da Silva *et al.*, 2013). We also noticed a lower activity of AGAT in the kidney of the creatine supplemented group. As a result, GAA production should be reduced; but we did not see any reduction in plasma GAA or kidney and hepatic GAA in the creatine supplemented group. This may be because the amount of creatine fed was not high enough to alter the plasma GAA concentration, or more time was needed to see an effect on this outcome. We could not expaline the kinetic properties of AGAT as there is no data available on Michaelis-Menten constant (Km) of AGAT for pig. In contrast, creatine supplementation reduced the plasma GAA concentration in rats (Edison *et al.*, 2007; Da Silva *et al.*, 2009) and humans (Derave *et al.*, 2004). In those studies, rats were fed 4 g/kg of creatine for 2 weeks and humans were fed 20 g/day for 1 week followed by 5 g/day for 19 weeks, but these creatine supplementation levels are likely higher than the respective body accretion rates.

Consistent with other studies in rats (Walker, 1979; Da Silva *et al.*, 2009), there were no changes in the GAMT activity in the liver with creatine supplementation. This suggests that creatine supplementation did not down-regulate GAMT activity, in contrast to the down-regulation of AGAT activity. The reduction in

plasma homocysteine concentrations in the creatine-supplemented group compared to the base group also suggests that less transmethylation occurs with creatine supplementation. Consistently, it has previously been shown that dietary creatine lowers plasma homocysteine in rats (Stead *et al.*, 2001; Deminice *et al.*, 2009) and humans (Korzun, 2003).

The fractional synthetic rate of creatine represents the fraction of the total creatine pool that was synthesized. The estimated fractional synthetic rate is represented by the ratio of the SRAs of creatine and SAM. The lower hepatic fractional synthetic rate of creatine in creatine supplemented pigs could be due to either less creatine synthesis or due to a larger pool of creatine. However, I did not observe any difference in creatine DPM/g of liver in the creatine supplemented group compared to the base group, suggesting the larger creatine pool is likely diluting newly synthesized creatine thereby reducing its SRA. The similar DPM/g also suggests that creatine synthesis as a percent of methionine flux did not change (ie as much label ended up in creatine after 6 h regardless of how much creatine was fed). Moreover, stable isotope studies on these same animals demonstrated that there was no difference in the whole body rate of arginine conversion to creatine or GAA conversion to creatine in creatine-supplemented pigs compared to the base group (data from O. Chandani Dinesh thesis). Combined, these data suggest that creatine supplementation did not alter de novo creatine synthesis flux in spite of a down-regulation of AGAT specific activity. This disconnect between AGAT activity or capacity and actual whole body flux through AGAT warrants further investigation.

An increase in the SRA of hepatic PC was observed in the creatine supplemented piglets compared to the Base group; however, in spite of a similar trend,

the fractional synthetic rate of PC was not different. This higher PC SRA suggests that supplementing creatine spares methyl groups for PC synthesis via the PEMT pathway. Approximately 70% of the PC synthesis occurs via CDP-choline: 1,2-diacylglycerol choline phosphotransferase enzyme. The remaining 30% is synthesized via the PEMT pathway (DeLong *et al.*, 1999; Reo *et al.*, 2002; Li & Vance, 2008). Although the hepatic total PC concentration was not different in the creatine-supplemented group, it is possible that this alternate pathway compensates for higher PC synthesis via PEMT. Creatine supplementation did not increase protein synthesis or DNA methylation. This observation that creatine can spare methionine for PC synthesis, but not for protein synthesis, suggests that hepatic protein synthesis is conserved over transmethylation reactions when methionine is limiting.

Surprisingly, muscle tissue did not accumulate greater creatine in the creatine supplemented group. Supplementing creatine led to higher creatine concentrations in plasma and the liver, suggesting that the supplemented creatine was absorbed by the neonatal intestinal tract. In both animals and humans, total creatine pool in the muscle is increased when creatine is continuously supplemented (Harris *et al.*, 1992; Ipsiroglu *et al.*, 2001). Since the storage capacity of creatine is limited in skeletal muscles (Harris *et al.,* 1992) and the creatine concentration of biceps femoris muscle is relatively high (Mora *et al.,* 2008), it can be speculated that despite the ~2.5 fold increase in plasma creatine concentrations, muscle creatine concentration was at a maximal creatine storage capacity prior to supplementation. The creatine transporter, a high affinity transporter in the plasma membrane, has a Km of 20-50 μM, thus can accumulate creatine efficiently into the cell leading to an intracellular concentration in muscle in the range of 20-40 mM (Speer *et al.,* 2004). The plasma creatine

concentration was 360 μM in the creatine supplemented group, so it was also likely that the transporter was saturated, limiting creatine uptake into muscle. Therefore, supplementing creatine at a level that fulfills the daily accretion 'requirement' to marginally methionine deficient neonatal piglets was likely not significant enough to affect the massive creatine pool in the muscles in the short time frame of the study. Brosnan et al. reported that the liver has the highest GAMT activity in piglets with only 3% of that specific activity in muscle (Brosnan *et al.*, 2009). Therefore, muscle does not likely synthesize significant amounts of creatine, but should still be the main target tissue for creatine synthesized in the liver. Indeed, the pattern of plasma creatine SRA matches that of muscle creatine SRA suggesting that creatine in muscle is transported from the liver via the plasma.

4.3. Effects of GAA supplementation and methyl demand in neonatal piglets

Creatine synthesis is proportional to GAA availability in rats (Da Silva *et al.*, 2009) and piglets (McBreairty *et al.*, 2013). The liver is the primary organ for creatine synthesis. With GAA supplementation, plasma and liver creatine concentrations were 3-fold and 5-fold higher, respectively, when the methionine supply was in excess, compared to the Base-GAA group. Also, a significantly higher specific activity of GAMT in the GAA/Excess Met group compared to the Base-GAA group was observed, suggesting that enzyme activity was induced to accommodate the higher rate of creatine synthesis. This is best interpreted in terms of the kinetic properties of GAMT and the substrate concentrations. GAMT activity in the pig follows Michaelis-Menten kinetics and Km for SAM is 49 μM and for GAA is 27 μM (Walker, 1979). The plasma GAA concentration was ~6 μM in Base-GAA and GAA/Excess Met groups, so GAMT was not saturated. On the other hand, supplementing GAA to

methionine deficient diets limited conversion of GAA to creatine according to metabolite concentrations. However, there was no increase in the fractional synthetic rate of creatine in the GAA/Excess Met group compared to the Base-GAA group, in spite of a significantly higher DPM creatine/g of tissue in the GAA/Excess Met group. These data suggest that GAA/Excess Met led to rapid creatine synthesis and this newly synthesized creatine accumulated to a greater extent in the liver; however, when measured as a fraction of the massive creatine pool, the percent of this creatine pool synthesized per hour was not different.

Interestingly, GAA supplementation did not lead to higher plasma GAA or hepatic GAA suggesting that it is utilized immediately in the intestinal tract and/or liver to synthesize creatine, when the methyl supply is excess. However, when the methyl supply was limited and creatine was not being synthesized, supplemented GAA still did not increase the GAA concentration in plasma or liver. It is possible that GAA accumulated in other tissues not measured. GAA is known to be neurotoxic to the brain (Schulze *et al.*, 1997), so it is also possible that the body rapidly excreted unmetabolized GAA via urine or bile to prevent toxicity. In humans, it has been shown that GAA is excreted in urine (Ostojic *et al.*, 2013). Although not measured in the present study, it would be interesting to see the effect of dietary GAA on urinary GAA excretion.

GAA can spare arginine for growth in chicks (Almquist *et al.*, 1941). More recently, Dilger et al. demonstrated that GAA was as effective as creatine in sparing arginine for growth in broiler chicks, but only when arginine is deficient in diets (Dilger *et al.*, 2013). In enterally fed piglets, first-pass arginine synthesis does not change when arginine is deficient or in excess in the diet (Wilkinson *et al.*, 2004). The

lack of change in growth rate in our study suggests that GAA did not spare arginine for growth when arginine was deficient. This may be due to the fact that first-pass arginine synthesis was sufficient to maintain the growth rate, even with deficient dietary arginine. To support this hypothesis, no increases in plasma or hepatic arginine concentrations was observed when GAA was supplemented to the arginine deficient diet. Interestingly, a 2 h constant infusion of GAA via the portal vein increased hepatic arginine in piglets (McBreairty *et al.*, 2013). This higher hepatic arginine may have been utilized for non-protein functions in the liver including urea or polyamine synthesis.

GAA supplementation was also more successful at increasing hepatic creatine concentrations compared to creatine supplementation (Table 3.2). However, similar to the effect of creatine supplementation, muscle tissue did not accumulate creatine when creatine synthesis was increased in the liver, suggesting that the muscle creatine pool is maximized in neonatal piglets, or the pool size is too large to see a difference in only 5 days.

4.4. Methionine metabolism and methyl demand in neonatal piglets

Hepatic methionine concentrations did not change with deficient or excess dietary methionine. However, hepatic SAM concentration was higher when methionine was supplemented, suggesting that excess methionine was rapidly converted to SAM, perhaps as a mechanism to prevent hepatic methionine levels from becoming toxic. As a result, a higher SAM:SAH ratio was also observed when excess methionine was supplemented in the diet, compared to deficient methionine diets. The SAM:SAH ratio is often used as a transmethylation index (Rowling *et al.*, 2002). A lower ratio suggests a higher transmethylation rate. However, when comparing low

and high dietary intakes of methionine, the SAM:SAH ratio is likely dictated by methionine supply, rather than transmethylation rate. When comparing the two excess methionine diets, significantly lower hepatic SAM and SAM:SAH ratio were observed in the GAA/Excess Met diet, compared to the Excess Arg/Met diet, reflecting the higher transmethylation flux from GAA to creatine in that diet.

Approximately 70% of PC is synthesized via the Kennedy pathway and the remaining 30% is synthesized via the PEMT pathway. The hepatic PC concentration was similar among methionine-deficient and methionine-excess diets, but there was a significantly higher fractional synthetic rate of PC with excess dietary Arg/Met. This suggests that PC synthesis via PEMT was higher when the methyl supply was expanded with more dietary methionine. Excess Arg/Met led to proportionately more methyl incorporation into PC (~56%) versus creatine (~27%) (Table 3.8). This diet condition was considered a control situation with excess nutrients. However, supplementing GAA to this excess diet significantly reduced the fractional synthetic rate of PC in the liver, even though the methyl supply was excess as reflected in the change in hepatic distribution of methyl label into PC (~38%) versus creatine (~48%). This suggests GAA supplementation led to higher creatine synthesis which was facilitated at the expense of PC synthesis. Limited PC synthesis may lead to reduce VLDL secretion by hepatocytes, rsulting in hepatic triacylglycerol accumulation.

We also observed a 2 fold higher concentration of plasma GAA in the Excess Arg/Met group compared to the GAA/Excess Met group, but no difference in hepatic GAA. This suggests dietary GAA is immediately utilized by liver to synthesize creatine, as has been previously demonstrated in rats (Da Silva *et al.*, 2009); but in contrast to that study in rats, dietary GAA did not reach the post-hepatic circulation in

our piglets, perhaps because our dietary level was supplemented at a rate that was efficiently metabolized by the liver. In neonatal piglets, ~20-30% of dietary methionine is used by the gut including protein, transmethylation and transsulfuration reactions (Shoveller *et al.*, 2003b; Riedijk *et al.*, 2007). However, most of the remaining methionine is distributed in the body with a substantial proportion sequestered by the liver. Endogenous GAA is thought to be mainly synthesized in the kidney from arginine and exported to the blood for circulation to the liver via the renal vein and the caudal vena cava (Edison *et al.*, 2007). So higher plasma GAA concentrations in the Excess Arg/Met group are likely due to the fact that samples were taken from the 'post-renal' caudal vena cava where circulating GAA is higher, compared to the dietary GAA groups where little GAA appears in the post-hepatic vena cava.

Supplementing excess methionine did not change the fractional synthetic rate of hepatic protein compared to the deficient methionine diets, but it did change transmethylation rates. This suggests that a similar amount of dietary methionine is partitioned to hepatic protein synthesis, even when marginally deficient. Therefore, we might conclude that hepatic protein synthesis is conserved at the expense of transmethylation, when methionine is limiting. The hepatic protein synthesis result is in agreement with the work of Bauchart-Thevret and coworkers, who suggested that hepatic protein synthesis is preserved over methionine transmethylation in neonatal piglets when fed a methionine/cysteine free diet (Bauchart-Thevret *et al.*, 2009). If we use hepatic protein synthesis as a biomarker of methionine deficiency, then it could be argued that the Base diet was not 'deficient' in methionine. However, if

transmethylation was used as a marker, then methionine was deficient since PC synthesis via PEMT did respond to dietary methionine.

Hepatic protein turnover is higher than muscle protein turnover (Millward & Garlick, 1972) in rapidly growing piglets. We observed higher hepatic protein turnover than muscle protein turnover only in Excess Met/Arg and GAA/Excess Met groups. Unexpectedly, the deficient methionine diets increased the fractional synthetic rate of the muscle protein compared to the excess methionine diets. The reason why a deficient methionine diet results in more muscle protein remains unexplained, but this was a consistent observation that was verified by a separate study conducted in our lab using the same diets.

As the precursor for homocysteine, high dietary methionine generally induces hyperhomocysteinemia in rats (Stead *et al.*, 2001; Fukada *et al.*, 2006a). GAA-induced hyperhomocysteinemia has also been observed in rats (Stead *et al.*, 2001; Fukada *et al.*, 2006a) and in humans (Ostojic *et al.*, 2013). Interestingly, no difference was observed in plasma or hepatic homocysteine concentrations in GAA/Excess Met group compared to other groups. This may be due to interspecies differences or because GAA-induced hyperhomocysteinemia can be overcome by providing excess methionine. However, hepatic taurine concentration was higher in excess methionine groups compared to deficient methionine groups. Taurine is synthesized from cysteine, which is the only precursor for taurine synthesis and a limiting amino acid for glutathione synthesis (Tappaz, 2004). Higher hepatic and plasma taurine concentrations indicate that the transsulfuration pathway is more active when excess methionine is supplemented, which was expected. SAM is known as the allosteric activator of CBS (Prudova *et al.*, 2006; Riedijk *et al.*, 2007), the rate limiting enzyme

in the transsulfuration pathway (Riedijk *et al.*, 2007). Although I did not measure CBS activity, a higher SAM concentration in the excess methionine groups may have led to up-regulated CBS activity thereby facilitating transsulfuration. Also, lower homocysteine concentrations in plasma in the Excess Arg/Met group compared to the Base group may also be due to the activation of CBS by SAM. Moreover, methionine-induced hyperhomocysteinemia can be suppressed by serine and glycine as reported in rats (Fukada *et al.*, 2006b). Serine is a substrate for CBS enzyme. A lower hepatic serine concentration in the GAA/Excess Met group may suggest that serine was utilized by CBS in this group. Overall, these hepatic metabolite concentrations suggest that methionine deficiency leads to reduced hepatic and plasma taurine and reduced available methyl groups for SAM dependent methylation.

4.5. Arginine metabolism in neonatal piglets

In contrast to plasma, where arginine concentrations were higher with arginine supplementation, hepatic arginine concentrations did not change with deficient or excess arginine in diets. However, the hepatic ornithine concentrations were higher with excess arginine supplementation. This suggests that arginine entering the liver is disposed immediately via urea synthesis, similar to how hepatic methionine seems to be rapidly converted to SAM with supplemental methionine. The excess arginine supplementation also led to lower hepatic glycine. Because the arginine amidino group is transferred to glycine to produce GAA, these data are consistent with the significantly higher GAA and lower glycine in plasma in these groups, compared to arginine-deficient groups. Also, excess arginine led to lower serine concentrations which may be due to higher glycine synthesis from serine (Schlupen *et al.*, 2003).

Glutamine synthesis is the alternative pathway for ammonia disposal when arginine is deficient in the diet. Rapid increases in glutamine concentrations are observed in enterally fed neonatal piglets with no arginine in their diet (Brunton *et al.*, 1999). When arginine is limited in the diet, urea synthesis is hampered and resulting ammonia is removed by the glutamate/glutamine mechanism. This may lead to significantly higher hepatic glutamine concentrations, as observed in pigs on the low arginine diets compared to the excess arginine group. This also validates our model that presumes a dietary arginine intake at 0.3 g/kg/day is marginally deficient in neonatal piglets. Moreover, plasma GAA concentrations were also significantly lower in arginine deficient animals compared to animals with excess dietary arginine, which suggests that GAA synthesis is reduced when arginine is deficient.

In summary, supplementing creatine led to higher hepatic and plasma creatine concentrations but did not increase muscle creatine. It also led to a reduction of the fractional synthetic rate of creatine in the liver. For the other groups, higher hepatic and plasma creatine concentrations were observed only when the supply of methyl groups was expanded in the diet. The fractional synthetic rate of hepatic protein was not affected by supplementation with GAA and creatine, demonstrating that hepatic protein synthesis was conserved over transmethylation reactions when methionine was limiting.

5. Conclusion and future directions

Amino acids are expensive and half of the feed costs to domestic animal producers are comprised of amino acids and proteins. Therefore, feeding precursors or products of these amino acids could potentially reduce the cost of the production. In

this present study, we intended to measure the sparing effect of creatine and GAA on methionine and arginine.

In piglets, GAA supplementation is effective at increasing creatine stores, but only when methionine is excess, and at the expense of PC synthesis, but not hepatic protein. However, GAA supplementation does not seem to spare arginine for growth. We conclude that the demand for methionine should be considered when GAA is added to the diet so that methyl groups are not potentially limited. Another potential strategy is to provide excess remethylation precursors (ie betaine, choline, folate, serine) which could increase methionine availability by facilitating enhanced remethylation from homocysteine to methionine.

Creatine supplementation is effective, but not as effective as GAA, at increasing creatine stores, but it does not seem to spare methionine or arginine for growth and/or protein synthesis, in spite of feeding creatine and GAA well in excess of normal intakes from sow's milk. We fed creatine and GAA at levels that would have replaced any need for de novo synthesis of these metabolites, so methionine and arginine should have been spared from those pathways. At this time, the metabolic consequences of creatine supplementation on methionine metabolism remain unknown, especially on transmethylation flux. The lower PC synthesis has several implications for neonatal pigs. Although PC can be synthesized from preformed choline, PEMT-derived PC contains higher amounts of essential long chain fatty acids than PC synthesized from choline. Also, the sparing effects of dietary creatine and GAA on methionine and arginine for muscle protein synthesis and/or growth remain unknown in piglets. Therefore, future studies should investigate the effect of excess creatine supplementation on methionine interchange between transmethylation and

protein synthesis when methionine is excess in diet. Also, the effect of excess GAA supplementation on arginine interchange between protein synthesis and other metabolic pathways when arginine is excess in the diet needs to be investigated. Further studies are warranted in the field to observe the sparing effect of the above amino acids for protein synthesis and/or growth by simply measuring the growth rate and nitrogen balance. Because protein synthesis is easier to measure than protein breakdown, it is easy to misinterpret protein synthesis as protein balance. But if breakdown decreases proportionate to synthesis, then there is no net balance change. So a more complete investigation of protein balance is warranted before making assumptions about protein synthesis data.

The piglet amino acid requirement data are transferrable to the human infant after adjustments are made for the rapid growth rate of the piglets. Some of the commercial infant formulas (ie soy) are virtually creatine-free, while cow-milk-based formulas vary in creatine content. Therefore, supplemental arginine and methionine could be considered in such formulas to accommodate the potentially higher amino acid requirements for added creatine synthesis.

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Appendices

Appendix I: Amino acid profiles of adaptation diet and experimental diet

Amino acids	Adaptation diet		Base		Base+GAA		Base+CRE		Excess Arg/Met		GAA/Excess Met	
	g/kg/day	g/l	g/kg/day	g/l	g/kg/day	g/l	g/kg/day	g/l	g/kg/day	g/l	g/kg/day	g/l
Alanine	1.45	5.89	3.04	11.16	2.82	10.39	2.82	10.38	0.00	0.00	2.61	9.61
Arginine	0.94	3.65	0.30	1.10	0.30	1.10	0.30	1.10	1.80	6.62	0.30	1.10
Aspartate	0.94	3.32	0.94	3.47	0.94	3.47	0.94	3.47	0.63	2.31	0.94	3.47
Cysteine	0.22	0.76	0.22	0.83	0.22	0.83	0.22	0.83	0.22	0.83	0.22	0.83
Glutamate	1.63	5.72	1.63	6.00	1.63	6.00	1.63	6.00	1.63	6.00	1.63	6.00
Glycine	0.48	1.47	0.36	1.31	0.36	1.31	0.36	1.31	0.36	1.31	0.36	1.31
Histidine	0.48	1.69	0.48	1.76	0.48	1.76	0.48	1.76	0.48	1.76	0.48	1.76
Isoleucine	0.72	2.51	0.72	2.64	0.72	2.64	0.72	2.64	0.72	2.64	0.72	2.64
Leucine	1.62	5.67	1.62	5.94	1.62	5.94	1.62	5.94	1.62	5.94	1.62	5.94
Lysine-HCl	1.10	5.58	1.29	5.91	1.29	5.91	1.29	5.91	1.29	5.91	1.29	5.91
Methionine	0.30	1.04	0.20	0.74	0.20	0.74	0.20	0.74	0.50	1.84	0.50	1.84
Phenylalanine	0.82	3.00	0.61	2.24	0.61	2.24	0.61	2.24	0.61	2.24	0.61	2.24
Proline	1.29	4.52	1.29	4.73	1.29	4.73	1.29	4.73	1.29	4.73	1.29	4.73
Serine	0.87	3.11	0.87	3.19	0.87	3.19	0.87	3.19	0.87	3.19	0.87	3.19
Taurine	0.07	0.27	0.07	0.28	0.07	0.28	0.07	0.28	0.07	0.28	0.07	0.28
Tryptophan	0.33	1.14	0.33	1.21	0.33	1.21	0.33	1.21	0.33	1.21	0.33	1.21
Tyrosine	0.12	0.44	0.12	0.43	0.12	0.43	0.12	0.43	0.12	0.43	0.12	0.43
Valine	0.82	2.89	0.82	3.03	0.82	3.03	0.82	3.03	0.82	3.03	0.82	3.03
Threonine	0.82	2.23	0.82	3.03	0.82	3.03	0.82	3.03	0.82	3.03	0.82	3.03
GT*	0.00	0.00	0.38	1.63	0.38	1.63	0.38	1.63	0.38	1.63	0.38	1.63
Creatine (CMH**)	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.44	0.00	0.00	0.00	0.00
GAA	0.00	0.00	0.00	0.00	0.09	0.34	0.00	0.00	0.00	0.00	0.09	0.34

* Glycine-Tyrosine dipeptide

** Creatine monohydrate

Appendix II: D-glucose and major minerals concentrations in the diets

Composition	g/l of diet
D-glucose	90.3
Trihydrate K_2HPO_4	1.57
Monobasic KH_2PO_4	1.09
Potassium acetate	1.47
MgCl	2.17
$MgSO_4$	0.78
Calcium gluconate	6.41

Appendix III: Trace mineral concentrations in the diets

Elements	Minerals supplied	mg/kg of BW/day	g/l of diet
Zine	$ZnSO_4.7H_2O$	10.09	40.78
Copper	$CuSO_4.5H_2O$	0.86	3.12
Manganese	$MnSO_4.H_2O$	0.66	1.89
Chromium	$CrCl_3.6H_2O$	0.01	0.05
Selenium	SeO_2	0.05	0.06
Iodine	NaI	0.02	0.02

Appendix IV: Commercial multi-vitamin mix composition

Vitamins	Each 4 ml of vial 1 contains	Dose (per kg of BW/day)
Ascorbic acid (vitamin C)	80 mg	17.41
Vitamin A (as palmitate)	2300 IU	500.48
Vitamin D_3 (cholecalciferol)	400 IU	87.04
Thiamine (Vitamin B_1) (as the hydrochloride)	1.2 mg	0.26
Riboflavin (Vitamin B2) (as riboflavin-5-phosphate sodium)	1.4 mg	0.30
Pyridoxine HCl (Vitamin B6)	1 mg	0.22
Niacinamide	17 mg	3.70
Dexpanthenol (as d-pantathenyl alcohol)	5 mg	1.1
Vitamin E (dl-α-tocopheryl acetate)	7 IU	1.52
Vitamin K_1	0.2 mg	0.04
	Each 1 ml of vial 2 contains	
Folic acid	140 µg	4.35
Biotin	20 µg	30.46
Vitamin B_{12} (cyanocobalamin)	1 µg	0.22

Inactive ingredients in vial 1: 50 mg polysorbate 80, sodium hydroxide and/or hydrochloric acid for pH adjustment.

Inactive ingredient in vial 2: 75 mg mannitol, citric acid and/or sodium citrate for pH adjustment

Vial 2 was mixed with vial 1 just prior to use. 3 ml of the mixed solution was added to each 750 ml diet bag, to deliver the vitamin dose.