The effects of dietary methyl donors on methionine partitioning in the neonatal piglet

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Abstract

The metabolism of the indispensable amino acid methionine is critical during development. Methionine is used to synthesize protein for growth and, using the methionine cycle, it is the precursor of >50 critical nutrients and contributes to epigenetic regulation. Therefore, the dietary methionine requirement must factor all the potential roles of methioine. Three major processes summarize the methionine cycle: transmethylation (TM), which transfers methyl groups to nutrient precursors and DNA; transsulfuration (TS), which represents methionine disposal; and remethylation (RM), which resynthesizes methionine using the dietary methyl donors folate and choline (*via* betaine). Dietary intakes of folate vary drastically, and choline intakes are often below the adequate intakes during preganancy and in early life, which we hypothesized would influence the methionine requirement. To test our hypothesis, we fed 4-8 day old neonatal piglets a low-methionine diet that was either deficient (**MD-**), or replete (**MS+**), in dietary methyl donors. We evaluated how methionine was balanced between the major TM reactions and protein synthesis. The **MD-** group exhibited marked differences in TM as creatine synthesis was ≈30% less ($p<0.05$), and phosphatidylcholine synthesis was ≈60% more ($p<0.05$) during **MD-** feeding. Interestingly, while **MD-** feeding did not affect liver protein synthesis, the methionine availablity and protein synthesis were lower in skeletal muscle of the **MD-** *vs.* **MS+** animals ($p<0.05$). Furthermore, whole body protein turnover was also reduced during **MD-** feeding ($p<0.05$), which is significant as protein turnover is especially critical during infancy. Next, we measured the effect of methyl donors on the rates of TM, TS, and RM. The rates of RM and TM were

reduced by ≈75% in the **MD-** group ($p<0.05$), while TS was unchanged. In order to evaluate the effectiveness of individual methyl donors on RM, we 'rescued' a second group of **MD-** animals with betaine (**MD+B**), folate (**MD+F**) or both (**MD+FB**). The rate of RM and TM increased by ≈2-fold after rescue ($p<0.05$) and reduced protein breakdown ($p<0.05$). These studies showed that dietary methyl donors affect neonatal methionine metabolism, which should be considered when defining the dietary requirements of methionine during development.

For grandpa Reno,

Fiż-żgħażagħ nitgħallmu, fl-età nifhmu

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List of Abbreviations

5-CH_3-THF – 5-methyl tetrahydrofolate

AGAT – L-arginine: glycine amidinotransferase

AI – adequate intake

APE – atom percent excess

ASR – absolute synthetic rate

BHMT – betaine homocysteine methyl transferase

BSA – bovine serum albumin

CBS – cystathionine β synthase

CDP – cytidine diphosphate-choline

CGL – cystathionine ϒ lyase

DMG – dimethylglycine

DPM – disintegrations per minute

DTT - dithiothreitol

DNMT – DNA methyltransferase

EI – electron impact

FNC – fractional net conversion

FPF – fraction of product flux

FSR – fractional synthetic rate

GAA – guanadinoacetic acid

GAMT – guanidinoacetic acid methyltransferase

GCMS – gas chromatography mass spectrometry

GNMT – glycine N-methyltransferase

Hcy – homocysteine

HPLC – high-pressure liquid chromatography

IAAO – indicator amino acid oxidation

IUGR – intrauterine growth restriction

Ks – rate of tissue protein synthesis

MAT – methionine adenosyltransferase

Met – methionine

MPE – mole percent excess

MSyn – methionine synthase

MTHF - metyltetrahydrofolate

MTHFR – methyltetrahydrofolate reductase

PB – protein breakdown

PC – phosphatidylcholine

PD – protein deposition

PE – phosphatidylethanolamine

PEMT – phosphatidylethanolamine methyltransferase

PFBBr – pentafluorobenzyl bromide

Phe – phenylalanine

PS – protein synthesis

Q – flux

Q_C – [^{13}C] methionine flux

Q_M – [^{2}H-methyl] methionine flux

Q_{Met} – [^{3}H-methyl] methionine flux

Q_{Phe} – [^{13}C] phenylalanine flux

RM – remethylation

SAA – sulfur amino acids

SAH – S-adenosylhomocysteine

SAHH – S-adenosylhomocysteine hydrolase

SAM – S-adenosylmethionine

SHMT – serine hydroxymethyltransferase

SDH – sarcosine dehydrogenase

SRA – specific radioactivity (*ie.* DPM/amount)

THF – tetrahydrofolate

TM – transmethylation

TS - transsulfuration

1.0 Introduction

1.1 Methionine

Methionine is an indispensable, sulfur-containing amino acid required to synthesize protein, as well as a number of other critical nutrients during the methionine cycle (Figure 1.1). The methionine cycle transfers the terminal methyl group from methionine to various methylated products *via* transmethylation to form homocysteine, which can irreversibly transfer its sulfur atom for cysteine synthesis *via* transsulfuration. Therefore, a dietary methionine requirement must incorporate the demands of protein synthesis, transmethylation and transsulfuration. In addition, a methionine requirement must also consider that methionine can be replenished by preformed dietary methyl groups that remethylate homocysteine (Keller et al., 1949). Indeed, intakes of methionine and preformed methyl groups affect the methionine cycle in adults (Mudd and Poole, 1975), and rats can survive with homocysteine and preformed methyl groups in the absence of methionine (Du Vigneaud, 1952). However, it is presently unclear the extent that dietary methyl groups contribute to the methionine requirement.

In the body, homocysteine is methylated to methionine by methyl groups that are either derived from choline, which acts *via* betaine, or by methylneogenesis *via* folate. Therefore, the dietary supply of choline, betaine and folate should govern the rate of homocysteine remethylation, and affect the availability of methionine for partitioning into transmethylation and protein synthesis. Those processes are critical during development, and thus it is important to determine how dietary methyl donors contribute to the methionine requirement of the neonate. This

chapter will focus on the metabolism of dietary methyl donors, and rationalize their contribution and importance to methionine partitioning in early life.

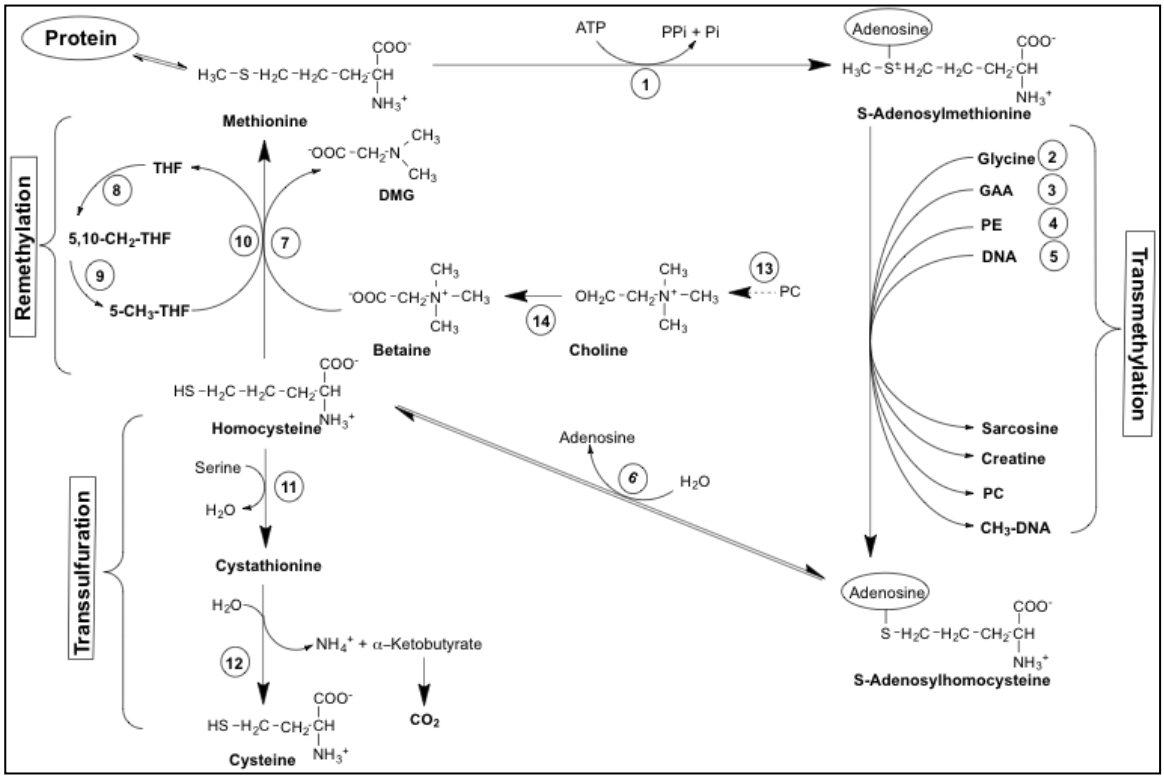

Figure 1.1 A schematic diagram of the relevant partitioning sites of methionine metabolism. The most obvious site of methionine partitioning is into protein. It is expected that the majority of dietary methionine is incorporated into proteins, however methionine is also made available after protein breakdown *ie.* protein turnover. Methionine is partitioned into the methionine cycle when methionine is irreversibly adenosylated to form *S*-adenosylmethionine (SAM). The labile methyl group of SAM is prone to enzymatic transfer during the process of "transmethylation". The majority of methyl groups are transferred to DNA and used to synthesize sarcosine, creatine and phosphatidylcholine (PC) during transmethylation. The common product of transmethylation is S-adenosylhomocysteine (SAH), which is maintained in equilibrium with homocysteine. Homocysteine has two fates, it can either be irreversibly oxidized towards cysteine during "transsulfuration", or it can receive a methyl group and reform methionine in a process called "remethylation". Remethylation essentially completes the methionine cycle and provides an additional source of methionine for subsequent partitioning into protein, or back into the methionine cycle. The ability of remethylation to affect methionine partitioning is the focus of this chapter. Circled numbers in the diagram indicate the enzymes: 1) Methionine adenosyltransferase (MAT; EC 2.5.1.6), 2) Glycine N-methyltransferase (GNMT; EC 2.1.1.20), 3) Guanidinoacetic acid methyltransferase (GAMT; EC 2.1.1.2), 4) Phosphatidylcholine methyltransferase (PEMT; EC 2.1.1.17), 5) DNA methyltransferase (DNMT; EC 2.1.1.204/37), 6) S-Adenosylhomocysteine hydrolase (SAHH; EC 3.3.1.1), 7) Betaine homocysteine methyltransferase (BHMT; EC 2.1.1.5), 8) Serine hydroxymethyltransferase (SHMT; EC 2.1.2.1), 9) Methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.15), 10) Methionine synthase (MSyn; EC 2.1.1.13), 11) Cystathionine β synthase (CBS;) 12) Cystathionine Y lyase (CGL; EC 4.4.1.1), 13) various phospholipases (mostly D; EC 3.1.4.4), 14) represents both choline dehydrogenase (EC 1.2.1.8) and betaine aldehyde dehydrogenase (EC 1.1.3.17).

1.2 Remethylation

The term "remethylation" refers to the enzyme-mediated transfer of methyl groups to homocysteine and the subsequent appearance of methionine. Under normal dietary conditions in adults, it was estimated that methionine proceeds through remethylation ≈1.5-2.0 times before being oxidized, but this estimation was approximately doubled during methionine-restriction (Mudd and Poole, 1975). Indeed, remethylation is a major metabolic process that is regulated by methionine flux. For instance, remethylation in neonatal piglets contributed ≈20% of whole body methionine flux regardless of whether or not dietary methionine and cysteine were provided (Bauchart-Thevret et al., 2009). Similarly, children derive ≈25% of methionine flux from remethylation regardless of nutritional status (Jahoor et al., 2006). Therefore, while remethylation contributes significantly to methionine metabolism, it may be limited by the supply of dietary methionine; and thus the extent that remethylation contributes to the methionine requirement is not straightforward.

Remethylation has garnered significant clinical interest due to the consequent removal of homocysteine. Indeed, a homocysteine elevation in blood (*ie.* hyperhomocysteinemia) is an independent risk factor of several chronic diseases (Wierzbicki, 2007), and furthermore, children that present with hyperhomocysteinemia are at significant risk of developing obesity and hypertension by adulthood (Osganian et al., 1999). While the sequelae of hyperhomocysteinemia are not within the scope of this chapter, a simple strategy to mitigate the risks associated with hyperhomocysteinemia is to eliminate

homocysteine by remethylation. Therefore, supplements of the dietary methyl donors folate (R. Clarke et al., 2010), betaine (Olthof and Verhoef, 2005) and choline (Olthof et al., 2005) have been administered to hyperhomocysteinemic patients to try to increase the rate of homocysteine elimination. While those trials have had mixed effectiveness at mitigating disease risk (R. Clarke et al., 2010; Strain et al., 2004), the study of homocysteine reduction has furthered understanding of how dietary methyl donors contributes to remethylation.

1.2.1 Folate

The generic term "folate" is used here to collectively describe the tetrahydrofolate (vitamin B9) compounds that deliver a labile methyl group to homocysteine from the one-carbon pool by methylneogenesis. Remethylation only represents a portion of folate biochemistry, and a broader review of folate metabolism is found elsewhere (Bailey and Gregory, 1999). Folate, as 5-methyltetrahydrofolate (5-CH_3-THF) provides a methyl group to homocysteine *via* the vitamin B12-containing enzyme methionine synthase (MSyn; EC 2.1.1.13). The loss of a methyl group from 5-CH_3-THF yields tetrahydrofolate (THF), which subsequently forms 5,10-methylenetetrahydrofolate (5,10-CH_2-THF) upon gaining a hydroxymethyl group largely from serine by the vitamin B6-dependent enzyme serine hydroxymethyltransferase (SHMT; EC 2.1.2.1). To a lesser extent, 5,10-CH_2-THF can also be formed from sarcosine (S. R. Davis et al., 2004; Du Vigneaud et al., 1946) by the riboflavin dependent enzyme sarcosine dehydrogenase (SDH; EC 1.5.8.3). Finally, a methyl group becomes available for remethylation when 5,10-

CH_2-THF is irreversibly reduced to 5-CH_3-THF by methylenetetrahydrofolate reductase (MTHFR; EC 1.5.1.15). Therefore, while serine is the predominant one carbon source for methylneogenesis (S. R. Davis et al., 2004), folate is required to deliver a labile methyl group to MSyn for remethylation.

The consumption of folate during development has long been a topical ussue due to its association with neural tube defects, such as spina bifida and anencephaly. As a result, the United States Centre for Disease Control recommended that foodstuff fortification with folate become mandatory (Houk et al., 1992). In Canada, this was realized in 1998 after which cereal grain products were fortified with folic acid, a synthetic form of folate. Today, clinical folate deficiencies are rare among Canadians (Gudgeon and Cavalcanti, 2014) as folate intakes are considered to be adequate, yet highly variable (Shakur et al., 2010). Furthermore, it is expected that most Canadian neonates are folate replete due to the folic acid fortification program, and folic acid supplementation during pregnancy and periconception (Houghton et al., 2009). However, this does not necessarily mean that remethylation demands are consistently fulfilled as it cannot be assumed that remethylation is an asymptotic function of folate intake. Moreover, it was demonstrated that homocysteine concentrations were unaffected by moderate vitamin B6 restriction in adults, which should have affected methylneogenesis (*via* SHMT) as well as the elimination of homocysteine by conversion to cysteine (*via* CBS) (Lamers et al., 2011). Furthermore, in an average adult, folate consumption provides ≈5-10 mmol methyl/d (Niculescu and Zeisel, 2002), which in a 70-kg person equates to ≈3-6 μmol/(kg•h) of preformed methyl groups. That alone is inadequate to fulfill

remethylation demands in pregnant women (17-27 μmol methionine/(kg•h)) (Dasarathy et al., 2010), infants (19-26 μmol methionine/(kg•h)) (Thomas et al., 2008) and piglets (10-49 μmol methionine/(kg•h))(Bauchart-Thevret et al., 2009). The point is that while dietary folate is a source of labile methyl groups, remethylation by MSyn relies upon methylneogenesis which involves no fewer than 4 vitamins and 5 enzymes. The contribution and significance of MSyn will be addressed in more detail herein.

The enzymes of folate metabolism are susceptible to numerous genetic polymorphisms that predict the preponderance of hyperhomocysteinemia (Frosst et al., 1995; Jacques et al., 1996), and thus presents an additional complexity to understanding the contribution of MSyn to methionine partitioning. Indeed, polymorphisms of the MTHFR gene are associated with fetal viability (Isotalo et al., 2000), and MTHFR knockout mice exhibit an 80% mortality rate (Schwahn et al., 2004). This is of concern due to the frequency of various MTHFR polymorphisms which are >50% in some populations (Guéant-Rodriguez et al., 2006); moreover, the frequency of MTHFR polymorphisms is increasing in populations with high folic acid intakes (Guéant-Rodriguez et al., 2006; Lucock and Yates, 2005). Regardless, any polymorphism of folate metabolism might hinder the capacity of MSyn to perform remethylation, as would low intakes of folate, riboflavin, vitamin B6 and vitamin B12. Of particular relevance is vitamin B12, which forms the MSyn cofactor, cyanocobalamin. Low vitamin B12 status is a risk for vegetarians and is common among residents of developing countries (Allen, 2009), and because maternal vitamin B12 intake is predictive of fetal B12 status (Hussein et al., 2009), it is

important to study the early life consequences of reduced MSyn activity. However, because B12 deficiencies take months to develop, and because serine is dispensable in neonates, the acute manipulation of dietary folate represents the best means of understanding how MSyn contributes to methionine partitioning in early life.

1.2.2 Choline

Choline (N,N,N-triethylethanamonium) is an essential nutrient with a complex metabolism as it does not only arise from methionine *de novo* (see 1.4.1), but it is also obtained in the diet as either free choline, glycerophosphocholine, phosphocholine or phosphatidylcholine (PC) (Zeisel et al., 2003). The capacity of choline to contribute to remethylation was first recognized when Simmonds *et al.* discovered that feeding rats a methionine-deficient diet with homocysteine and methyl-labeled choline supported growth, and moreover, that the methyl moiety of choline appeared in methionine (Simmonds et al., 1943).

Endogenous choline synthesis cannot fulfill the demand for this nutrient alone (Zeisel et al., 1991), and thus requirement levels have been determined for males (550 mg/d), females (425 mg/d), pregnancy (450 mg/d), lactation (550 mg/d), and infants (200 mg/d) (Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline, 1998; Zeisel, 2006). Choline is critical during development as evidenced by the massive transport of choline from placenta to fetus (McMahon and Farrell, 1985; Sweiry et al., 1986), the constant hepatic release of maternal choline into circulation throughout gestation and lactation (Velzing-Aarts et al.,

2005; Zeisel, 2006; Zeisel et al., 1995), and the high concentration of choline esters in breast milk, which are estimated to be ≈1500 μM (Holmes-McNary et al., 1996). Furthermore, like folate, periconceptional choline intake is predictive of neural tube defects (Shaw et al., 2004) and indeed, choline is critical for the developing rodent brain as evidenced by the permanent reduction in cognitive ability in response to early life choline restriction (Meck and C. L. Williams, 2003; Zeisel and da Costa, 2009). However, the choline content of breast milk depends on maternal choline intakes (Fischer et al., 2010; Ilcol et al., 2005), which is of concern because two-thirds of lactating women were reported as consuming choline well below requirement levels (Fischer et al., 2010). Furthermore, infant formulas provide a wide range of choline esters with soy-based formulas providing less than half the amount of choline compared to breast milk (Holmes-McNary et al., 1996).

While choline intakes are generally considered adequate, they are highly variable in many populations. Indeed, as much as 25% of an otherwise choline-replete population was shown to consume less than half the choline requirement (Zeisel, 2009). Furthermore, when factoring in genetic polymorphisms, as much as 50% of the population may have choline demands that are greater than current requirements (da Costa et al., 2006; Kohlmeier et al., 2005; Niculescu and Zeisel, 2002). While choline restriction alone results in liver damage (Buchman et al., 1995; Zeisel et al., 1991), the consequence of low or variable choline status on methionine partitioning is difficult to interpret due to the numerous fates of choline. Indeed, choline is the limiting substrate for PC synthesis by the CDP-choline pathway (reviewed by (Gibellini and Smith, 2010)), is the main component of the

neurotransmitter acetylcholine (Blusztajn and Wurtman, 1983), and is a major precursor of the labile methyl donor betaine (Weinhold and Sanders, 1973). Therefore, while the provision of choline has been shown to enhance remethylation in humans (Mudd and Poole, 1975), rats (Shinohara et al., 2006; Simmonds et al., 1943), and sheep (Lobley and Connell, 1996), the capacity of choline to participate as a methyl donor relies upon its oxidation to betaine.

1.2.3 Betaine

Betaine (N,N,N-trimethylglycine) is synthesized *de novo* in the mitochondria of liver and kidneys by the irreversible two-step oxidation of choline, by choline dehydrogenase (EC 1.1.99.1,) and betaine aldehyde dehydrogenase (EC 1.2.1.8) (Chern and Pietruszko, 1999). Therefore, while choline can theoretically meet betaine demands, dietary betaine can only spare its own synthesis by choline (Dilger et al., 2007). While it was estimated that a significant portion of ingested choline is converted to betaine in rats (Cheng et al., 1996; Weinhold and Sanders, 1973), the primary source of betaine in the body is through betaine consumption (Clow et al., 2008; Ross et al., 2014). The majority of betaine in the body is found within cells, where it contributes to osmotic balance (Lang, 2007), but the physiological importance of betaine metabolism has remained somewhat of an enigma in mammals. Recently, clinical applications of betaine metabolism have emerged that associate betaine intakes with various diseases (Craig, 2004; Lever and Slow, 2010). Indeed, in some cases of hyperhomocysteinemia, betaine supplementation was

effective at reducing homocysteine when folate supplementation was ineffective (Olthof and Verhoef, 2005; Ronge and Kjellman, 1996; Schwahn et al., 2004).

Betaine donates a methyl group to homocysteine by the zinc-dependent cytosolic enzyme betaine homocysteine methyltransferase (BHMT; EC 2.1.1.5), which yields methionine and dimethylglycine (DMG). BHMT is mostly active in the liver, but renal activity has also been reported (Finkelstein et al., 1971; McKeever et al., 1991; Sunden et al., 1997). Polymorphisms of the BHMT gene have been described (Feng et al., 2011; Park and Garrow, 1999), and are considered to occur at a high frequency within the population (da Costa et al., 2006; Morin et al., 2003). However, the utility of BHMT polymorphisms as a disease marker is currently unclear, but will likely change as the importance of betaine metabolism is linked to numerous pathologies (Ueland, 2010). Recently, BHMT knockout mice have been developed that exhibit a phenotype marked by the development of fatty liver and hepatocellular carcinoma. BHMT knockout mice are sexually viable but weigh less in early life compared to wild-type littermates (Teng et al., 2011), which is in agreement with the intrauterine, growth-restricted (IUGR) piglet model of fetal programming that exhibit low hepatic BHMT capacity (MacKay et al., 2012).

The developmental roles of betaine are not completely understood, but betaine metabolism changes drastically during pregnancy and early development. For example, in rats and humans, massive quantities of betaine are excreted in the urine after birth, and continue until urinary betaine excretion rates gradually diminish to adult levels by adolescence (Davies et al., 1988; Lever and Slow, 2010). While it is not clear if betaine excretion is due to immature kidney formation (Lever

and Slow, 2010), rat pups also accumulate dietary betaine in the kidney and liver until well after weaning (Clow et al., 2008). Because BHMT is detected in mammals after 10 days of gestation (Feng et al., 2011; Fisher et al., 2002) and is critical for inner cell mass formation (Lee et al., 2012), it does not appear that urinary betaine excretion is due to a lack of BHMT activity in early-life. Indeed, BHMT knockout mice present with massive intracellular betaine concentrations compared to wild-type mice after 5 weeks (Teng et al., 2011). Because BHMT is the only known mechanism of betaine catabolism, betaine excretion is only expected to occur once remethylation demands are fulfilled; however, the contribution of BHMT to remethylation in early life has not been confirmed *in vivo*, and the importance of betaine accretion and excretion in early life remains unknown.

During pregnancy, plasma betaine is a determinant of fasting homocysteine concentrations, and it has been suggested that betaine is a greater source of methyl groups in late pregnancy compared to folate (Ueland et al., 2005; Velzing-Aarts et al., 2005). Indeed, maternal plasma choline levels increase continuously during pregnancy, whereas plasma betaine levels decrease initially, and are maintained low until after birth (Velzing-Aarts et al., 2005). Meanwhile, preimplantation mouse embryos rapidly accumulate betaine, and blastocyst formation depends on the presence of betaine and BHMT (Lee et al., 2012). However, unlike choline and folate, there is no association between maternal betaine intake and neural tube defects (Fisher et al., 2002), and the importance of dietary betaine during gestation and early life is unknown. Regardless, dietary betaine should be considered both for its capacity to spare choline (Clow et al., 2008; Dilger et al., 2007; Shinohara et al.,

2006) and to enhance methionine availability. Moreover, reduced intakes of choline, folate and methionine are expected to enhance BHMT demands (Finkelstein et al., 1971; Park and Garrow, 1999), and it is suggested that all polymorphisms of methyl metabolism, which occur at a significant frequency within the population (da Costa et al., 2006; Guéant-Rodriguez et al., 2006; Zeisel and da Costa, 2009), would also enhance the metabolic demand for betaine. Further investigations into the fate of dietary betaine during gestation and early life are warranted.

Dietary intakes of betaine are not well described, but estimates suggest that it is highly variable. In the USA and New Zealand, betaine intakes were estimated as 1-2.5 g/d (Craig, 2004) and ≈0.5 g/d (Slow et al., 2005), respectively. However, a more recent estimate of 131 mg betaine/d was reported based on the consumption of a western diet (Ross et al., 2014). The situation in neonates is similar to adults, as betaine intakes are estimated to be highly variable but low during suckling (30-60 mg/d). Indeed, the betaine content of infant formulas were reported to be within the range of breast milk, which was largely dependent on maternal betaine intakes (Sakamoto et al., 2001). Taken together, all of the reported estimates of betaine intake are well below supplemental levels that often provide 3-6 g betaine/d (Olthof and Verhoef, 2005; Ronge and Kjellman, 1996; Storch et al., 1991). However, because there is no dietary betaine requirement, it is unclear how variable intakes of this methyl donor affect methyl metabolism, or if there is a demand for dietary betaine in early life.

The effects of betaine supplementation are complicated and indicate that remethylation by BHMT is tightly regulated. For example, in men supplemented

with 3 g betaine/d under otherwise normal dietary conditions, the whole body rate of remethylation was unchanged despite increased transmethylation and decreased protein synthesis (Storch et al., 1991). This is confusing due to convincing evidence that betaine is effective at enhancing remethylation in adults (Mudd et al., 1980; Mudd and Poole, 1975) and protein deposition in swine (Fernández-Fígares et al., 2002). Furthermore, betaine supplements enhanced remethylation in chickens (Pillai et al., 2006) and diminished circulating homocysteine concentrations in humans (Olthof and Verhoef, 2005). However, there are very few data to describe the *in vivo* effects of variable betaine intake in early life, especially in its capacity to furnish a portion of the methionine requirement.

As a final remark regarding the contribution of BHMT to methionine availability, it is important to note that betaine can also provide a labile methyl group for MSyn. Indeed, a product of BHMT, DMG, inhibits BHMT activity (Garrow, 1996) and participates in methylneogenesis upon conversion to sarcosine. This might explain why betaine (or choline) supplementation in chickens enhanced remethylation by MSyn, and *not* BHMT (Pillai et al., 2006). The point is that studies focused on how dietary methyl donors furnish remethylation, must also consider how folate, choline and betaine work alone as well as in concert to replenish methionine.

1.2.4 Dietary methyl donors

Remethylation is an intricate process that relies on the compensatory regulation of BHMT and MSyn *in vivo*. For example, studies in rats demonstrate that

a low-choline diet depletes hepatic folate (Varela-Moreiras et al., 1992), and that a low folate intake depletes hepatic choline (Kim et al., 1994). Furthermore, lowering folate availability either by dietary restriction or folate antagonists resulted in the reduction of hepatic betaine, and the rise in BHMT activity in rats (Barak et al., 1984; Barak and Kemmy, 1982). And when methylneogenesis was restricted in humans, BHMT flux compensated to maintain remethylation (S. R. Davis et al., 2005b), which is in agreement with others (Jacob et al., 1999). However, BHMT activity does not only contribute significantly to remethylation during folate restriction. For example, in rats the *in vivo* BHMT activity was conserved regardless of folate status (Du Vigneaud, 1952), and BHMT and MSyn were shown to contribute to remethylation equally (Finkelstein and Martin, 1984). Furthermore, choline restriction reduced remethylation more than folate restriction in rats (Shinohara et al., 2006), and betaine was more effective than folate at mitigating a homocysteine rise after a methionine-load in chickens (Pillai et al., 2006). It is clear that remethylation is a well-controlled process that is at least somewhat adaptive to substrate availability under normal conditions. However, the extent that BHMT or MSyn compensate for whole body remethylation is not known, nor is the potential sparing capacity of remethylation on the methionine requirement. Because methionine is a precursor for creatine, phosphatidylcholine, carnitine, sarcosine, sphingomyelin, polyamines, methylated DNA and moreover, tissue protein, it is critical that studies address how dietary methyl donors affect methionine partitioning for growth, metabolite synthesis and gene regulation. The remainder of this chapter will focus on the specific sites of methionine partitioning.

1.3 Methionine partitioning

1.3.1 Transmethylation

Transmethylation represents the rate of methyl group transfer from methionine to various methylated products, and can be considered as the nonprotein requirement of methionine (Bertolo and McBreairty, 2013). Transmethylation commences when the sulfur atom of methionine is irreversibly adenosylated to form S-adenosylmethionine (SAM) by the enzyme methionine adenosyltransferase (MAT; EC 2.5.1.6). SAM is considered to be the primary biological methyl donor (Catoni, 1953), a feature partially attributed to the presence of a sulfonium ion that causes the terminal methyl group to become labile due to the electrophilic nature of carbon. This labile methyl group of SAM is transferred to a myraid of metabolic precursors by methyltransferase enzymes that yield the common product, S-adenosylhomocysteine (SAH), which is in equilibrium with homocysteine by S-adenosylhomocysteine hydrolase (SAHH; EC 3.3.1.1).

The SAM molecule exerts allosteric control over methionine metabolism and its concentration reflects the current supply of methyl groups. Indeed an elevation in hepatic SAM occurs after a high-methionine intake (Finkelstein and Martin, 1986) and indicates that methyl groups are plentiful, and thus methionine disposal (*ie.* transsulfuration) is favoured over remethylation (Finkelstein, 2006). Furthermore, the SAM and SAH concentrations are often expressed as a ratio to provide an index of methyl status. Because SAH is an allosteric inhibitor of all known methyltransferase reactions (J. T. Brosnan et al., 2010), the SAM/SAH ratio provides

an indication of the rate of transmethylation, *ie.* a greater SAM/SAH ratio indicates that methyl groups are plentiful, and that homocysteine clearance is rapid. However, the SAM/SAH ratio must be interpreted with caution (Uthus and Brown-Borg, 2003; K. T. Williams and Schalinske, 2007), and cannot be assumed reflective of *in vivo* transmethylation. The point is that both transmethylation and the methionine cycle are governed by methyl group availability, and thus the supply of dietary methyl donors is expected to make a significant contribution to the transmethylation requirement.

Transmethylation represents a significant metabolic burden on dietary methionine. Indeed, 75% of hepatic methionine participates in transmethylation (McBreairty et al., 2013), and a methionine molecule is estimated to proceed through transmethylation 2-4 times in adults depending on the availability of dietary methyl (Mudd and Poole, 1975). Within the scope of development, the plasticity of transmethylation is of concern. Indeed, *in vivo* transmethylation rates are responsive to dietary and physiological changes such as betaine supplementation in adults (Storch et al., 1991), progression of pregnancy (Dasarathy et al., 2010), and sulfur-amino acid restriction in piglets (Bauchart-Thevret et al., 2009). If dietary methyl intake is adequate, then it is assumed that remethylation can overcome enhanced transmethylation demands under normal conditions. However, if intakes of methyl groups in the form of methionine, folate, betaine or choline are low or variable, can remethylation compensate for these deficiencies? Moreover, if methyl supply is low, are specific reactions of transmethylation prioritized?

There are at least 50 described reactions of transmethylation (Schubert et al., 2003), however it has been estimated that there may be >300 methyltransferase enzymes that employ SAM as substrate (J. T. Brosnan and M. E. Brosnan, 2006; Katz et al., 2003). Due to its vast nature it is difficult to predict the effects of a global reduction in transmethylation during development. Therefore, it is of critical importance to quantify the minimum portion of the pediatric methionine requirement that is required to furnish transmethylation *in vivo,* as well as to identify potential sites of methyl partitioning during transmethylation. Like all synthetic reactions, transmethylation devotes a quantitatively greater amount of methyl groups to specific products, and thus represents an opportunity to measure transmethylation partitioning. The-products of these reactions include, but are not limited to, sarcosine, creatine, phosphatidylcholine and DNA (Mudd et al., 2007; Stead et al., 2006).

Sarcosine

The formation of sarcosine during transmethylation is regarded as an overflow pathway when methyl groups are plentiful (Mudd et al., 1980), and sarcosine is synthesized by methyl transfer to glycine, *via* glycine N-methyltransferase (GNMT; EC 2.1.1.20). However, quantifying the methyl burden of sarcosine synthesis during transmethylation is complicated by the fact that sarcosine is a methylneogenesis precursor. Furthermore, because sarcosine is also synthesized from DMG, the *in vivo* measure of sarcosine synthesis is ambiguous.

Therefore, GNMT activity it is not considered to contribute significantly to the transmethylation requirement during infancy.

Creatine

The quantitative importance of remethylation for creatine synthesis was first described by tracing methyl-labeled choline into creatine, and, discovering that more methyl-label was found in creatine than methionine (Du Vigneaud et al., 1941). Creatine synthesis is a multi-step process that occurs in the pancreas, kidneys and liver. In rats and piglets, the process commences in the pancreas and kidneys when L-arginine: glycine amidinotransferase (AGAT; EC 2.1.4.1) catalyzes the formation of guanidinoacetatic acid (GAA) and ornithine (J. T. Brosnan et al., 2009; da Silva et al., 2009). GAA is the precursor for creatine (Bloch and Schoenheimer, 1941) when it is transported into the liver and methylated by guanidinoacetate N-methyltransferase (GAMT; EC 2.1.1.2) (J. T. Brosnan et al., 2009; da Silva et al., 2009). It has been widely reported that creatine is a major product of transmethylation, and that creatine synthesis is a critical process during development (J. T. Brosnan et al., 2011; Edison et al., 2013; Stead et al., 2006). Indeed, sow-fed neonatal piglets devote ≈35% of dietary methionine to creatine synthesis (J. T. Brosnan et al., 2009). Meanwhile, it was estimated that only ≈20% of portally infused [^{3}H-methyl] methionine flowed through GAMT (McBreairty et al., 2013), and thus remethylation contributes significantly to creatine synthesis. Furthermore, GAMT is sensitive to enhanced methyl demand and its activity was increased after GAA supplementation (Stead et al., 2001), and a portal GAA infusion

(McBreairty et al., 2013). Therefore, enhanced GAMT activity was furnished either by enhanced remethylation, reduced protein synthesis, or the sacrifice of other transmethylation reactions. The rate of transmethylation was not measured in those studies, but clearly creatine relies heavily on dietary methyl donors for its synthesis, and is sensitive to enhanced methyl demand.

Creatine synthesis has also been shown to be sensitive to reduced methylation demands in rodents as evidenced by creatine supplementation studies. Creatine supplementation effectively reduced plasma homocysteine concentrations (Deminice et al., 2009; Stead et al., 2001), and in a model of high-fat feeding; creatine supplementation also increased hepatic SAM and led to a reduction in the SAM/SAH ratio (Deminice et al., 2011). However, it is yet unclear what the consequences of variable creatine supply are on *in vivo* transmethylation partitioning. This is of particular relevance as breastfed infants are estimated to synthesize ≈90% of their creatine needs, whereas formula-fed infants are only required to synthesize ≈65% of creatine *de novo* (Edison et al., 2013). Therefore, it is assumed that creatine synthesis exhibits plasticity during development depending on the supply of dietary creatine, and that enhanced creatine demands are furnished either by dietary methyl donors, or the sacrifice of other transmethylation reactions. However, the priority of creatine synthesis among other transmethylation reactions is currently unknown.

Phosphatidylcholine

PC synthesis is especially complex as two different pathways contribute to its production under normal conditions. If preformed choline is available, then PC can be formed by the CDP-choline pathway, which is also known as the "Kennedy pathway" in honour of the pathway's discoverer, Eugene Kennedy (Kresge et al., 2005). The Kennedy pathway occurs in all tissues but because it does not require labile methyl groups to synthesize PC, the biochemistry of this synthetic pathway does not pertain to this discussion, and is reviewed elsewhere (Fagone and Jackowski, 2013; Gibellini and Smith, 2010). When dietary choline is absent, then PC synthesis must occur exclusively by the sequential addition of three methyl groups from SAM to phosphatidylethanolamine, by the hepatic enzyme, phosphatidylethanolamine methyltransferase (PEMT; EC 2.1.1.17) (Schneider and Vance, 1979). Indeed, PEMT has been postulated as an evolutionary mechanism to overcome acute choline restriction (Walkey et al., 1998). Because PEMT requires 3 moles of SAM to form 1 mole of PC, the methyl demand of the PEMT pathway is considered to be quantitatively greater than creatine, and potentially all other transmethylation reactions (McBreairty et al., 2013; Stead et al., 2006).

While both PEMT and the Kennedy Pathway synthesize PC, the two pathways work in concert under normal conditions to cover the costs of PC synthesis. For example, mice lacking the PEMT gene exhibit normal growth when choline is provided, but feeding a choline-deficient diet to PEMT knockouts is lethal (Walkey et al., 1998). However, PEMT knockout mice exhibit hepatosteatosis and lower hepatic PC concentrations despite the provision of dietary choline (Zhu et al., 2003). Indeed,

the Kennedy pathway can only furnish 70% of PC demands, and thus PEMT consumes a significant portion of the transmethylation requirement under all conditions (Vance et al., 2007).

The metabolic consequences of choline-restriction represent an interesting point of metabolic control. PEMT activity is increased during choline-restriction in rats (Schneider and Vance, 1978), and presumably the rate of PC oxidation to choline was also enhanced in those animals to supply the Kennedy pathway. However, the interplay between PEMT and the Kennedy pathway is extremely complex, and is discussed elsewhere (Vance et al., 1997). The point is that because PC is a major transmethylation product (Stead et al., 2006) that is sensitive to choline supply (Schneider and Vance, 1978), then whole body transmethylation should be responsive to the presence or absence of choline.

As previously mentioned, two-thirds of lactating women do not consume adequate choline (Fischer et al., 2010), and as much as 50% of the population are expected to have a genetic preponderance for enhanced choline demands (da Costa et al., 2006; Kohlmeier et al., 2005; Niculescu and Zeisel, 2002). Included among those genetic abnormalities are PEMT polymorphisms that occur at a relatively high frequency, such as in one study population where 20% of women exhibited a specific PEMT mutation (Zeisel, 2012). Therefore, we consider it likely that neonates commonly experience choline restriction, but it is unclear how much of the neonatal choline requirement can be fulfilled by PEMT, especially if there is an inherent PEMT mutation. Furthermore, it is also unclear whether global transmethylation or remethylation are increased to accommodate enhanced PC synthesis during choline

restriction, or if other transmethylation reactions are sacrificed to furnish PEMT demands, such as creatine synthesis or DNA methylation.

DNA

One of the most intriguing sites of early life methylation is of cytosine guanine dinucleotides located in the promoter regions of DNA. The methylation of DNA occurs by a number of DNA methyltransferase enzymes (DNMT 1,2,3; EC 2.1.1.37) and in general, DNA methylation represses gene expression (Jones and Takai, 2001; Niculescu and Zeisel, 2002) and persists throughout the lifetime of an organism (Kotsopoulos et al., 2008). Indeed, DNA methylation is an important locus of study for the fetal origins of adult disease hypothesis (reviewed by (McMillen and Robinson, 2005)). The hypothesis establishes that early life exposure to nutrients will permanently affect gene expression, and thus "program" an organism to be more or less susceptible to certain diseases later in life (Godfrey and Barker, 2000). Because dietary reductions in methionine, choline and/or folate have been shown to reduce DNA methylation (Burdge et al., 2009; Cordero et al., 2013; Kotsopoulos et al., 2008; Niculescu and Zeisel, 2002), it is likely that dietary methyl donors affect methyl group partitioning onto DNA.

1.3.2 Transsulfuration

Transsulfuration represents the sulfur transfer from homocysteine to cysteine, and provides an irreversible means of homocysteine elimination. Transsulfuration commences upon condensation of homocysteine with serine by the

vitamin B6-dependent enzyme cystathionine β synthase (CBS; EC 4.2.1.22) to form cystathionine, which is converted to cysteine by an additional vitamin B6-dependent enzyme, cystathionine γ lyase (CGL; EC 4.4.1.1). Indeed, transsulfuration accounts for the nutritional observation that dietary cysteine spares the methionine requirement by 40% in piglets (Shoveller et al., 2003a; 2003b), and 55% in children (Humayun et al., 2006).

The capacity of cysteine to spare the methionine requirement provides a rationale that dietary methyl donors can also spare the methionine requirement. Furthermore, it is possible that dietary methyl donors will enhance transsulfuration (*ie.* methionine disposal) once the requirements of transmethylation and protein synthesis are met. Indeed, transsulfuration was sacrificed for transmethylation and protein synthesis in piglets when sulfur amino acids were removed from the diet (Bauchart-Thevret et al., 2009). Therefore, dietary methyl is not expected to affect partitioning towards transsulfuration until SAM concentrations accumulate (Finkelstein et al., 1971), and thus dietary methyl donors should only enhance the partitioning of methionine towards transsulfuration after the requirements of transmethylation and protein synthesis are met.

1.3.3 Protein

Perhaps the most critical site of methionine partitioning during development is into protein, which is considered here as a surrogate measure of growth. Indeed, like all amino acids, methionine was evolutionarily selected for a role in protein incorporation before its nonprotein role was exploited (*ie.* the methionine cycle). In

proteins, methionine plays an important structural role due to its highly hydrophobic nature (J. T. Brosnan and M. E. Brosnan, 2006); and interestingly, methionine occupies a highly conserved position in the substrate recognition pocket of all protein kinases (da Costa et al., 2006; Morin et al., 2003; Torkamani, 2008) and is thought to play a major role in mitigating oxidative stress in bacterial cells (Luo and Levine, 2009). It is not known how dietary methyl donors contribute to methionine availability for protein incorporation, but because methionine is cycled ≈2 times prior to disposal, and because the provision of methyl affects methionine cycling (Mudd and Poole, 1975), it is likely that if the methyl supply is restricted, then so is the availability of methionine for protein incorporation.

1.5 Neonatal Methionine Requirements

The recommended methionine intake for infants is currently based on breast milk, which is 28 mg/(kg•d) (WHO, 2007), however a recent minimum estimate of 38 mg/(kg•d) was determined by feeding graded levels of methionine to infants (Huang et al., 2012). While those values are similar, and widespread methionine deficiencies are not expected in the population, this discrepancy demonstrates the complexity in determining the methionine requirement, as breast milk may contain different quantities of methyl donors or transmethylation products than commercial infant formulations. Therefore, the methionine requirement must consider that methionine metabolism services protein synthesis, transmethylation and transsulfuration; but also that transmethylation and transsulfuration are affected by the supply of their precursors or products (Finkelstein and Mudd, 1967; Schneider

and Vance, 1978; Stead et al., 2001). Furthermore, it stands to reason that remethylation also contributes to the methionine requirement, and thus it is important to quantify the contribution of remethylation to methionine partitioning in early life. The following series of studies investigates the impacts of dietary methyl donors on the partitioning of methionine into protein, as well as the other major sites of methionine partitioning such as transsulfuration and transmethylation in the neonatal piglet.

2.0 Experimental Approach

2.1 Objectives

The major objective of this dissertation was to determine the effects of dietary methyl donors on neonatal methionine metabolism. This was accomplished by administering a series of isotope infusions to determine how the presence or absence of dietary methyl donors affected tracer kinetics. We also focused our objective to clarify how the individual dietary methyl donors affect methionine metabolism when provided alone, as well as in concert. The purpose of this chapter is to describe how a nutritional model of methyl restriction was formulated, as well as to describe the isotopic infusions.

2.2 The piglet model

The neonatal piglet is considered the closest non-primate model of the human infant (Miller and Ullrey, 1987). The piglet offers significant advantages compared to rodent models due to their larger size, and tolerance to multiple catheterizations for rapid blood sampling and the feeding of experimental diets (Bertolo et al., 1999). Furthermore, nutrient requirements are well defined in swine due to their extensive use in agriculture, which permits the formulation of elemental diets (Lee et al., 2012; National Research Council, 2012). The piglet has been used extensively for amino acid requirement studies, and their metabolism is responsive to acute dietary manipulations such as a change in dietary amino acid composition (Brunton et al., 2007). Indeed, a methionine requirement has been reported in domestic piglets that was sensitive to the presence of dietary cysteine (Shoveller et

al., 2003b), and furthermore, runt Yucatan miniature piglets exhibit perturbed sulfur amino acid metabolism (MacKay et al., 2012). Therefore, we postulated that Yucatan miniature piglets would be sensitive to acute manipulations in the supply of dietary methyl groups.

2.3 Diets

To study the contribution of remethylation to methionine partitioning required a diet that restricted methyl availability, *ie.* a methyl-deficient diet. The dietary methyl donors, folate, choline and betaine were not added to the methyl-deficient diet formulation, and it was necessary to ensure that dietary lipid was not a significant source of choline (or betaine). At the time of diet formulation, there was little information on the betaine content of lipid sources, but the commonly used emulsified lipid formulation is synthesized from egg yolk phospholipids (Intralipid, Baxter, MA), and is estimated to provide dietary choline at the requirement for the piglet (Lee et al., 2012; National Research Council, 2012; Wykes et al., 1993). Therefore, we opted to use soybean oil as it contains linoleic acid which is essential for the piglet (Fisher et al., 2002; National Research Council, 2012), and very low levels of choline (Engel, 1943). While it has not been reported, it is suspected that the betaine content of soybean oil is low. It was also necessary to ensure that dietary methionine was incapable of furnishing methyl demands, and thus a pilot study was performed with methionine fed at 40% below the piglet requirement (44 μmol/(kg•h)) (Shoveller et al., 2003a). However, feeding methionine at such a low level was not well tolerated by 3-7 day old piglets from our herd without folate,

choline and betaine. Therefore, we opted to progressively restrict methyl group availability by initially providing methionine at the level of requirement, followed by restricting methionine to ≈80% of the piglet requirement (Shoveller et al., 2003a). The level of 80% allowed our model to detect the effects of sparing methionine, while still being at a tolerable level for the neonatal piglet.

To measure the contribution of dietary methyl donors on methionine partitioning required the formulation of methionine-restricted control diets that were replete with dietary methyl donors. In chapters **3.0**, **4.0** and **5.0**, a methyl-sufficient control diet is described that was formulated to provide choline at the piglet requirement (Craig, 2004; National Research Council, 2012), folate at the level provided by a commercial vitamin formulation (Infuvite Pediatric, Baxter, MA), and betaine at the level of methionine. The level of dietary betaine was chosen to ensure methyl demands could theoretically be met by betaine alone. The methyl-replete piglets were compared to the methyl-restricted piglets described above.

To focus the effects of individual methyl donors on methionine partitioning, methyl-restricted piglets were subsequently rescued with folate, betaine or both (folate + betaine). We chose only betaine and folate for the rescue study (**6.0**), as they are the most direct remethylation precursors. The provision of choline would have complicated the elucidation of the contribution of BHMT to remethylation, and affected PEMT (Schneider and Vance, 1978). While it is acknowledged that choline is a source of preformed methyl groups, the term "labile methyl donors" will be used to distinguish betaine and folate from choline in the methyl rescue chapter (**6.0**).

2.4 In vivo labeling studies

2.4.1 Hepatic partitioning of methionine

To measure the effects of dietary methyl donors on tissue specific methionine partitioning, [^{3}H-methyl] methionine was provided orally to piglets for 6 hours. To our knowledge, this is the first study to trace the methyl moiety of dietary methionine into muscle and liver protein, as well as through hepatic transmethylation. All of the tracer infusions presented in this dissertation were provided gastrically to factor nutrient absorption by the gut on amino acid kinetics. Because enteral feeding is the primary nutrient source for the healthy neonate, we assert that the enteral provision of tracers provides comprehensive information on the nutritional fate of methionine under various states of nutriture.

2.4.2 Whole body protein synthesis

To measure the effects of dietary methyl donors on whole body protein dynamics (Chapter **4.0**), we measured the oxidation of an indicating amino acid (*ie.* phenylalanine) with and without dietary methyl donors. The principle of the phenylalanine infusion was similar to the indicator amino acid oxidation (IAAO) technique that has been described elsewhere (Brunton et al., 2007; Pencharz and Ball, 2003; Zello et al., 1995). IAAO has been used to calculate amino acid requirements (Ball and Bayley, 1984; Brunton et al., 1998), and is based on the principle that protein synthesis is governed by the availability of the most limiting amino acid. While we do not report a methionine requirement, the IAAO technique is used to calculate whole body protein dynamics in piglets by relating the flux of an

indicating amino acid to the rate of isotope infusion and oxidation (Zello et al., 1990). This technique offers significant advantages over other methods such as nitrogen balance as it is non-invasive, and also permits repeated infusions in the same animal.

2.4.3 Whole body methionine kinetics

The whole body rates of transsulfuration, transmethylation and remethylation were measured (see Chapter **5.0** and **6.0**) by an isotope dilution technique that was first developed by Storch and Young (Storch et al., 1988). The infusion relies on the differential kinetic behaviour between [$^{13}C_1$] methionine and [^{2}H-methyl] methionine to calculate methionine cycle flux (Figure 2.1). Because only [^{2}H-methyl] methionine is diluted by transmethylation, then remethylation is equivalent to the flux differences between the two isotopes. Transsulfuration is calculated by the oxidation of [$^{13}C_1$] methionine in breath samples upon hydrolysis of cystathionine to α-ketobutyrate, which is converted to propionyl CoA and subsequently succinate. The sum of remethylation and transsulfuration equates to the rate of whole body transmethylation (Storch et al., 1988). This method has been used by others, and was shown to be sensitive to dietary manipulations (Bauchart-Thevret et al., 2009; Lamers et al., 2011; Riedijk et al., 2007). Since the method was first reported, it has been adjusted to account for intracellular methionine levels by utilizing labeled homocysteine as a surrogate of intracellular methionine (MacCoss et al., 2001). The strength of this approach is that it provides a measure of whole

body methionine kinetics *in vivo*, and thus accounts for the contributions of all organ systems to give a holistic view of methionine metabolism.

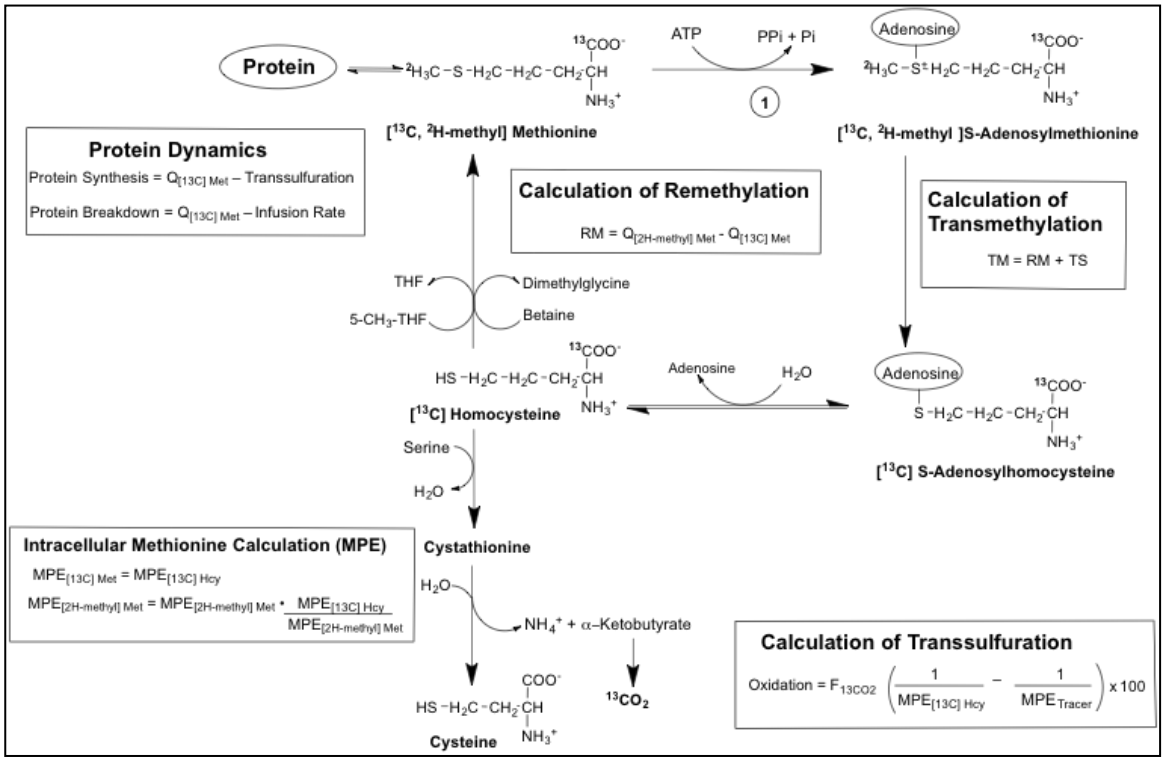

Figure 2.1 A depiction of the labeling pattern during the [^{13}C, ^{2}H-methyl] methionine infusion and the resultant calculations of transmethylation (TM), remethylation (RM), transsulfuration (TS), protein synthesis and protein breakdown (Storch et al., 1988). Where Q represents tracer flux (µmol met/(kg•h)). In order to factor for intracellular methionine, the mole percent excess (MPE) of homocysteine (Hcy) was used (MacCoss et al., 2001).

3.0 The effects of dietary methyl donors on hepatic methionine partitioning during transmethylation in the neonatal piglet

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3.1 Introduction

Methionine is an essential amino acid that is not only incorporated into protein, but is also in high demand for non-protein metabolism in what is commonly known as the methionine cycle. The cycle functions to transfer methyl groups in order to synthesize critical nutrients as well as to regulate gene expression. The methionine used during this process of transmethylation can be considered the non-protein requirement of methionine. The partitioning of methionine between protein incorporation and transmethylation, as well as the further partitioning among the >50 transmethylation reactions (Schubert et al., 2003), is of great clinical importance. This is especially true in neonates that not only require methionine for rapid tissue expansion (*ie.* growth), but also to provide substrate for rapidly turning over transmethylation reactions. Despite this obvious importance, the balance of dietary methionine that is used for protein synthesis *versus* transmethylation is unknown.

[1] JLR wrote manuscript and performed all animal procedures, infusions, diet formulation, data analysis, statistics and contributed to study design. LEM trained JLR on methods for hepatic [^{3}H-methyl] analysis, and assisted in the measure of plasma choline, betaine, DMG, folate and B12. EWR assisted in development of LCMSMS methods. JAB and RFB contributed to study design, analysis and project funding as JLRs co-supervisors.

In the liver, transmethylation is thought to utilize a significant portion of dietary methionine. Indeed, during first-pass metabolism, ≈80% of dietary methionine is directed to the liver (Riedijk et al., 2007), which is a major site of transmethylation in young pigs (J. T. Brosnan et al., 2009; McBreairty et al., 2013; Riedijk et al., 2007). Methionine that is partitioned towards transmethylation is adenylated to form S-adenosylmethionine (SAM). SAM becomes the primary biological methyl donor when it is demethylated to S-adenosylhomocysteine (SAH), which is in equilibrium with homocysteine. The majority of transmethylation occurs to synthesize creatine and phosphatidylcholine (PC), as well as to methylate DNA (Bertolo and McBreairty, 2013; Stead et al., 2006). Recently, we demonstrated that hepatic methionine partitioning towards transmethylation is readily manipulated by a portal infusion of guanidinoacetic acid (GAA), which is the demethylated precursor for creatine (McBreairty et al., 2013). Moreover, compared to normal weight littermates, piglets experiencing intrauterine growth restriction (IUGR) exhibit alterations in hepatic methionine partitioning, as well as circulating methionine cycle intermediates (MacKay et al., 2012; McBreairty et al., 2013), suggesting growth rate has profound effects on methionine partitioning.

Methyl groups donated during transmethylation can be replenished by remethylation. This process involves the transfer of a methyl group to homocysteine from the dietary methyl donors, 5-CH_3-tetrahydrofolate and betaine. 5-CH_3-tetrahydrofolate (or folate) regenerates methionine through donation of a methyl group to homocysteine *via* methionine synthase. Independent of this pathway, betaine (trimethylglycine), which is the irreversibly oxidized product of the

essential nutrient choline, can remethylate homocysteine *via* betaine:homocysteine methyltransferase. The potential for dietary methyl donors to quantitatively spare methionine is great. For example, ≈50% of hepatic methionine is partitioned towards transmethylation (McBreairty et al., 2013) in the piglet and indeed, ≈25% of whole body methionine flux is derived from remethylation (Riedijk et al., 2007). Furthermore, remethylation was able to maintain protein synthesis in piglets that were fed a methionine-deficient diet; this was accomplished by partitioning methionine away from transmethylation (Bauchart-Thevret et al., 2009). Therefore, remethylation is capable of sparing a significant portion of methionine for protein synthesis. The aim of this study was to determine how remethylation impacts hepatic methionine partitioning between protein synthesis and transmethylation, and moreover, which transmethylation reactions are conserved or sacrificed during dietary methyl restriction.

To study the effects of limiting remethylation, neonatal piglets were fed diets that were deficient (**MD-**), or replete (**MS+**) in the dietary methyl donors folate, choline and betaine. To focus the effects of dietary methyl donors on methionine partitioning, methionine was made limiting by providing dietary methionine at 80% of the requirement (Shoveller et al., 2003b). We hypothesized that methyl restriction (*ie.* **MD-** feeding) would decrease circulating concentrations of the methionine cycle intermediates and lower the rates of hepatic methionine partitioning towards creatine and DNA, in order to conserve hepatic protein synthesis. It was further hypothesized that PC synthesis would be sensitive to choline-restriction.

3.2 Materials and Methods

3.2.1 Chemical reagents and isotopes

All chemicals and reagents were of the highest available purity and obtained from Sigma (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ) or Alfa Aesar (Ward Hill, MA). Amino acids were from Ajinomoto, Co (Tokyo, Japan). [^{3}H-methyl] methionine was obtained from American Radiochemicals, Inc. (St. Louis, MO). [$^{2}H_{9}$-trimethyl] choline chloride and [$^{2}H_{11}$] betaine were obtained from Cambridge Isotope Laboratories (Tewksbury, MA).

3.2.2 Animals

3.2.2.1 Surgical procedures and study protocol

The animal care committee at Memorial University of Newfoundland approved all animal protocols performed herein. On study day 0, 4-9 day old Yucatan miniature piglets were transported to the animal care facility from the University vivarium. The piglets were weighed and then anesthetized with an intramuscular injection of acepromazine (0.5 mg/kg; Atravet; Ayerst Laboratories, Montreal, QC) and ketamine hydrochloride (20 mg/kg; Rogarsetic Rogar STB, Montreal, QC). Prior to endotracheal intubation, piglets were given an intramuscular injection of atropine (20 mg/kg; Rafter Dex Canada, Calgary, AB) to reduce airway secretions during the procedure. Anesthesia was maintained during surgery by 1-2% isoflurane (Abbott Laboratories Ltd, Abbott Park, IL), delivered with medical grade oxygen via the endotracheal tube. All piglets were fitted with two venous catheters (jugular and femoral) as well as a gastric catheter for enteral feeding. Post-

operatively, animals received intravenous doses of analgesic (0.03 mg/kg buprenorphine; Temgesic, Schering-Plough Ltd, Montreal, QC) and antibiotics (trimethoprim and sulfadoxine; Borgal, Intervet, Whitby, ON). Animals were transported to metabolic cages after being fitted with a mesh jacket (Lomir Biomedical Inc., Notre-Dame-de-L'Île Perrot, Canada). The dorsal section of the jacket contained a swivel attachment for a flexible metal tether. The tether allowed for the infusion of diet and gave the piglets free movement within their circular cages that measured 75 cm in diameter. The wire-mesh cages allowed the piglets full visual and aural contact. The piglets were monitored closely throughout the study and surgical incision sites were treated daily with topical antibiotics (Hibitane Veterinary Ointment, Ayerst Laboratories, Radnor, PA).

Intravenous feeding began immediately after surgery to provide an energy source during adaptation. Diet was provided using a medical-grade, pressure-sensitive infusion pump (Baxter Corporation, Mississauga, ON). Initially, a lipid-free elemental diet formulation (see 3.2.2.2) was provided intravenously at a rate of 5.7 mL/(kg•h) overnight. On the morning of study day 1, the rate of intravenous feeding was increased to 8.5 mL/(kg•h) and that evening, enteral feeding was initiated at the full rate of 11.3 mL/(kg•h) until the end of the study. Animal weights were recorded daily.

3.2.2.2 Dietary Regimen

All animals were fed 1.1 MJ/kg of metabolizable energy with dextrose and lipid each supplying 50% of the non-protein energy intake. The amino acid

compositions are summarized in table 3.1. From Day 0 to 5, the dietary amino acid composition was complete and similar to others (Shoveller et al., 2003b; Wykes et al., 1993). On the evening of Day 5, dietary methionine was restricted to 80% of the estimated requirement for a piglet (Shoveller et al., 2003a). Diets remained isonitrogenous by providing an equimolar amount of alanine; piglets were fed this methionine-restricted diet for the remainder of the study. Piglets were provided minerals at >200% of the NRC requirements (National Research Council, 2012). Soybean oil (J.M. Smucker Company, Orrville, OH) was provided as the lipid source during gastric feeding, and was chosen due to low concentrations of choline and betaine . The lipid was delivered intragastrically with the elemental diet *via* a syringe pump at a rate of 0.4 mL/(kg•h).

One group of piglets (N = 8) was maintained on the methyl-deficient (**MD-**) diet (*ie.* devoid of folate, choline and betaine) from day 0 to 8. A second group of piglets (N = 7) was fed the same diet but were made methyl sufficient (**MS+**) by providing folate (38 μg/(kg•day)), choline (60 mg/(kg•day)) and betaine (238 mg/(kg•day)) in the diet; the dietary concentrations of folate and choline were based on the NRC requirements for pigs of this age (National Research Council, 2012). The concentration of betaine was chosen to provide an equimolar amount of methyl groups that would be provided if dietary methionine were fed at its requirement. All vitamins, were provided to the **MS+** piglets based on a pediatric commercial solution (Infuvite Pediatric: Baxter Corporation, Mississauga, ON) at >110% of the requirement. The **MD-** piglets received all vitamins from the same

commercial solution except a solution of biotin (20 µg/mL) and cyanocoblamine (1 µg/mL) was provided separately, at the same rates as the **MS+** piglets.

Table 3.1 Amino acid concentrations fed to neonatal piglets during acclimation (days 0 to 5), and during methionine restriction (day 5 until the end of the study).

	Acclimation Period (day 0-5)	Methionine Restriction (day 5-end of study)
Amino acid	**Concentration (g/L)**	
Alanine	6.10	6.32
Arginine	3.46	3.46
Aspartic acid	3.46	3.46
Cysteine	0.82	0.82
Glutamic acid	5.99	5.99
Glycine	1.76	1.76
Histidine	1.76	1.76
Isoleucine	2.64	2.64
Leucine	5.93	5.93
Lysine-HCl	5.90	5.90
Methionine	1.10	0.73
Phenylalanine	3.02	3.02
Proline	4.72	4.72
Serine	3.19	3.19
Taurine	0.28	0.28
Tryptophan	1.21	1.21
Tyrosine	0.43	0.43
Valine	3.02	3.02
Threonine	3.02	3.02

3.2.3 Isotope infusions and calculations

3.2.3.1 Infusion protocol

On the morning of study day 8, the animals received an enteral infusion of [^{3}H-methyl] methionine. The infusion was initiated with a priming dose of 0.3 µCi/kg, and continued at 0.3 µCi/(kg•h). Blood samples were taken every 30

minutes for 6 hours. Blood was transferred immediately to tubes containing heparin, and centrifuged at 3000 x g, for 5 minutes. The plasma fraction was frozen for later use. Immediately after the final blood sample was taken, animals were anesthetized using 3% isoflurane delivered with oxygen. Once animals were anesthetized, the liver and a piece of biceps femoris muscle were removed, weighed and freeze clamped. Tissue samples remained at -80 °C until analyzed.

3.2.3.3 Calculation of specific radioactivity (SRA)

The enrichment of [^{3}H-methyl] products (including methionine) in tissue and the time course values in plasma were expressed as the specific radioactivity (SRA), which is defined as follows:

$$SRA = DPM/\mu mol\ methionine$$

Where DPM is the disintegrations per minute of [^{3}H-methyl] product, or [^{3}H-methyl] methionine.

3.2.3.2 Calculation of fractional synthesis rate of transmethylation products and protein

The rate of [^{3}H-methyl] incorporation into creatine, PC and DNA was determined as the fractional synthesis rate (FSR) using the following equation:

$$FSR\ (\%/h) = (SRA_{Product}/SRA_{Precursor})/time \times 100$$

Where the $SRA_{Product}$ was DPM/μmol for creatine and PC, and DPM/μg for DNA. The $SRA_{Precursor}$ was DPM/μmol of hepatic SAM. In the case of liver protein synthesis (Ks), $SRA_{Product}$ was DPM/μmol methionine from hydrolyzed liver protein, and

$SRA_{Precursor}$ was DPM/μmol of intracellular hepatic free methionine. The Ks is expressed as percent per day (%/d).

3.2.4 Analytical procedures and Instrumentation

3.2.4.1 Plasma concentrations and enrichments of choline, betaine and dimethylglycine (DMG)

Concentrations of choline, betaine and dimethylglycine (DMG) were quantified using an HPLC MS/MS as described elsewhere (Holm et al., 2003; Kirsch et al., 2010). Briefly, plasma samples were mixed with 3X volume of acetonitrile containing 100 μM of [$^2H_{11}$] betaine and [$^2H_9$-methyl] choline. Samples were well mixed and centrifuged at 5000 x g for 5 minutes. The supernatant was removed and added to a glass vial, or a polyethylene 96 well micro plate. Samples were added to the autosampler and 1 μL was injected into a normal-phase HPLC column (Atlantis HILIC Silica 3 μM, 2.1x100 mm, Waters Corporation, Milford, MA). The quaternary ammonium compounds were eluted in an isocratic solvent system consisting of ammonium formate (15 mM, pH 3.5; 17.5%) and acetonitrile (82.5%) for 6 minutes at 0.6 mL/min with the HPLC (Waters Alliance 2795, Waters Corporation, Milford, MA). The column effluent was split at a ratio of 1:4 and delivered at a rate of 150 μL/min to the tandem mass spectrometer (Micromass Ultima Triple-Quad MS, Waters Corporation, Milford, MA). The compounds were detected in multiple-reaction monitoring mode using the following m/z transitions: [$^2H_{11}$] betaine 129 → 68, betaine 118 → 59, [$^2H_9$-methyl] choline 113 → 69, choline 104 → 60, DMG 104 → 58.

Plasma concentrations were calculated using calibrating standards made using dialyzed plasma spiked with choline, betaine and DMG standards, and using [$^2H_{11}$] betaine and [$^2H_9$-methyl] choline as internal standards. The [$^2H_9$-methyl] choline was also employed as the internal standard for DMG as described elsewhere (Holm et al., 2003). Concentrations were computed using MassLynx Software (Waters Corporation, Milford, MA).

3.2.4.2 Plasma amino acids and glutathionine

Plasma amino acids were quantified using phenylisothiocyanate derivatives (PITC) as described elsewhere (Bidlingmeyer et al., 1984). Briefly, 100 uL of plasma was combined with 20 µL of 2.5 µmol/mL norleucine standard, and 1 mL TFA (0.5% TFA in methanol). Samples were mixed and centrifuged for 5 minutes. The supernatants were transferred to 3 mL plastic tubes and freeze dried overnight. Dried samples were combined with 50 µL of 20:20:60 mixture of triethylamine (TEA):methanol:water and freeze dried for 1 hour. Samples were removed and derivatized using 50 µL of water:TEA:methanol:PITC (10:10:70:10) for 35 minutes and dried overnight with a vaccum pump and cold trap. Samples were resuspended with 200 uL of sample diluent (710mg of Na_2HPO_4 into 1 L of water, pH 7.4 with 10 % H_3PO_4 acid; and replace 5% of the volume with acetonitrile), and centrifuged at 5000 x g for 5 minutes. Supernatants were transferred to a glass/plastic vial and kept at 4 °C.

Detection of amino acids was through an HPLC fitted with a PicoTag column (3.9 mm x 150 mm, 4 µm) from Waters (Milford, MA). The column was equilibrated

using “buffer A” (0.1 M Sodium acetate, and 0.05% acetonitrile) and “buffer B” (45% acetonitrile, 15% methanol) at a flow rate of 1 mL/min. Amino acids were eluted using a gradient system (Bidlingmeyer et al., 1984) and were detected with a UV detector and quantitated using Breeze software from Waters (Milford, MA). The methionine fraction was collected for 3 minutes and the DPM were measured using a scintillation counter (Perkin Elmer, Canada).

Plasma concentrations of homocysteine, cysteine and glutathione were determined using HPLC as described elsewhere (Vester and Rasmussen, 1991). Briefly, 150 µL of plasma was combined with 0.2 mM 8-amino-naphthalene-1,3,6-trisulfonic acid as an internal standard. Samples were reduced by adding 30 µL of tris(2-carboxyethyl)phosphine and incubated at room temperature for 30 minutes. Proteins were then precipitated with 125 µL of 0.6 M perchloric acid. The supernatant was removed and derivatized with 100 µL of 7-fluorobenzo-2,1,3-oxadiazole-sulfonic acid ammonium salt (1 mg/mL) for 1 hour at 60 °C. Samples were placed on ice for 5 minutes, and centrifuged twice at 7000 rpm for 5 minutes with the supernatant extracted each time with an autopipette. Samples were analyzed by HPLC using a gradient buffer system starting with 100% “buffer A” (0.1 M acetate, pH 4.0 containing 0.02% methanol) and changing to 100% “buffer B” (0.1 M phosphate buffer, pH 6.0 containing 0.05% methanol) by 12 minutes, in a linear fashion, and held for 2.5 minutes. The buffer system was changed back to 100% buffer A over 2 minutes; and held for 3 minutes until the next injection. Homocysteine, glutathione and cysteine were detected with fluorescence (Ex/EM 385/515 nm) (Vester and Rasmussen, 1991).

3.2.4.3 Measurement of Plasma B12 and folate

B12 and folate concentrations were quantified using an automated immunoassay analyzer (Architect i2000 Immunoassay Analyzer, Abbott, IL) by competitive immunochemiluminescence technique. Concentrations of plasma B12 and folate were measured using kits purchased from Abbott Diagnostics (Abbott, IL).

3.2.4.4 Hepatic concentrations and SRA of various metabolites and DNA

Hepatic SAM and SAH

The concentrations of SAM and SAH in liver were quantified using a previously described HPLC method (Ratnam et al., 2006). Liver was homogenized in ice-cold 8% trichloroacetic acid and centrifuged for 5 minute at 13,000 x g at 4 °C. The supernatant was filtered and immediately transferred to a plastic vial and placed in a refrigerated HPLC autosampler. SAM and SAH concentrations were detected using an HPLC (Waters 1525 HPLC, Milford, MA) fitted with a Vydac column (250 mm x 2.1 mm, Cat # 218TP54; Grace Scientific, Columbia, MD). The column was equilibrated with 96% "buffer A" (50 mM NaH_2PO_4, 10 mM heptanesulfonic acid, pH 3.2) and 4% "buffer B" (acetonitrile). SAM and SAH were separated with the following gradient: 96-80% buffer A, and 4-20% buffer B over 15 minutes at a flow rate of 1 mL/min. After 15 minutes column conditions immediately transitioned back to 96% buffer A for 5 minutes prior to the next injection. SAM and SAH were detected at 258 nm and quantitated using Breeze

software (Waters, Milford, MA). During SAM elution, the column effluent was collected into 4x30 second fractions, and each fraction was combined with 4 mL of Scintiverse (Cocktail E, Fisher Scientific, Fair Lawn, NJ). DPM were measured for 10 minutes with a liquid scintillation counter (Perkin Elmer, Canada).

Hepatic Creatine

Hepatic creatine concentrations were measured using the methods of others (Lamarre et al., 2010). Briefly, 100 mg of liver (or muscle) was homogenized in 450 µL Tris buffer (50 mM, pH 7.4) and poured into a microcentrifuge tube containing 50 µL of trifluoroacetic acid. Tubes were mixed by inversion and put on ice for 10 minutes. Samples were then centrifuged for 10 minutes at 5000 x g at 4 °C. Samples were prepared for HPLC using BondElut C18 solid phase extraction cartridges (Life Technologies, Burlington, ON) primed with one volume of methanol followed by 2 volumes of water. Samples were passed through the C18 for injection on HPLC. Creatine was separated using a graphite column (Hypercarb, 100 mm x 4.6 mm; Thermo Scientific) and isocratic flow (0.5% TFA and 3% methanol) at 1 mL per minute. The creatine was collected using a fraction collector and DPM was measured using a liquid scintillation counter (Perkin Elmer, Canada).

Hepatic PC

The concentrations of hepatic PC were determined as described elsewhere (Folch et al., 1957). Briefly, 100 mg of liver was homogenized in 300 µL of 50 mM NaCl. A 1.5 mL aliquot of chloroform:methanol (2:1) was added, samples were well-

mixed and stored at 4 °C overnight. The next day samples were centrifuged at 3000 x g for 10 minutes and the bottom layer was extracted and evaporated under N_2. The dried samples were solubilized with 100 µL isopropanol. A 20 µL aliquot of sample and PC standard were loaded onto GelG-60 TLC plates and placed into a TLC chamber containing mobile phase (chloroform:methanol:acetic acid:water, 25:15:4:2). Once the plate was ≈¾ saturated with mobile phase, the plate was left in the fume hood for 10 minutes to dry; and then placed into a TLC chamber containing iodine to visualize lipid bands. The band corresponding to PC was carefully scraped into acid washed vials, and 300 µL of 70% perchloric acid was added. Samples were placed at 180 °C until clear and allowed to cool. The extracted PC samples were mixed with 500 µL of water and 50 µL of 5% ammonium molybdate and 50-µL aliquot of a 1-amino-2-naphthol-6-sulphonic acid solution; the samples were mixed, and placed in boiling water for 12 minutes. Phosphate concentrations were detected at 815 nm as compared to phosphate standards (Bartlett, 1959). DPM were measured for 10 minutes in a portion of sample with a liquid scintillation counter (Perkin Elmer, Canada).

Hepatic DNA

The hepatic DNA was isolated using a phenol:chloroform extraction. A ≈0.5 g piece of tissue was homogenized in ≈2.5 mL of 50 mM Tris (ph 8), 1% SDS, 100 mM EDTA, and 100 mM NaCl. Liver homogenates were combined with 225 µL of proteinase K (10 mg/mL) and placed in a 56 °C water bath overnight to digest proteins. The following day, an equal volume of a phenol:chloroform:isoamyl alcohol

(PCI) solution (25:24:1 v/v) was added to the homogenate and mixed by rapid inversion. Samples were centrifuged at 5000 x g for 15 minutes. The aqueous layer was transferred to a new tube, the same volume of PCI was added again, and the procedure was repeated until the top phase was clear. Once achieved, an equal volume of chloroform isoamyl alcohol solution (24:1 v/v) was added to the aqueous phase and centrifuged. The aqueous phase was again recovered and combined with an equal volume of isopropanol and ⅕ volume of sodium acetate. The tube was rocked for 5 minutes and an autopipette tip was used to collect the precipitated DNA. A 1000 µL aliquot of a 10 mM Tris (pH 8) and 0.1 mM EDTA solution was added, and samples were gently heated and mixed to solubilize the DNA. DNA concentrations were quantified using spectrophotometry and 2 µL of the DNA solution. DPM were measured in 1 mL of the DNA solution for 10 minutes with a liquid scintillation counter (Perkin Elmer, Canada).

Hepatic methionine

The concentration and SRA of intracellular and protein bound fractions of methionine were determined in liver as described elsewhere (Bidlingmeyer et al., 1984). Briefly, 0.5 g of liver tissue was homogenized in 1.5 mL PCA (2%) and centrifuged at 3000 x g for 15 minutes to pellet proteins. The supernatant was kept, and the protein pellet was homogenized with 1.5 mL of PCA (2%) and centrifuged two additional times. The supernatants were pooled and spiked with the internal standard norleucine (25 µmol/mL). The resultant supernatant and pellet (protein) fractions were stored at -20 °C.

The SRA of intracellular methionine was determined by combining 3 mL of the supernatant with 375 µL of 2 M K_2CO_3 and mixed by inversion. Samples were centrifuged at 4500 x g for 3 minutes and dried overnight. The resulting samples were derivatized with PITC (see above) and methionine was separated and fractioned using HPLC. DPM in the methionine fraction was measured for 10 minutes with a liquid scintillation counter (Perkin Elmer, Canada).

The SRA of bound methionine was determined through hydrolysis of the protein fraction in 3 mL HCl (6 M). Samples were hydrolyzed for 24 hours in screw top vials at 110 °C. Hydrolysates were diluted to 12.5 mL with water and passed through a 2 µm syringe filter. Samples could be stored at -20 °C; however, methionine should not be considered stable while stored in strong acid, and thus it is prudent to analyze hydrolysates fresh. When ready for HPLC, 1 mL of hydrolysate was placed in a vacuum over overnight, and derivatized with PITC (see above). DPM were measured for 10 minutes with a liquid scintillation counter (Perkin Elmer, Canada).

Hepatic GAA

The concentration of GAA in liver was quantified by separation and detection with an HPLC and fluorescence detector after derivatization with ninhydrin. The method is detailed elsewhere (Buchberger and Ferdig, 2004). Briefly, ≈0.25 g of liver was homogenized in 4X the volume of 1 M perchloric acid (PCA). Samples were immediately centrifuged (at 4 °C) at 12,750 rpm for 20 minutes. The supernatant was extracted and weighed. A 25 µL aliquot of 50% (w/v) of K_2CO_3, 10 µL of

universal indicator and 125 µL of 20% (w/v) KOH was added to each supernatant and mixed. Samples were left on ice for approximately 10 minutes. The pH of each sample was adjusted to 6.5-7 using KOH. Neutralized samples were then centrifuged (at 4°C) for 10 minutes at 3,500 rpm. Supernatants were weighed and stored at -80°C until needed.

Neutralized liver extracts were thawed on ice and a 400 µL aliquot was mixed with fresh 0.9% ninhydrin and 300 µL of 1.3 M KOH. Derivitization took place in the dark for 15 minutes at room temperature, followed by mixing with 100 µL of fresh 5% ascorbic acid and 100 µL of 5 M phosphoric acid, and incubated in the dark for 30 minutes at 90°C. Samples were cooled and filtered for injection into an HPLC equipped with a C18 column (150 mm x 4.6 mm, Hypersil ODS; YMC). A 50 µL aliquot of filtrate was injected into the HPLC and the GAA was eluted with gradient flow of degassed 5% buffer A (50 mM formic acid) and 95% buffer B (100% methanol). The gradient transitioned to 90% buffer B within 15 minutes. The flow rate was continuous at 1 mL/min. GAA was detected using fluorescence (Ex/EM 290/470) and quantified with Waters Breeze software.

3.2.5 Statistics

An unpaired t-test was used to compare values between the **MD-** and **MS+** piglets. Normality was confirmed in all cases using the D'Agastino and Pearson test. For plasma B12 concentrations over time, a two-way ANOVA was performed with a Bonferroni post-test. Statistics were calculated using Prism software 5.0b (La Jolla CA). A p-value of <0.05 was considered to be significant in all cases.

3.3 Results

3.3.1 Animal Growth

Weight gain between the **MD-** and **MS+** piglets did not differ during the course of the 8 day study (303.2 ± 66.0 g/kg **MD-** *vs.* 320.3 ± 81.8 g/kg **MS+**; p = 0.6), after methionine-restriction (141.2 ± 50.2 g/kg **MD-** *vs.* 155.0 ± 63.4 g/kg **MS+**; p = 0.6), and piglet weights were the same between groups at the end of the study (2.45 ± 0.26 kg **MD-** *vs.* 2.40 ± 0.37 kg **MS+**). There were no differences in liver weight (96.5 ± 14.2 g **MD-** *vs.* 98.3 ± 16.2 g **MS+**).

3.3.2 Plasma Amino Acids

Plasma amino acid concentrations on study day 8 are displayed in Table 3.2. The concentrations of circulating amino acids were unchanged between the two study groups.

Table 3.2 Circulating concentrations of plasma amino acids on study day 8[1]

	MD-	MS+
	μM Plasma	
Aspartic Acid	84.2 ± 27.6	92.0 ± 20.2
Glutamic Acid	90.6 ± 42.0	97.7 ± 27.0
Hydroxyproline	61.3 ± 26.9	57.1 ± 15.5
Serine	396.6 ± 135.5	326.0 ± 86.5
Glycine	911.3 ± 340.8	754.3 ± 282.8
Asparagine	228.9 ± 58.0	176.3 ± 34.8
Taurine	106.9 ± 23.4	114.9 ± 20.6
Histidine	15.7 ± 26.8	13.0 ± 20.2
Citrulline	87.5 ± 58.4	55.2 ± 53.3
Threonine	397.5 ± 143.0	266.5 ± 104.1
Alanine	583.2 ± 105.7	449.4 ± 135.5
Arginine	102.2 ± 57.9	139.0 ± 79.6
Proline	509.4 ± 106.6	380.7 ± 143.1
Tyrosine	51.3 ± 37.4	145.4 ± 115.0
Valine	363.3 ± 76.7	221.6 ± 174.9
Methionine	50.0 ± 15.6	37.8 ± 14.7
Isoleucine	187.0 ± 51.4	145.0 ± 55.3
Leucine	354.4 ± 96.4	299.6 ± 91.2
Phenylalanine	213.0 ± 125.4	136.3 ± 35.5
Tryptophan	74.2 ± 64.8	86.4 ± 54.3
Ornithine	157.8 ± 43.0	100.3 ± 41.3
Lysine	336.9 ± 98.0	282.0 ± 138.2

[1] Values are mean concentration ± SD. MS+, methyl replete, MD-, methyl restricted. N = 7 for MS+ and N = 8 for MD- piglets.

3.3.3 Plasma concentrations of methyl donors and related metabolites in plasma

The methyl deficient diet was validated by analyzing metabolites related to the methionine cycle. The plasma homocysteine concentrations were elevated by more than 2-fold during methyl restriction ($p<0.0001$), and the concentrations of the methyl donors folate ($p<0.0001$), choline ($p< 0.04$) and betaine ($p<0.0001$) were all significantly lower in the **MD-** animals (Table 3.3). Plasma DMG concentrations were also diminished in the **MD-** piglets, which suggests lower remethylation. Glutathione and cysteine concentrations were not different between the two groups.

Interestingly, the plasma concentrations of B12 were elevated in the **MD-** piglets on study day 8, which was confirmed when values were log-transformed to achieve normality (3.0 ± 0.2 **MD-** *vs.* 2.3 ± 0.3 **MS+**; $p < 0.0003$). To help explain this result, plasma B12 concentrations were compared on study days 5 (405.8 ± 87.6 pg/mL **MD-** *vs.* 186.4 ± 38.6 pg/mL **MS+**), 7 (713.1 ± 313.6 pg/mL **MD-** *vs.* 180.1 ± 46.1 pg/mL **MS+**) and 8 (1108.4 ± 401.3 pg/mL **MD-** *vs.* 284.6 ± 225.5 pg/mL **MS+**) (Figure 3.1). There was an effect of time on log-transformed B12 values ($p < 0.05$), and thus plasma B12 was accumulating over time in the **MD-** piglets.

Table 3.3 Plasma concentrations of metabolites in the MD- and MS+ piglets[1]

	MD-	**MS+**	p-value
Folate (ng/mL)	24.5 ± 8.3	41.0 ± 10.7*	0.0001
B12 (ng/mL)	1.1 ± 0.4 *	0.3 ± 0.2	0.003
Choline (μM)	2.2 ± 2.9	7.5 ± 4.9*	0.04
Betaine (μM)	0.7 ± 0.4	162.1 ± 51.2*	0.0001
DMG (μM)	0.1 ± 1.0x10^2	4.0 ± 1.6*	0.0001
Glutathione (μM)	1.2 ± 0.7	1.1 ± 0.3	0.6
Cysteine (μM)	104.2 ± 25.5	84.1 ± 33.7	0.09
Homocysteine (μM)	36.4 ± 10.2*	13.2 ± 4.3	0.0001

[1] Values are means ± SD; p-values < 0.05 were considered significant; MS+, methyl replete, MD-, methyl restricted. N = 7 for MS+ and N = 8 for MD- piglets.

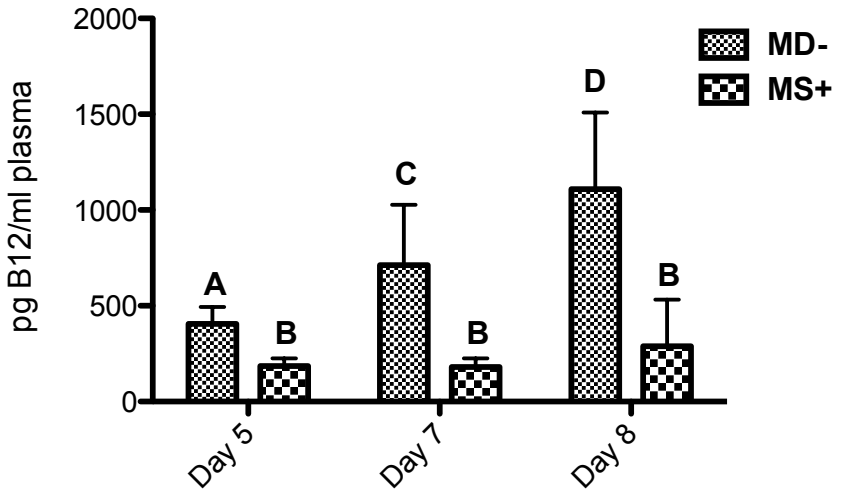

Figure 3.1 Daily concentrations of plasma B12 in MD- and MS+ piglets after 5, 7 and 8 days of feeding. Letters indicate statistically significant groupings ($p<0.05$). N = 7 for MS+ and N = 8 for MD-. Values are means ± SD. Note that MS+ values are not normally distributed.

3.3.4 Plasma enrichment of [^{3}H-methyl] methionine and achievement of steady state

Steady state is required to apply the equations used for the quantification of hepatic methionine kinetics during the [^{3}H-methyl] methionine infusion. After 4 hours, the SRA of plasma methionine had slopes not different from zero ($p>0.05$) for all pigs (typical example shown in Figure 3.2).

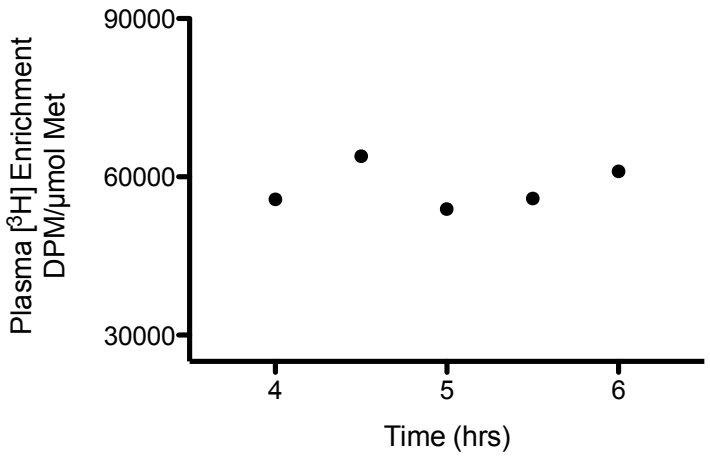

Figure 3.2 Time course values of plasma SRA during a typical [^{3}H-methyl] methionine infusion in piglets.

3.3.5 Hepatic concentrations and SRA (when applicable) of SAM, SAH, creatine, PC, DNA, methionine and GAA

The concentrations and applicable SRA for hepatic metabolites are found in Table 3.4. Concentrations of SAM did not differ between the two groups, nor did the SRA of SAM, which was used to calculate the FSR of the various transmethylation products. However, the concentration of SAH in the liver was significantly greater in the **MD-** piglets compared to the **MS+** piglets ($p<0.002$). In the **MD-** piglets, the SAM/SAH ratio was lower by more than 50% compared to the **MS+** group ($p<0.01$).

The feeding of the **MD-** diet did not affect the hepatic concentrations of creatine, GAA or PC, nor the calculated SRA for PC and DNA (Table 3.4). However, the SRA of creatine was significantly lower in the **MD-** groups compared to the **MS+** piglets. Hepatic methionine concentration and SRA were unchanged in the **MD-** group compared to **MS+** piglets. In order to expand on the measurement of creatine dynamics, the creatine concentration and SRA was expanded to the skeletal muscle.

The creatine concentration and SRA in skeletal muscle was unaffected by methyl restriction

Table 3.4 Tissue concentrations and SRA values of metabolites in MD- and MS+ piglets after a 6-hour infusion of [^{3}H-methyl] methionine

	MD-	**MS+**	p-value
Concentration			
Hepatic SAM (nmol/g)	50.9 ± 11.8	55.1 ± 28.3	0.7
Hepatic SAH (nmol/g)	32.8 ± 8.7*	16.7 ± 7.1	0.002
Hepatic SAM/SAH	1.66 ± 0.6	3.6 ± 1.6*	0.0095
Hepatic Creatine (µmol/g)	0.59 ± 0.35	0.52 ± 0.24	0.7
Hepatic GAA (nmol/g)	29.1 ± 1.1	27.2 ± 1.8	0.8
Hepatic PC (µmol/g)	4.0 ± 0.6	4.4 ± 0.5	0.15
Hepatic Methionine (µmol/g)	0.14 ± 0.05	0.16 ± 0.02	0.3
Muscle Creatine	32.3 ± 7.1	35.8 ± 7.3	0.4
SRA			
Hepatic Creatine (DPM/nmol)	56.5 ± 9.2	88.1 ± 31.5*	0.03
Hepatic PC (DPM/nmol)	28.6 ± 10.1	23.8 ± 7.9	0.3
Hepatic DNA (DPM/µg)	1.4 ± 0.4	1.4 ± 0.3	0.8
Hepatic SAM (DPM/nmol)	131.8 ± 11.6	161.2 ± 55.4	0.1
Hepatic Methionine (DPM/nmol)	80.8 ± 26.7	76.7 ± 23.5	0.7
Muscle Creatine (DPM/µmol)	502.5 ± 207.6	548.5 ± 101.8	0.6

[1] Values are means ± SD; p-values < 0.05 were considered significant and are indicated with *; MS+, methyl replete, MD-, methyl restricted. N = 7 for MS+ and N = 8 for MD- piglets.

3.3.6 Hepatic partitioning of dietary methionine

The SRA of each of the methyl products (including liver protein) was used to calculate total hepatic distribution of dietary methionine ($DPM_{Product}$/g liver), and compared as a percentage of the total DPMs in all of these products combined (Table 3.5). PC was the largest consumer of methyl groups from dietary methionine and collectively, the transmethylation products (*ie*. PC, creatine, DNA) accounted for >50% of the dietary [^{3}H methyl] in the liver. Hepatic protein accounted for over a third of dietary [^{3}H methyl], while free methionine, DNA and SAM contained relatively low amounts of [^{3}H methyl]. There was no effect of diet on any of these partitioning percentages.

Table 3.5 Percentage of [^{3}H-methyl] products in piglet livers after a 6-h enteral infusion of [^{3}H-methyl] methionine[1]

($DPM_{Product}$/g)/(DPM_{Total}/g) x 100	**MD-** (%)	**MS+** (%)	p-value
Creatine	10.5 ± 4.2	18.2± 11.8	0.1
PC	43.0 ± 10.4	41.2 ± 6.7	0.7
DNA	1.8 ± 0.7	1.6 ± 0.5	0.6
SAM	2.1 ± 0.9	2.2 ± 1.1	0.7
Liver Protein Hydrolysate	36.5 ± 7.0	37.8 ± 7.4	0.8
Tissue Free Methionine	3.6 ± 1.5	3.3 ± 1.3	0.7
Total DPM/g liver	$3.5E^5$ ± $8.4E^4$	$3.5E^5$ ± $7.6E^4$	0.5

[1] Values are mean percentages ± SD. MS+, methyl replete, MD-, methyl restricted. N = 7 for MS+ and N = 8 for MD- piglets.

3.3.7 Rate of incorporation of [^{3}H-methyl] to creatine, PC, DNA and protein

The FSR of the major hepatic transmethylation products were calculated (Figure 3.3). The FSR of creatine decreased by ≈30% in the **MD-** piglets compared to the **MS+** group (6.42 ± 2.7 %/h *vs.* 9.18 ± 1.7 %/h, respectively; $p<0.04$) (Figure 3.3a). On the contrary, the FSR of PC was ≈60% greater in the **MD-** piglets compared to **MS+** group (3.64 ± 1.3 %/h *vs.* 2.26 ± 0.4 %/h, respectively; $p<0.02$) (Figure

3.3b). The FSR of DNA was unaffected by methyl restriction (1.04 ± 0.36 %/h **MD-** *vs.* 0.85 ± 0.26 %/h **MS+**; p>0.05) (Figure 3.3c).

The FSR of hepatic protein synthesis (Ks) was also quantified using the SRA of tissue free methionine as the precursor (Figure 3.3d). There was no significant difference between the two dietary conditions (37.1 ± 19 %/d **MD-** *vs.* 49 ± 22 %/d **MS+**; p = 0.3). Furthermore, the SRA of bound methionine was unchanged (8665 ± 3570 DPM/µmol **MD-** *vs.* 8350 ± 2375 DPM/µmol **MS+**).

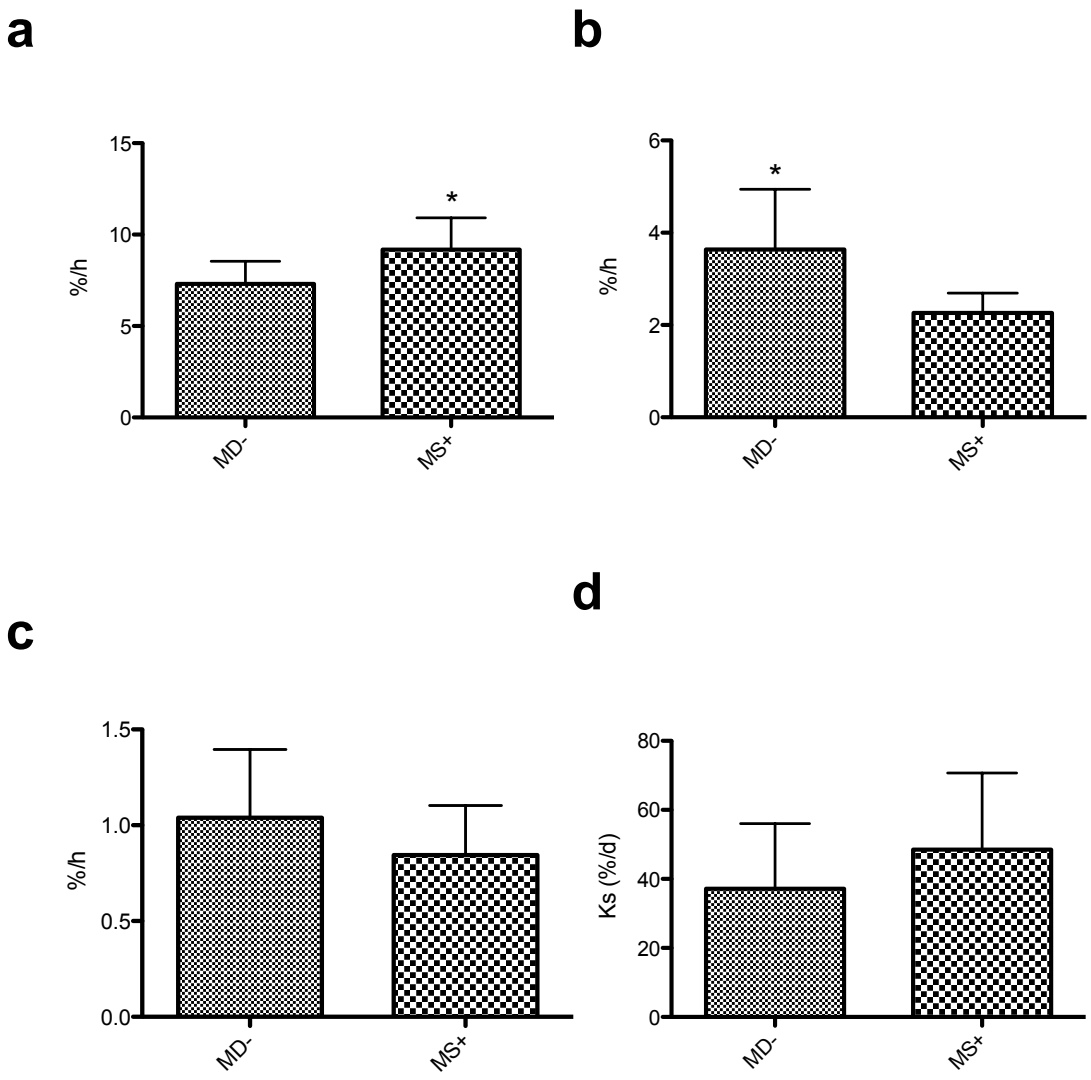

Figure 3.3 The FSR of creatine (a), PC (b), DNA (c) and protein (d) in MD- and MS+ piglets after an enteral infusion of [^{3}H-methyl] methionine. The transmethylation products are expressed as %/h, and Ks is shown as %/d. All values are ± SD; N = 8 for MD- and N = 7 for MS+. * Denotes a significant difference between the two dietary conditions ($p<0.05$).

3.4 Discussion

The objectives of this study were to determine the extent remethylation spares methionine for hepatic protein synthesis and transmethylation, and to determine which hepatic transmethylation pathway is conserved or sacrificed during dietary methyl restriction. These objectives were accomplished by feeding piglets a methionine-restricted diet that was either deficient or replete in the dietary methyl donors folate, choline and betaine, followed by an enteral infusion of [^{3}H-methyl] methionine. Indeed, the methyl deficient **MD-** piglets had diminished plasma concentrations of folate, betaine, choline and DMG, and increased plasma homocysteine concentration, validating the model. The elimination of dietary methyl donors led to lower creatine synthesis, with the apparent prioritization of dietary methyl groups for PC synthesis with no change in hepatic protein synthesis.

The fractional synthesis rates (FSR) of the three major transmethylation products were measured in liver. We found that both PC and creatine syntheses were sensitive to dietary methyl supply. The synthesis of creatine was sacrificed in the liver of **MD-** animals, which is of clinical significance as creatine accretion represents a significant metabolic process in growing animals; indeed, it has been estimated that ≈30% of dietary methionine is used to synthesize creatine. A sow-fed piglet is estimated to endogenously synthesize 77% of whole body creatine accretion, at a rate of 25.5 μmol/(kg•h) (J. T. Brosnan et al., 2009). If **MS+** piglets synthesized 30% less creatine, then methyl donors spared ≈7.7 μmol methionine/(kg•h) in these piglets, which is approximately 10% of dietary methionine. In other words, methyl donors contribute an equivalent of 10% of

dietary methionine, just for creatine synthesis. However, this level of sparing should be interpreted with caution as the rate of *de novo* creatine accretion was calculated in sow-fed piglets, and may be different in these methionine-restricted piglets that were provided no dietary creatine. In order to confirm this, the rate of *de novo* creatine synthesis, and the total creatine pool size must be calculated in these piglets.

On average, hepatic creatine synthesis was found to consume ≈15% of the [^{3}H-methyl] label in the two groups of piglets. If this estimate is applied to the dietary provision of 88 μmol methionine/(kg•h) fed here, then that equates to 13 μmol creatine/(kg•h). That is 50% lower than the rate of whole body creatine accretion in sow-fed piglets of a similar age (J. T. Brosnan et al., 2009). The rapid efflux of creatine from the liver may explain this discrepancy (da Silva et al., 2009), but it is impossible to calculate given the available data. It is also likely that creatine synthesis is not maximized in the **MS+** group given all pigs were limited in dietary methionine. Regardless, dietary methyl donors contribute a significant amount of methionine for creatine synthesis as evidenced by the diminished FSR and SRA of hepatic creatine.

In contrast to creatine, PC synthesis was enhanced in the absence of dietary methyl donors. PC can be synthesized by the sequential methylation of phosphatidylethanolamine (PE) with 3 methyl groups *via* PE methyltransferase (PEMT); thus, for every mole of PC synthesized by PEMT, 3 moles of methyl groups are required. Indeed, the fraction of dietary methyl groups ending up in PC/g liver was the largest of all products, including DNA, creatine and protein. This direct

calculation validates previous estimates discussed elsewhere (McBreairty et al., 2013). The hepatic PC concentration was 7.6 μmol/g liver, which equates to ≈730 μmol of PC in the entire liver of these piglets. If it is assumed that the hepatic PC pool is recycled daily (Walkey et al., 1998), then that requires 30 μmol PC/h in these piglets, which would consume up to 90 μmol methionine/h for PC synthesis, if synthesized exclusively *via* PEMT. While this calculation is based on conjecture, PC synthesis could theoretically consume up to ≈42% of the dietary methionine that was provided to these 2.4 kg piglets. Indeed, this is in agreement with the hepatic partitioning of methionine, where 42% of the hepatic [^{3}H methyl] was found in PC. Furthermore, the FSR of PC was significantly enhanced by ≈60% in the **MD-** piglets, likely as a result of omitting dietary choline (Schnieder and Vance, 1978) thus preventing PC synthesis via the cytidine diphosphate-choline pathway, which uses preformed choline. Therefore, methyl donors spared an estimated 18 μmol methionine/h, which equates to the sparing of 20% of the dietary methionine fed in this study. The increased FSR of PC was in concert with a decreased FSR for creatine and demonstrates that during methyl restriction, piglets prioritize PC synthesis over creatine synthesis. This result demands the following question: can the sparing of creatine synthesis provide adequate methyl groups to accommodate the enhanced PC synthesis during methyl restriction?

Based on the above calculations, it does not seem feasible that the sparing of creatine synthesis during methyl restriction was able to furnish sufficient methyl groups for enhanced PC synthesis. However, this is difficult to confirm as hepatic release of PC and creatine are independently complex (J. T. Brosnan et al., 2009;

Vance, 2008). This is especially true for PC, which can be synthesized in all tissues by the cytidine diphosphate-choline pathway (Gibellini and Smith, 2010), and so whole body PC metabolism and regulation must be considered. Future investigations should compare rates of flux and hepatic release of creatine and PC during methyl restriction.

An additional factor to consider is whether protein synthesis was sacrificed to spare methionine and maintain transmethylation during methyl restriction. This is a daunting possibility in the neonate where protein synthesis is a major consumer of dietary methionine during rapid growth. Overall, protein consumed approximately 37% of dietary methionine in the liver, which is similar to previous estimates (McBreairty et al., 2013). However, there was no apparent change in tissue-bound methionine SRA during methyl restriction, nor was there a change in tissue-free methionine SRA in the liver; therefore, Ks of hepatic protein was not affected by methyl restriction. This is an interesting finding as it suggests that **MD-** animals conserved protein synthesis during methyl restriction, while enhancing PC synthesis at the expense of creatine synthesis. It is clear that a more holistic approach is required to further understand neonatal methionine partitioning into protein such as whole body protein synthesis using isotopic tracers, or measuring plasma proteins to get a measure of hepatic protein release.

Despite liver being a major site of protein synthesis in the pig (Baumann et al., 1994), it is not necessarily a reflection of what is occurring in other metabolically active tissues, such as muscle and gut, or the piglet as a whole. Furthermore, transmethylation activity is greatest in the liver (J. T. Brosnan et al., 2009;

McBreairty et al., 2013; Riedijk et al., 2007), and it is the major site of PC (Vance et al., 2007) and creatine (Bertolo and McBreairty, 2013; J. T. Brosnan et al., 2009; da Silva et al., 2009; Tachikawa et al., 2012) syntheses. Indeed, >50% of dietary methionine was used in hepatic transmethylation. Therefore, the use of the [^{3}H-methyl] methionine label to measure hepatic protein synthesis is complicated by the high transmethylation demand in that tissue. It is suggested that protein synthesis in tissues with low transmethylation activity may have been attenuated to accommodate the hepatic demand for dietary methyl groups. It is hypothesized that the restriction of dietary methyl donors affected whole body methionine kinetics, as reflected by the repartitioning of methionine during hepatic transmethylation. In order to test this hypothesis, whole body protein synthesis, remethylation, transmethylation, and transsulfuration should be quantified during methyl restriction in order to determine if tissues other than liver reduced protein synthesis to spare methionine for transmethylation reactions.

It has been suggested that the hepatic concentrations of SAM and SAH provide an indication of overall transmethylation. It was hypothesized that the absence of methyl groups would decrease remethylation and result in less available methionine for conversion to SAM. This hypothesis was derived from rat studies that observed decreases in hepatic SAM when animals were fed diets deficient in choline (Zeisel et al., 1989) and folate (Balaghi et al., 1993). However in this study, methyl restriction did not change the hepatic SAM concentration or SRA. The corollary to this observation is that dietary methyl donors alone cannot spare methionine from a state of deficiency to a state of excess and, that any extra

methionine is immediately metabolized. This assertion is further supported by no changes in circulating and hepatic methionine concentrations between the two dietary conditions.

In contrast, the concentration of hepatic SAH, like plasma homocysteine, was markedly increased during methyl restriction. The SAH concentration has been suggested to be a more robust measure of methylation status (Schatz et al., 1977), and indeed the higher SAH concentration resulted in halving the hepatic SAM/SAH ratio during methyl restriction (**MD-**). This ratio has been suggested to reflect a broad range of methylation reactions and to positively correlate with SAM concentrations (Schatz et al., 1977) and remethylation (Lamarre et al., 2012; Stam et al., 2005). The metabolic situation in the **MD-** piglets is similar to adult Yucatan minipigs that were made folate-deficient (Halsted et al., 2002). Those animals had elevated SAH concentrations and hypomethylated DNA, with no changes in hepatic SAM concentration. Therefore, it is hypothesized that a low SAM/SAH ratio that is caused solely by changing hepatic SAH concentrations is due to decreased transmethylation. Based on the decreased SAM/SAH ratio observed here, this suggests that methyl restriction reduced transmethylation (Lamarre et al., 2012; Stam et al., 2005). However, it is necessary to directly quantify the rates of transmethylation in these animals to confirm this assertion.

The rate of methyl incorporation into DNA was not sensitive to methyl supply. This is in agreement with observations made by others in IUGR piglets (MacKay et al., 2012), as well as in IUGR piglets receiving a bolus infusion of [^{3}H-methyl] methionine into the portal vein (McBreairty et al., 2013). On the other hand,

rats that were chronically fed diets without methionine or choline exhibited rapid DNA hypomethylation (Wainfan et al., 1989; Wainfan and Poirier, 1992). Hypomethylated DNA was also observed during folate deficiency in adult Yucatan minipigs. It has been suggested that the fine-control mechanisms of DNA methyl transfer are controlled by hepatic SAM concentrations (Yi et al., 2000). Indeed, in chronically stressed rats, increasing cranial SAM concentrations via an intracerbroventricular infusion of methionine was found to ameliorate DNA hypomethylation. What is even more remarkable was that restoring DNA methylation status also restored the behaviour of early-life programmed rats, well into adulthood (Weaver et al., 2005). Because SAM concentrations were unchanged between the **MD-** and **MS+** piglets, it is not surprising that DNA methylation was unchanged. Chronic feeding of the **MD-** diet is considered more likely to have affected methylation of DNA, as dietary methyl donor concentrations were still detectable in the **MD-** piglets.

An unexpected finding of this study was that B12 concentrations were elevated during methyl restriction. To the knowledge of the author, this is the first time that this has been described. However, patients with suboptimal levels of plasma folate, tended to have elevated B12 concentrations; but because this elevation was not statistically significant, it was not discussed by those authors (Kang et al., 1987). We hypothesize that a lower rate of flux through methionine synthase during methyl restriction resulted in diminished requirement for B12. This could have stimulated the release of B12 into circulation for disposal, and presented

as an increased concentration in plasma. Further investigations are required to explain this observation.

Because of the effects of methyl restriction on hepatic creatine synthesis (Figure 3.3a), it was postulated that creatine accumulation may have been affected in the **MD-** piglets. Therefore, intramuscular creatine was measured because skeletal muscle is the major site of mammalian creatine deposition (J. T. Brosnan et al., 2009; da Silva et al., 2009). There were no effects of methyl-restriction on muscle creatine concentrations or SRA. Plasma and urinary creatine/creatinine concentrations were not measured, and thus it is not clear if hepatic creatine release was affected by methyl-restriction. However, it should be considered that a considerable amount of labeled creatine reached muscle, and thus creatine may be a quantitatively greater consumer of dietary methionine than shown here in liver. Future studies are required to focus on the potential effects of methyl deficiency on hepatic creatine release.

3.5 General Conclusions

This study demonstrated the capacity for dietary methyl donors to affect methionine partitioning during hepatic transmethylation. This has broad implications for pediatric nutrition as dietary methyl donors had a significant impact on hepatic methyl partitioning, and attenuated elevations in risk factors of chronic disease during acute methionine-restriction. Dietary methyl donor intake must be considered when estimating nutrient requirements in the neonate, and it is asserted that global transmethylation is affected by methyl-restriction; and thus

other products of transmethylation are sensitive to methyl supply. It is hypothesized that feeding a methyl deficient diet affects whole body methionine partitioning.

4.0 Dietary methyl donors have a significant impact on whole body protein turnover and skeletal muscle protein synthesis in methionine restricted neonatal piglets

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4.1 Introduction

Methionine is one of four sulfur-containing amino acids (SAA). It is the only SAA that is considered essential, and indeed, methionine is a precursor for the endogenous synthesis of the other SAA, which include taurine, homocysteine and cysteine. Due to its pivotal role in SAA metabolism, methionine cycle activity is significantly impacted when the SAA intake is variable. For example, piglets fed SAA-deficient diets maintained protein synthesis by enhancing remethylation and sacrificing transsulfuration (Bauchart-Thevret et al., 2009). Moreover, the presence of dietary cysteine has a significant impact on the rate of transsulfuration, and indeed, dietary cysteine spares the methionine requirement by ≈40% in neonatal piglets (Shoveller et al., 2003a) and ≈55% in children (Humayun et al., 2006). It follows that, similar to cysteine, dietary methyl donors can also potentially spare methionine for protein synthesis and methionine cycle activity, and thus affect the methionine requirement.

It has been shown here that the provision of dietary methyl donors affected the partitioning of methionine among transmethylation pathways, but did not change hepatic protein synthesis in neonatal piglets. The lack of effect on hepatic

[2] JLR wrote manuscript and performed all animal procedures, infusions, diet formulation, data analysis, statistics and contributed to study design. SVH measured enrichment of breath samples by IRMS. JAB and RFB contributed to study design, analysis and project funding as JLRs co-supervisors.

protein synthesis confounded our initial hypothesis, although methionine partitioning to protein could have been sacrificed in other tissues. The liver is a major site of metabolism and has a tight relationship with other organ systems. Due to its importance in whole body metabolism and regulation, liver growth is assumed to be highly conserved in early life. Indeed, studies have shown a significant relationship between abnormal liver growth *in utero* and adult disease (Barker et al., 1995). We hypothesized that protein synthesis in non-hepatic tissues is likely impacted during methyl restriction. Thus we took a multi-faceted approach to measure protein synthesis in the whole body, as well as in skeletal muscle which is not only the site of most protein synthesis (T. A. Davis et al., 1996; 1989), but has also been shown to be highly sensitive to nutritional perturbations (T. A. Davis et al., 1996; Yin et al., 2010). The aim of this study was to determine the effects of methyl supply on whole body protein dynamics and skeletal muscle protein synthesis during methionine restriction. Hepatic rates of protein synthesis are also presented again for the purposes of discussion.

In order to describe the experimental approach, a brief discussion regarding the employed methods is necessary. The current method of choice for calculating amino acid requirements in human infants is the indicator amino acid oxidation (IAAO) technique. This technique provides a measure of *in vivo* whole body protein synthesis and breakdown in a relatively non-invasive and rapid manner. This is compared to nitrogen-balance studies, which can take several days (Irwin and Hegsted, 1971; Zello et al., 1995), and tissue-specific measures such as arterio-venous techniques, which require invasive biopsies (Paddon-Jones et al., 2004). A

drawback of IAAO is that it does not allow for the calculation of tissue-specific protein synthesis (Ks) in metabolically active organs such as the liver or skeletal muscle. In order to capture comprehensive data on protein dynamics during methionine restriction, a multi-isotope infusion was employed to measure both whole body protein dynamics using IAAO, as well as tissue-specific protein synthesis using the direct incorporation of tracer.

We hypothesized that dietary methyl donors spare a significant amount of methionine for protein synthesis in non-hepatic tissues in the neonate. Animals were fed diets that were either deficient (**MD-**) or replete of dietary methyl donors (**MS+**) and protein synthesis was measured using enteral infusions of [^{13}C] phenylalanine and [^{3}H-methyl] methionine. Both groups of animals were fed adequate cysteine and 80% of the methionine requirement for a piglet (based on (Shoveller et al., 2003b)).

4.2 Methods

4.2.1 Chemical reagents and isotopes

All chemicals and reagents were of the highest available purity and obtained from Sigma (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ) or Alfa Aesar (Ward Hill, MA). Amino acids were from Ajinomoto, Co (Tokyo, Japan). [^{13}C] phenylalanine was obtained from Cambridge Isotope Laboratories (Tewksbury, MA). [^{3}H-Methyl] methionine was obtained from ARC Radiolabeled Chemicals (St. Louis, MO).

4.2.2 Animals

4.2.2.1 Surgical procedures and study protocol

Surgical and animal care procedures were previously described in Section 3.2.2.1.

4.2.2.2 Dietary Regimen

The dietary regimen was previously described in 3.2.2.2. The dietary amino acid profile is found in Table 3.1.

4.2.3 Isotope infusions and calculations

4.2.3.1 Infusion protocols

On the morning of Day 6, animals (N = 8) were transported to an oxidation facility where they were placed into a 30 x 30 x 90 cm, plexiglass chamber. The airtight chambers were equipped with a swivel-tether system that allowed for the constant pumping of diet as well as access to venous catheters from the chamber exterior. Fresh air was supplied using a plastic hose that was attached to one end of the chamber. An air pump was attached to the other end, which served to pull fresh air through the chamber and expired air from the piglet towards the air pump. Mass flow controllers (Qubit Systems: Kingston, ON) were fixed between the chambers and the air pump, which controlled the rate of airflow through the chamber. A Multi-Channel gas exchange system (Qubit Systems: Kingston, ON) was fixed in upstream of the flow controllers and facilitated access to chamber exhaust as well as a steady flow of fresh air. The chamber exhaust (*ie.* pig breath) could either be sampled by experimenters or directed toward CO_2 and O_2 analyzers (AEI Technologies: Pittsburgh, USA). The gas exchange system allowed access to the fresh air that was

flowing into the chambers as reference gas. The reference gas was used to correct measurements of O_2 consumption (VO_2) and CO_2 production (VCO_2) rates by the piglet. The VCO_2 and VO_2 concentrations in piglet breath were calculated with commercial software (Qubit Systems: Kingston, ON).

Once piglets were sealed inside the chamber, air was pulled through the chamber for approximately 1 h to equilibrate the system. Prior to the [^{13}C] phenylalanine infusion (t_0), the piglet exhaust was collected using a midget bubbler into 1 M NaOH for 20 minutes to trap the expired CO_2. Concurrently, blood was sampled *via* the jugular catheter and transferred immediately to a Vacutainer containing heparin and centrifuged for 10 minutes at 3000 x g. Plasma was removed and stored at -20 °C for later analyses. Piglets received a primed:constant infusion of [^{13}C] phenylalanine at a rate of 20 μmol/kg:10 μmol/(kg•h), respectively. The gastric infusion was delivered with the pig's elemental diet, which was modified to account for the infused phenylalanine. The infusion lasted 6 h; breath and blood samples were collected every 30 minutes and compared to t_0. After the infusion, animals were transported back to their metabolic cages and continued on their diet.

On Day 8, a gastric infusion of [^{3}H-methyl] methionine was performed (see 3.2.3.1). Note that for this infusion, N = 7 for the **MS+** piglets and N = 8 for **MD-** piglets. At necropsy, a sample of liver and biceps femoris muscle were excised and rapidly freeze-clamped. Tissue samples were stored at -80°C.

4.2.3.2 Isotopic enrichment and flux

In order to confirm steady state during the [^{13}C] phenylalanine infusion, the enrichment of plasma and breath samples were expressed as mole percent excess (MPE) and atom percent excess (APE), respectively. In plasma, the enrichment of [^{13}C] phenylalanine was calculated using the following equation:

$$MPE = [M+1]/([M+0] + [M+1]) \times 100\% ,$$

where [M+1] and [M+0] are the respective peak areas of [^{13}C] phenylalanine and the naturally occurring ion.

The $^{13}CO_2$ enrichment in breath samples was expressed as APE:

$$APE = AT_B - AT_{T(X)}/(1- (AT_B - AT_{T(X)})) ,$$

where AT_B and $AT_{T(X)}$ are the respective signal ratios of $^{13}CO_2$ and $^{12}CO_2$ at background and time = X. In the case of the [^{3}H-methyl] methionine infusion, the plasma steady state enrichment was measured as the specific radioactivity (SRA) as described in 3.2.3.3.

Steady state was identified by visual inspection and confirmed when the regression of a group of plasma or breath samples was not significantly different than zero for the time course values of MPE, APE and SRA ($p>0.05$). The average of these MPE and SRA values at steady state were used to calculate the flux (Q) of the various tracers in plasma. The flux of phenylalanine (Q_{Phe}) was calculated from the [^{13}C] phenylalanine infusion:

$$Q_{Phe} = i(E_{IT}/E_{Phe} -1) ,$$

where i is the tracer infusion rate, E_{IT} is the enrichment of the infused tracer in MPE and E_{Phe} is the average phenylalanine enrichment in plasma at steady state. The flux of methionine (Q_{Met}) was calculated from the [^{3}H-methyl] methionine infusion:

$$Q_{Met} = i/SRA_{Met},$$

where i is the tracer infusion rate (DPM/(kg•h)) and SRA_{Met} (DPM/μmol methionine) is the steady state SRA of the [^{3}H-methyl] methionine label in plasma.

4.2.3.3 Calculation of [^{13}C] phenylalanine oxidation and whole body protein synthesis

Whole body protein synthesis was calculated using a stochastic model (House et al., 1997; Matthews et al., 1980; Zello et al., 1990). Briefly, the phenylalanine flux (Q_{Phe}) was equated to the sum of the phenylalanine incorporation rate into protein (S) and the oxidation rate of phenylalanine (O) which equaled the sum of phenylalanine release from protein breakdown (B) and the phenylalanine intake (I). This relationship is summarized by the following equation:

$$Q_{Phe} = S + O = B + I$$

The rate of phenylalanine oxidation (μmol/kg•h) was calculated using the $^{13}CO_2$ production rate ($F_{^{13}CO2}$) from the phenylalanine tracer using the following equation:

$$F_{^{13}CO2} = (F_{CO2})(E_{CO2})(44.6\ \mu mol/cm^3)\ /(W)(BRF)(100),$$

where F_{CO2} is the CO_2 production rate (cm^3/h), E_{CO2} is the steady state $^{13}CO_2$ enrichment in breath (APE), W is the weight of the piglet (Zello et al., 1990) and BRF is the bicarbonate retention factor (0.93) that was previously determined in fed piglets (House et al., 1997). The constant 44.6 μmol/cm^3 converts CO_2 volume to

μmol and the factor of 100 converts APE to a fraction (Zello et al., 1990). This calculation converts the $^{13}CO_2$ production in breath to the appropriate unit (μmol CO_2/kg•h).

The rate of phenylalanine oxidation (O) was calculated by correcting the oxidation of the [^{13}C] phenylalanine tracer to the whole body rate of phenylalanine oxidation using the relationship:

$$O = F^{13}_{CO2}(1/E_{Phe} - 1/E_{IT}) \times 100$$

The rate of phenylalanine oxidation (O) was subsequently used to calculate the rate of phenylalanine incorporation into protein (PS) from the relationship:

$$PS = Q - O$$

Protein breakdown (PB) was calculated using the dietary provision of phenylalanine (I) where,

$$PB = Q - I$$

Lastly, net protein deposition (PD) was calculated using the relationship,

$$PD = PS - PB.$$

4.2.3.4 Calculation of tissue protein synthesis

The rate of tissue specific protein synthesis (Ks) was expressed for skeletal muscle and liver using the equation for fractional synthesis rate (FSR):

$$Ks\ (\%/d) = (SRA_{Product}/SRA_{Precursor})/time \times 100\ ,$$

where $SRA_{Product}$ was DPM/μmol of tissue-bound methionine and $SRA_{Precursor}$ was DPM/μmol of free methionine. For liver, the absolute synthesis rate (ASR) was also calculated as:

ASR (%/d) = FSR x liver mass (g)

4.2.4 Analytical procedures and instrumentation

4.2.4.1 Quantification of plasma enrichment of [^{13}C] phenylalanine

The plasma enrichment of [^{13}C] phenylalanine was measured by derivatization with pentafluorobenzyl bromide (PFBBr) and detection by GCMS. A 50 µL aliquot of plasma was well mixed with 135 µL of 133 mM PFBBr and 25 µL of 0.5 M phosphate buffer (pH 8) in an 1.5 mL microcentrifuge tube. Samples were placed into a 60 °C oven for 30 minutes to allow for alkylation of phenylalanine by PFBBr. The reaction was terminated by adding 335 µL of hexane. Samples were well mixed, and the organic phase was transferred to a glass vial.

Derivatized plasma samples were analyzed using a GCMS equipped with an autosampler (Agilent Technologies). A 2 µL aliquot of the organic phase was injected onto a DB-5MS column (0.25 mm x 30 m x 0.22 µm) (Agilent Technologies: Mississauga, ON). The GC (6890N Network GC System) oven was preheated to 50 °C and equipped with helium carrier gas. Oven temperature was maintained initially for 3 min and subsequently ramped at 30 °C/min until it reached 280 °C and held for 4 min. After column separation, samples were immediately ionized with 70 eV of EI collision energy upon entering a quadrupole MS (5973 *inert* Mass Selective Detector). The specific ions for phenylalanine were monitored (*m/z* = 434 and 435) in SIM mode. Area under the curve for each ion was determined and used to calculate the ratio of [M+1] phenylalanine compared to [M+0] phenylalanine.

4.2.4.2 Plasma and tissue metabolites

All remaining analytical procedures were described previously (3.2.4) and applied to skeletal muscle, where applicable.

4.2.5 Statistics

An unpaired t-test was used to compare values between the **MD-** and **MS+** piglets. Statistics were calculated using Prism software 5.0b (La Jolla, CA). A p-value of <0.05 was considered significant.

4.3 Results

4.3.1 Animals and diet validation

Animals were balanced for sex, age and weight at arrival. The rate of growth after 6 d was unchanged between dietary conditions (304.5 ± 72.2 g/kg **MD-** *vs.* 284.0 ± 61.3 g/kg **MS+**; p = 0.6). On day 6, the average piglet weight was 2.2 ± 0.3 kg. The effects of **MD-** feeding were previously described in section 3.3.1-3.3.3 and 3.3.5.

4.3.2 [^{13}C] phenylalanine and [^{3}H-methyl] methionine steady state and flux

In order to calculate tracer flux, steady state was confirmed during the [^{13}C] phenylalanine and [^{3}H-methyl] methionine infusions. During the phenylalanine infusion, plateau was achieved in plasma and breath by 4 h for all pigs (typical example shown in Figure 4.1). Steady state was also achieved by 4 h in plasma during the [^{3}H-methyl] methionine infusion (Figure 3.2).

The phenylalanine flux rate was ≈13% lower in the **MD-** piglets compared to the **MS+** group (p = 0.003). However, the methionine flux was unchanged between these two dietary conditions (Table 4.1).

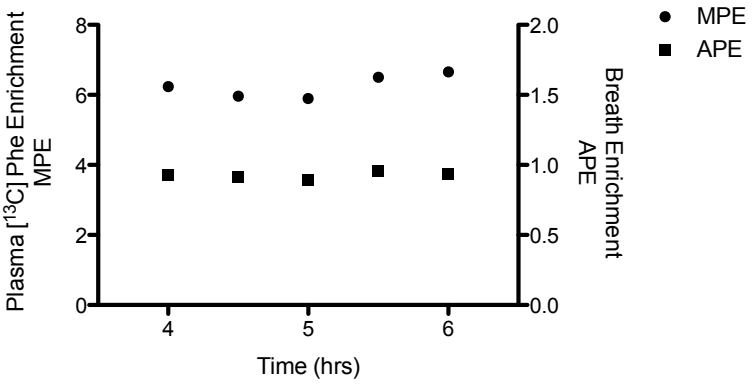

Figure 4.1 Time course values for plasma and breath enrichments for infusions of [^{13}C] phenylalanine. Plasma enrichments are expressed as mole percent excess (MPE). Breath enrichments are expressed as atom percent excess (APE).

Table 4.1 Whole body flux rates (Q) of methionine and phenylalanine during an intragastric infusion of [^{13}C] phenylalanine[1] and [^{3}H-methyl] methionine[2] tracers in piglets.

	MD-	**MS+**	p-value
$Q_{Phenylalanine}$	425.7 +/- 46.5	488.8 +/- 32.6	0.003
$Q_{Methionine}$	153.3 +/- 57.8	153.1 +/- 51.3	0.9

1 Values are μmol Phe/(kg•h) +/- SD; N = 8 for both groups
2 Values are μmol Met/(Kg•h) +/- SD; N = 8 for MD- and N = 7 for MS+

4.3.3 Quantification of whole body protein dynamics by IAAO

Whole body protein synthesis and breakdown were estimated stochastically using the intake, flux, and oxidation of phenylalanine (Figure 4.2). Whole body protein synthesis was ≈12% lower (p = 0.03) in the **MD-** animals (424.6 ± 44.0 μmol Phe/(kg•h)) relative to **MS+** (480.7 ± 37.1 μmol Phe/(kg•h) (Figure 4.2a). There was also a similar effect of **MD-** feeding on protein breakdown (Figure 4.2b), which was ≈22% lower (p = 0.01) in **MD-** piglets (221.7 ± 44.3 μmol Phe/(kg•h)) compared to

MS+ piglets (282.8 ± 35.2 μmol Phe/(kg•h)). Phenylalanine oxidation was unchanged between the groups (Figure 4.2c) (7.2 ± 3.7 μmol Phe/(kg•h) **MD-** *vs.* 9.9 ± 2.9 μmol Phe/(kg•h) **MS+;** p = 0.1) and the net deposition of protein (p = 0.9) was conserved during methyl restriction (Figure 4.2d) (200.7 ± 3.7 μmol Phe/(kg•h) **MD-** *vs.* 197.9 ± 2.9 μmol Phe/(kg•h) **MS+**).

4.3.4 Quantification of tissue-specific protein synthesis

The intracellular concentration of methionine in muscle was 50% lower in the **MD-** animals (p = 0.02), however there was no change in the SRA (p = 0.07). In the liver, the intracellular methionine concentration and SRA were unchanged (Table 4.2).

The fractional rate of protein synthesis (Ks) was calculated based on the fraction of intracellular [^{3}H-methyl] methionine that was incorporated into liver and muscle protein (Figure 4.3). In the liver, diet had no effect on Ks (37 ± 19 %/d **MD-** *vs.* 49 ± 22 %/d **MS+**; p = 0.3) (Figure 4.3a) or ASR (4126 ± 2745 %/d **MD-** *vs.* 6999 ± 1305 %/d **MS+**; p = 0.1) (Figure 4.3b). However, skeletal muscle Ks was ≈60% lower in the **MD-** animals (25.1 ± 15.0 %/d **MD-** *vs.* 59.7 ± 18.3 %/d **MS+**; p < 0.003) (Figure 4.3c).

Table 4.2 Methionine concentrations and SRA values in skeletal muscle of MD- and MS+ piglets after a 6-hour infusion of [^{3}H-methyl] methionine[1]

	MD-	MS+	*P* - value
		µmol/g tissue	
Hepatic Free Methionine	0.14 +/- 0.05	0.16 +/- 0.02	0.3
Muscle Free Methionine	0.10 ± 0.03	0.20 ± 0.09	0.02
		DPM/nmol	
Hepatic Methionine SRA	80.8 +/- 26.7	76.7 +/- 23.5	0.7
Muscle Methionine SRA	4.3 ± 2.4	2.2 ± 1.4	0.07

[1]Values are mean +/- SD of N = 8 for MD- and N = 7 for MS+

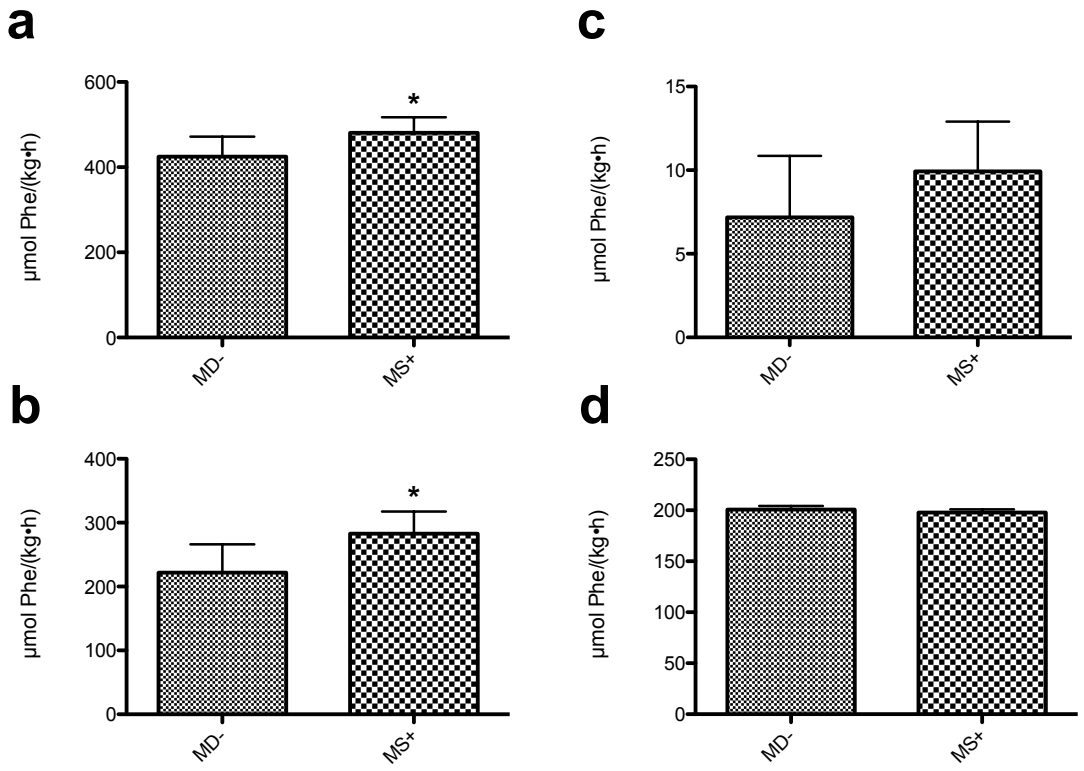

Figure 4.2 The rate of whole body protein synthesis (a), breakdown (b), phenylalanine oxidation (c) and net protein deposition (d) following a 6 hour enteral infusion of [^{13}C] phenylalanine. Values are expressed as µmol Phe/(kg•h). All values are means ± SD; N = 8 for both groups. * Denotes a significant difference between the two dietary conditions (p< 0.05).

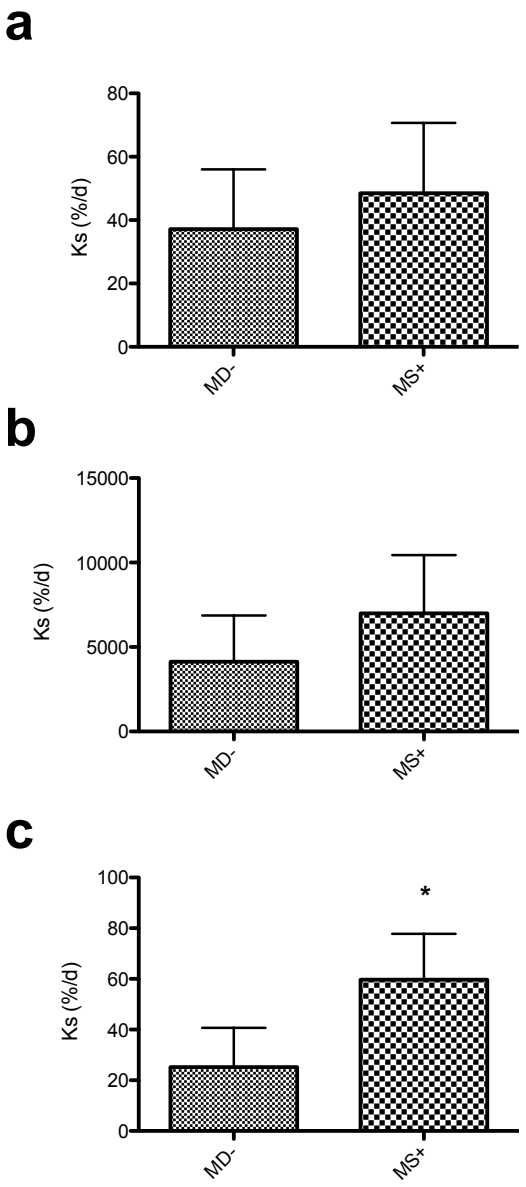

Figure 4.3 The rate of tissue specific protein synthesis (Ks) in the liver (a,b) and skeletal muscle (c) in the MD- and MS+ piglets after a 6 hour infusion of [^{3}H-methyl] methionine. Values are expressed as %/d in either a gram of liver (a) or muscle (c) or whole liver (b). All values are ± SD; N = 8 for MD- and N = 7 for MS+. * Denotes a significant difference between the two dietary conditions ($p < 0.05$).

4.4 Discussion

The objectives of this experiment were to measure the sparing effect of dietary methyl donors on methionine availability for whole body and tissue specific protein synthesis in methionine-restricted piglets. These objectives were achieved by administering enteral infusions of [^{13}C] phenylalanine and [^{3}H-methyl] methionine to methionine-restricted piglet diets that were either replete or deficient in dietary methyl donors (*ie.* choline, betaine and folate). Omitting dietary methyl donors significantly reduced whole body phenylalanine flux, protein synthesis and breakdown, as well as intracellular methionine and protein synthesis in skeletal muscle. This study demonstrated that dietary methyl donors can spare the neonatal whole body methionine requirement, likely by sparing methionine in muscle, rather than liver, for protein synthesis.

Whole body protein synthesis and breakdown were determined stochastically, as described elsewhere (Ball and Bayley, 1984; House et al., 1997; Zello et al., 1993). Whole body phenylalanine flux was reduced in the presence of dietary methyl donors; and since dietary tyrosine was provided, the decreased phenylalanine flux is significant. Our data suggest that dietary methyl donors have a significant impact on whole body phenylalanine disappearance (*ie.* protein deposition and oxidation) and appearance (*ie.* protein breakdown).

Whole body and skeletal muscle protein synthesis were significantly reduced in the absence of dietary methyl donors (**MD-**). Indeed, these observations demonstrate for the first time that dietary methyl donors can spare methionine for protein synthesis and potentially affect the neonatal methionine requirement. The

rate of whole body protein synthesis was ≈12% lower in the absence of methyl donors, which equates to ≈60 μmol Phe/(kg•h). When this rate is applied to the estimated phenylalanine content in human skeletal muscle (230 μmol Phe/g muscle) (Kaufman, 1999), methyl groups potentially spare ≈6 g muscle/(kg•d). However, this calculation is obviously an overestimate as it considers only skeletal muscle as the sole site of protein synthesis. But this calculation does put in perspective the reduction in protein synthesis that is affected by remethylation.

The absence of dietary methyl donors also resulted in a reduction of protein breakdown. Indeed, whole body protein breakdown was also ≈60 μmol Phe/(kg•h) during methyl restriction, which matches the difference in protein synthesis discussed above. Taken together, these data indicate that there was no change in net protein deposition but that protein turnover was lower during methyl restriction. This lower turnover is not surprising as rates of protein synthesis are ≈4X greater than available substrate in neonates (Dupont, 2003; Reeds et al., 2000). Protein turnover is important in early life as cellular machinery is being synthesized and rapidly degraded during tissue expansion (Dupont, 2003). Therefore, dietary methyl donors can help cover the nutritional costs of protein turnover during methionine restriction in the neonate. However, the percent of dietary methionine that was spared by methyl donors for protein turnover cannot be calculated using the current data because phenylalanine oxidation (*ie.* protein) as well as net protein deposition were unchanged between the two groups of piglets. Future studies might consider IAAO analysis while feeding graded levels of methionine to animals fed diets with

and without dietary methyl donors to calculate their relative contribution to the methionine requirement.

One of the most dramatic changes in the absence of methyl donors was a diminished rate of skeletal muscle protein synthesis (Ks). This observation is significant as skeletal muscle is a major site of protein synthesis and cell expansion in early life. In humans and piglets nitrogen accumulation in skeletal muscle approximately doubles from birth to weaning (Dickerson and Widdowson, 1960), and in rat pups, skeletal muscle constitutes 30% of total biomass at birth, and rapidly increases to 45% at weaning (T. A. Davis et al., 1989; Fiorotto et al., 2000). Furthermore, the linear increase in muscle accretion is accompanied by a linear *decrease* in muscle Ks during the same period of development (Fiorotto et al., 2000). Indeed, in piglets skeletal muscle Ks decreases markedly (≈50%) from 7 to 26 days. This Ks pattern was also observed in the liver, but to a much lesser extent (≈20%) (T. A. Davis et al., 1996). The implication of these observations is that skeletal muscle Ks early in life is largely for protein accretion (*ie.* growth), whereas liver Ks is largely for maintenance. Indeed, the rate of skeletal muscle Ks reported here was comparable to liver Ks, and thus when skeletal muscle is considered as a percentage of the whole body (≈30%), it is a more significant site of protein synthesis than liver, which was only ≈3% of total mass (see 3.3). However, it must be pointed out that the rate of skeletal muscle Ks in these piglets was comparable to rats (T. A. Davis and Fiorotto, 2009), but greater than domestic and miniature piglets of a similar age receiving an intravenous flooding dose of tracer (T. A. Davis et al., 1996; Wheatley et al., 2014) (Munasinghe, unpublished). The author has no explanation for the high

rate of skeletal muscle Ks found here, especially considering the constant tracer approach typically results in lower estimations of Ks than the bolus intravenous flooding dose (Jahoor et al., 1992). Because of the complex metabolism of methionine, this amino acid is often not recommended as a tracer for whole body protein synthesis. However, in our study, comparing the fate of the methionine tracer was the key objective; but we must acknowledge that results using this tracer may not be comparable to those from other studies using amino acid tracers with less complex metabolism. Nonetheless, methionine has been used before in this way. A constant intravenous infusion of [^{35}S] methionine administered to adult goats, resulted in muscle Ks rates that were lower than those observed here, but as adult animals, muscle Ks would be lower regardless of tracer choice (Champredon et al., 1990). With respect to neonates, other researchers in our group found similar rates of skeletal muscle Ks after administering the [^{3}H-methyl] methionine tracer to neonatal piglets (Thillayapalam, unpublished), which in turn were higher than rates in the same model using phenylalanine tracer flooding dose (Brunton et al., 2012). If we accept the higher Ks values as an artifact of the tracer choice, then we can say that the net effect of methyl donors on muscle protein synthesis is similar whether expressing muscle protein synthesis as Ks or as DPM/gram muscle protein ($p < 0.05$) (data not presented); thus, skeletal muscle protein accretion is sensitive to acute methyl restriction in early life.

The omission of dietary methyl donors also resulted in lower intracellular methionine concentrations in the skeletal muscle, an effect that was not found in the liver or plasma. These observations are in partial contrast to those in piglets fed a

SAA-free diet for 7 days that had lower free methionine in plasma (and jejunum), but no change in hepatic free methionine (Bauchart-Thevret et al., 2009). Because ≈80% of dietary methionine reaches the liver (Riedijk et al., 2007), it appears that hepatic release of methionine was sacrificed in our study to maintain hepatic Ks and transmethylation during methyl restriction. Furthermore, extrahepatic uptake of circulating methionine was likely rapid in both groups of animals, as there was no significant change in methionine flux between groups of methionine-restricted piglets. In other words, any excess methionine that was released into circulation from the liver in the **MS+** piglets was rapidly taken up by skeletal muscle, which led to an overt change in muscle intracellular methionine and protein synthesis. Interestingly, the SRA of intracellular skeletal muscle methionine was unchanged, and indeed tended to be higher in the **MD-** piglets ($p = 0.07$). While not significant, this trend reflects the lower intracellular methionine concentrations, likely as a result of decreased protein breakdown in **MD-** animals. Regardless, omission of dietary methyl donors resulted in a diminished supply of methionine for skeletal muscle, but not for the liver. This is interesting as betaine and folate were supplied in excess to the **MS+** piglets, and it was postulated that the methyl donors would have a greater rate of hepatic remethylation, resulting in an increase in methionine availability in the liver which could in turn be released to other tissues.

The rate of hepatic protein synthesis (Ks) was unchanged during methyl restriction, as was previously discussed (see 3.4). This disconnect between whole body and liver is in contrast to reports of a positive correlation between hepatic and whole body protein synthesis as detected by IAAO (Zello et al., 1995). However, it

should be noted that unlike other amino acids with simpler metabolism (*ie*. to protein or oxidation only), the vast majority of dietary methionine reaching the liver undergoes transmethylation (Table 3.4) (da Silva et al., 2009; Vance et al., 1997). Our data suggest that within the liver, rates of transmethylation were sacrificed to maintain hepatic Ks during methyl restriction, but this would not apply to the muscle where transmethylation is virtually non-existent relative to liver. Furthermore, in contrast to skeletal muscle in piglets and humans (Dickerson and Widdowson, 1960), hepatic Ks is not believed to change drastically from birth to weaning (Burrin et al., 1992), and thus it is not surprising that hepatic Ks was conserved in these piglets .

To the knowledge of the author, this is the first study that directly compared protein synthesis in specific tissues by direct incorporation of a label, as well as in the whole body using an indicator amino acid, and the strength of this approach is that it provides more holistic insight into neonatal protein synthesis during methyl deficiency, as both tracer approaches have different advantages. The indicator method is useful for determining the whole body amino acid or protein requirement (Elango et al., 2008); however, the utility of this method to strictly quantify the effects of non-protein precursors, *ie.* dietary methyl donors, on protein synthesis have not been evaluated. On the other hand, tissue-extraction after a constant infusion of a radiolabel allows for the calculation of tissue-specific protein synthesis; however, the key disadvantage is that it is impossible to extrapolate individual tissue data to whole body estimates of protein synthesis. Moreover, an enteral infusion of [^{3}H-methyl] methionine has not been previously used in this manner.

Interestingly, the two methods of calculating protein synthesis provide consistent results when comparing the treatment groups. The indicator amino acid infusion demonstrates that protein synthesis and breakdown are both equally affected by methyl restriction; and indeed, those outcomes correlate significantly with Ks of skeletal muscle ($R^2 = 0.5$; $p < 0.05$). Moreover, there were no differences in the rate of phenylalanine oxidation or net protein accretion, and these two measures also correlated significantly with the hepatic Ks ($R^2 = 0.35$; $p < 0.05$). These relationships make sense as liver is considered a major site of amino acid oxidation (Jungas et al., 1992), and muscle is the most rapidly expanding tissue in the neonate dictating growth (T. A. Davis and Fiorotto, 2009). Further studies might expand the comparison of an indicator amino acid with tissue-specific protein synthesis to confirm these relationships, and possibly expand the utility of the indicator amino acid oxidation technique.

4.5 General Conclusions

This experiment demonstrated that dietary methyl donors significantly impact whole body protein dynamics and alter methionine availability for protein synthesis in the skeletal muscle. This has significant clinical ramifications, as dietary methyl donors should be considered when evaluating the methionine requirement of a neonate. Despite a conservation of liver protein synthesis with methyl depletion, it is clear that dietary methyl donors significantly affect the partitioning of methionine for protein turnover, and potentially transmethylation. Due to the complexity of inter-organ methionine metabolism, and because different organs

receive and partition methionine differently, future studies should focus on the impact of dietary methyl donors on whole body methionine metabolism.

5.0 Dietary methyl donors have a significant impact on *in vivo* methionine partitioning between transmethylation and protein synthesis in the neonatal piglet

Robinson JL, Bartlett RK, Harding SV, Randell, EW, Brunton JA, Bertolo RF[3]

5.1 Introduction

The partitioning of methionine is of great clinical interest during infancy as both protein synthesis (PS) and transmethylation (TM) are significant metabolic processes which demand substantial dietary methionine. We have demonstrated that the absence or reduction of dietary methyl donors (*ie.* betaine, choline and folate) results in several metabolic perturbations in the methionine-restricted piglet. For example, dietary methyl donors affected the partitioning of dietary methionine through specific TM reactions in the liver (3.0), and they also affected methionine partitioning for whole body protein turnover and muscle PS (4.0). However, to this point, we have been unable to conclude that dietary methyl donors affect whole body rates of remethylation (RM), TM and transsulfuration (TS) during methionine-restriction.

The plasticity of the methionine cycle has long been established as intakes of labile methyl groups have been shown to alter methionine metabolism (Mudd and Poole, 1975). In the late 1980's, a method was developed to calculate *in vivo* rates of

[3] JLR wrote manuscript and performed all animal procedures, infusions, diet formulation, data analysis, statistics and contributed to study design. RKB measured hepatic enzyme activities under the supervision of JLR. SVH measured enrichment of breath samples by IRMS. EWR assisted in the measure of [^{2}H-methyl] moiety into choline and betaine. JAB and RFB contributed to study design, analysis and project funding as JLRs co-supervisors.

whole body RM, TM and TS using the dilution of two methionine isotopomers (Storch et al., 1988); the method was later modified to correct for intracellular methionine metabolism (MacCoss et al., 2001). The method readily detects changes in methionine metabolism after betaine supplementation in adult men (Storch et al., 1991), sulfur amino acid deficiency in piglets (Riedijk et al., 2007) and vitamin B_6 deficiency in adults (S. R. Davis et al., 2005b). In this study, we have employed this method to determine the capacity of dietary methyl donors to contribute to whole body RM and TM, and potentially overcoming methionine restriction.

Using an enteral [$^{13}C_1$, ^{2}H-methyl] methionine infusion, the objective of this study was to measure the *in vivo* effects of dietary methyl donors on whole body RM, TM and TS, as well as on the *in vitro* capacity of the hepatic enzymes responsible for homocysteine elimination. Lastly, we also investigated the utility of the [^{2}H-methyl] methionine infusion to trace the whole body catabolism of *de novo* synthesized phosphatidylcholine (PC) to free choline and betaine.

5.2 Materials and Methods

5.2.1 Chemical reagents and isotopes

All chemicals and reagents were of the highest available purity and were obtained from Sigma (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ) or Alfa Aesar (Ward Hill, MA). Amino acids were from Ajinomoto, Co (Tokyo, Japan). [^{14}C] Methyl-Tetrahydrofolic Acid ([^{14}C] MTHF), barium salt was from Amersham Biosciences, UK Limited (Buckinghamshire, UK). L-[3-^{14}C] Serine ([^{14}C] serine) and [^{14}C-methyl] N,N,N-Trimethyl Glycine ([^{14}C] betaine) were acquired from Moravek Biochemicals

(Brea, CA). [3,3,3',3',4,4,4',4'-^{2}H] homocystine ([^{2}H] homocystine), [$^{13}C_1$] methionine ([^{13}C] methionine) and [^{2}H-methyl] methionine were obtained from Cambridge isotopes (Tewksbury, MA).

5.2.2 Animals and infusion protocols

The dietary and surgical protocols were described in sections 3.2.2 and 4.2.2, and this study was approved by the Memorial University of Newfoundland Animal Care Committee.

On the morning of Day 7 the piglets received an equimolar infusion of [^{13}C, ^{2}H-methyl] methionine. The enteral methionine tracer infusion commenced with a 19.97 μmol/kg priming dose with a 9.97 μmol/(kg•h) constant infusion for 8 hours. The intake of dietary methionine was unchanged for the infusion protocol as dietary methionine was adjusted to account for the infusion of labeled methionine. The infusion took place in metabolic chambers; breath and blood were sampled throughout the procedure (See 4.2). After the infusion, piglets were returned to their metabolic cages and continued on their respective diets (*ie.* **MD-** or **MS+**). The following day, after a separate isotope infusion (See 3.0), liver samples were rapidly removed and freeze clamped during necropsy. Tissues remained at -80 °C until analysis.

5.2.3 Plasma enrichment of [^{13}C, ^{2}H methyl] methionine

The plasma enrichment of [^{13}C, ^{2}H methyl] methionine was quantified by GCMS. A 50 μL aliquot of plasma was mixed with 135 μL of 133 mM PFBBr, 15 μL of

0.5 M phosphate buffer (pH 8), 5 µL of 1 mM [^{2}H] homocystine and 5 µL of 10% tri-butylphosphine (in DMSO) in an eppendorf tube. Samples were left at room temperature for 30 minutes and subsequently placed in a 60 °C oven for 30 minutes to allow for alkylation. Thorough mixing with 335 µL of hexane terminated the reaction and a 2 µL aliquot of the organic fraction was analyzed using a GCMS (Agilent Technologies) fitted with a DB-5MS column (0.25 mm x 30 m x 0.22 µm) (Agilent Technologies: Mississauga, ON). The GC (6890N Network GC System) oven was preheated to 50 °C and equipped with helium carrier gas. Oven temperature was maintained initially for 3 minutes and subsequently ramped at 30 °C/min until it reached 280 °C, where it was held for 4 minutes. After column separation, samples were immediately ionized with 70 eV of EI collision energy upon entering a quadrupole MS (5973 *inert* Mass Selective Detector). The m/z for methionine was 328 [M+0], which was compared to 329 [M+1] and 331 [M+3] for [^{13}C] methionine and [^{2}H-methyl] methionine, respectively. The m/z of [^{13}C] homocysteine was also monitored during the infusion at 494 [M+0] and 495 [M+1]. Sample reduction was confirmed by monitoring reduction of the [^{2}H] homocystine internal standard at m/z 498 (*ie.* [M+4] homocysteine).

5.2.4 Hepatic enzyme activities

Liver homogenate preparation

Approximately 0.5 g of frozen liver was combined with 5X the volume of ice cold homogenization buffer (50 mM potassium phosphate dibasic buffer, pH 7.0). The samples were homogenized on ice using a Brinkmann Polytron homogenizer

(Delran, NJ) at half speed for approximately 30 seconds and then centrifuged at 20 000 *x g* for 30 minutes at 4 ˚C (Eppendorf Centrifuge 5804 R). The supernatant was immediately removed, and used to determine the protein concentration and enzyme activity. Protein concentrations were measured in 50 µL of liver supernatant mixed with 200 µL of 5% deoxycholic acid (DOC) and 750 µL of water. A 4 mL aliquot of "biuret reagent" (6.4 mM copper (II) sulfate pentahydrate, 21.3 mM potassium sodium tartrate tetrahydrate, 3% (w/v) sodium hydroxide) was added and samples were allowed to react for 30 minutes. Sample absorbance was measured at 560 nm using a plate reader (BioTek Powerwave X5). Protein concentrations were determined using a bovine serum albumin (BSA) standard curve (0-2 mg BSA).

Methionine synthase activity

The methionine synthase (MSyn) assay was adapted from Koblin *et al*. (Koblin et al., 1981). Ideally, protein concentrations of fresh liver were adjusted to 15-20 mg/mL using 50 mM potassium phosphate dibasic buffer (pH 7.0). The assay was linear with protein concentration (0-2.5 mg protein) and time (0-60 minutes) (MacKay et al., 2012).

MSyn activity was initiated by combining 100 µL of liver supernatant with 100 µL of MSyn substrate mixture (20 µM cyanocobalamin, 58 mM dithiothreitol (DTT), 0.5 mM S-adenosylmethionine (SAM), 15 mM DL-homocysteine, 14 mM β-mercaptoethanol, 1 mM methyltetrahydrofolate with 2.5 µCi of 5-[^{14}C] methyltetrahydrofolic acid and 175 mM potassium phosphate buffer, pH 7.5) and immediately blanketed under a flow of nitrogen, sealed, and mixed by inversion. The

samples were incubated in the dark for 30 minutes in a 37 ˚C shaking water bath. The reaction was stopped with 400 µL of cold deionized water and immediately stored on ice for immediate isolation of the reaction product.

The MSyn product ([^{14}C] methionine) was isolated from the reactant ([^{14}C] MTHF) by applying 400 µL of the reaction mixture to an ion-exchange resin (AG 1–x8 resin, 200–400 mesh, chloride form) from BioRad (St. Louis: USA). The resin-bound product was eluted using 3 volumes of 500 µL of deionized water. The effluent (1.5 mL) was collected in scintillation vials containing 10 mL Scintiverse scintillation cocktail E (Fisher Scientific) and counted for 10 minutes with a liquid scintillation counter (Perkin Elmer, Canada). The specific activity (DPM/nmol) of 10 µL of [^{14}C] MTHF acid standard was used to calculate the amount of product eluted in nmol. MSyn activity is expressed as nmol methionine/min•mg protein. The columns were regenerated using 5 volumes of 500 µL of 1 N HCl, followed by 5 volumes of 500 µL deionized water, or until resin was pH 5.

Blank samples were analyzed alongside each assay to correct for background radioactivity. The blanks contained 100 µL homogenization buffer *in lieu* of liver protein with MSyn substrate mixture. Samples were analyzed in triplicate.

Betaine homocysteine methyltransferase activity

The activity of betaine homocysteine methyltransferase (BHMT) was measured by modification of previously described methods (Finkelstein and Mudd, 1967; Garrow, 1996). Liver supernatant concentrations were diluted to 15 mg/mL

with homogenization buffer; the assay was linear with protein concentration (0.25-2.0 mg protein) and time (0-60 minutes) (MacKay et al., 2012).

BHMT activity was initiated upon addition of 100 µL of 10 mM [^{14}C] betaine to 100 µL of liver supernatant combined with 200 µL of 125 mM Tris (pH 7.5), and 100 µL of 25 mM DL-homocysteine. The reaction occurred in a 37 ˚C shaking water bath for 20 minutes. The reaction was terminated with 2.5 mL of cold deionized water and then immediately stored on ice. The products were separated immediately to prevent the loss of product.

The labelled products were separated by adding 2 mL of the terminated reaction solution to an ion-exchange column (Bio-Rad Poly-prep Chromatography Columns (0.8 x 4 cm), AG 1 – x4, 200 – 400 mesh, hydroxide form). The unreacted [^{14}C] betaine was washed from the column with deionized water (3 x 5 mL) and discarded. The bound [^{14}C] methionine and [^{14}C] dimethylglycine (DMG) were eluted into scintillation vials using 3 mL of 1.5 N HCl. Samples were measured for radioactivity by adding 10 mL of Scintiverse scintillation cocktail E (Fisher Scientific) and then counted for 10 minutes with a scintillation counter. The specific activity (DPM/nmol) of a [^{14}C] betaine standard was used to quantify the amount of product eluted in nmol. BHMT activity is expressed as nmol methionine/min•mg protein.

Blank samples were analyzed alongside each assay to correct for background radioactivity. The blanks contained 100 µL of homogenization buffer rather than liver protein with all enzyme substrates present. All samples were performed in triplicate.

Cystathionine β synthase activity

The activity of cystathionine β synthase (CBS) was measured by the methods of others (Mudd et al., 1965; Taoka et al., 1998). Fresh liver supernatants were diluted to 15 mg/mL using homogenization buffer; and the assay was linear with respect to time (0-60 minutes) and protein concentration (0-3 mg protein) (MacKay et al., 2012).

CBS activity was measured by mixing 150 µL of liver supernatant with 400 µL of CBS substrate mixture (0.219 mM L-cystathionine, 62.5 mM DL-homocysteine, 3.125 mM DL-propargylglycine, 0.38 mM SAM, and 0.625 mM pyridoxal-5-phosphate (PLP) in 187.5 mM Tris-HCl/3.125 mM EDTA, pH 8.3). The reaction mixture was equilibrated to 37 ˚C in a shaking water bath before initiating the reaction with 50 µL of 300 mM [^{14}C] serine (0.1 µCi). The reaction proceeded for 30 minutes at 37 ˚C and was terminated by the addition of 300 µL of cold 15% TCA. The precipitate was extracted by centrifuging the reaction mixture for 10 minutes at 9000 *x g*. A 500 µL aliquot of deproteinated sample was immediately applied to an ion exchange resin (AG 50w – x4 Resin, 200 – 400 mesh, H^+ form) from Bio-Rad (St. Louis, MO). The column was sequentially washed with deionized water (2 x 4 mL), 1 N HCl (6 x 4 mL), and then deionized water (4 x 4 mL). The effluent containing unreacted [^{14}C] serine was discarded. Finally, [^{14}C] cystathionine was eluted into a collection vial with 5 mL of 3 N ammonium hydroxide. A 1 mL aliquot of the [^{14}C] cystathionine-containing effluent was combined with 10 mL Scintiverse scintillation cocktail E (Fisher Scientific) and counted for 10 minutes with a liquid scintillation counter (Perkin Elmer, Canada). The counts were multiplied by 5 to correct for total

DPM in the 5 mL elution. The specific activity (DPM/nmol) of the [^{14}C] serine standard was used to measure nmol of product formed and CBS activity was expressed as nmol cystathionine/min•mg protein.

Blank samples were analyzed alongside each assay to correct for background radioactivity. The blanks combined 150 µL of homogenization buffer, in lieu of liver protein, with CBS substrate mixture. The mixture was allowed to equilibrate in the water bath alongside the other samples; however, the sample was removed and any activity was arrested with the cold 15% TCA prior to the addition [^{14}C] serine. All samples were analyzed in triplicate.

5.2.5 Calculations

5.2.5.1 Calculation of RM, TS and TM

The flux (Q) of the [^{13}C] methionine (Q_C) and [^{2}H-methyl] methionine (Q_M) tracers were calculated as:

$$Q_{Met} = i(E_{IT}/E_{Met} - 1),$$

where i is the isotope infusion rate, E_{IT} is the enrichment (MPE) of the isotope tracer and E_{Met} is the enrichment of methionine in plasma (MPE)**.** To account for intracellular methionine, the MPE of [^{13}C] homocysteine was used in place of the [^{13}C] methionine label in plasma to calculate Q_C. The loss of the [^{2}H-methyl] moiety during TM prevents the utilization of a homocysteine isotopomer as a surrogate for intracelluar [^{2}H-methyl] methionine enrichment, which was calculated by multiplying [^{2}H-methyl] methionine enrichment by the fractional difference of [^{13}C] homocysteine and [^{13}C] methionine enrichment using the equation:

$$E'_{[^2H\text{-}Met]} = E_{[^2H\text{-}Met]}(E_{[^{13}C\text{-}Hcy]}/E_{[^{13}C\text{-}Met]}) ,$$

where $E_{[^2H\text{-}Met]}$, $E_{[^{13}C\text{-}Hcy]}$, $E_{[^{13}C\text{-}Met]}$ are the plasma MPE enrichments of [^{2}H-methyl] methionine, [^{13}C] homocysteine, and [^{13}C] methionine, respectively. The intracellular enrichment of [^{2}H-methyl] methionine (*ie.* $E'_{[^2H\text{-}Met]}$) was used to calculate Q_M (MacCoss et al., 2001).

RM was calculated as: $RM = Q_M - Q_C$ and TS, which is related to methionine oxidation (Ox), was calculated by relating the $^{13}CO_2$ excretion in breath to the plasma enrichment of [^{13}C] homocysteine as in the equation:

$$TS = Ox = F_{^{13}CO2}(1/E_{[^{13}C\text{-}Hcy]} - 1/E_{IT}) \times 100 ,$$

where F^{13}_{CO2} is the rate of ^{13}C output in breath (Storch et al., 1988; 1990) after being adjusted by 0.93 for bicarbonate retention in neonatal piglets (House et al., 1997) and E_{IT} is the enrichment of the [^{13}C] methionine infusate. TM was calculated as the sum of TS and RM (*ie.* TM = TS + RM) and finally, the rate of whole body PS and protein breakdown (PB) were calculated using:

$$PS = Q_C - TS ,$$

$$PB = Q_C - I ,$$

where I is the dietary intake of methionine (Storch et al., 1990; 1988). Protein deposition (PD) was calculated as PS – PB = PD.

5.2.5.2 Fractional net conversion and fraction of product flux

To calculate the fraction of choline and betaine flux from [^{2}H-methyl] methionine, the following equation was first applied to express the % of product flux (Bertolo et al., 2003):

Fractional net conversion (FNC) = ($E_{Product}$/ $E_{Precursor}$) x 100%

The $E_{Product}$ was the steady state MPE enrichment of either [M+3] choline or [M+3] betaine. The $E_{Precursor}$ was $E'_{[^2H\text{-}Met]}$.

The term $E_{Product}/E_{Precursor}$ has been applied previously to calculate $Q_{Precursor \rightarrow Product}$ (μmol/(kg•h)) using the formula:

$$Q_{Precursor \rightarrow Product} = E_{Product}/E_{Precursor} \times Q_{Product} \times [Q_{Precursor}/(I + Q_{Precursor})] ,$$

where in this case I is the precursor infusion rate (*ie.* [^{2}H-methyl] methionine) and the product was either choline or betaine. The term $[Q_{Precursor}/(I + Q_{Precursor})]$ corrects for the contribution of the precursor infusion to the $Q_{Precursor \rightarrow Product}$ calculation (J. T. Clarke and Bier, 1982; Thompson et al., 1989; Urschel et al., 2007).

Because the above equation requires a separate infusion of labeled product to calculate $Q_{Product}$, this was factored out of the equation to generate "*fraction of product flux"* (FPF), and is considered the stable isotope equivalent to FNC as it corrects for the contribution of the [^{2}H-methyl] infusion to $Q_{Precursor \rightarrow Product}/ Q_{Product}$. FPF was defined as follows:

$$FPF = \frac{Q_{Precursor \rightarrow Product}}{Q_{Product}} = FNC \times \frac{Q_{Precursor}}{(I + Q_{Precursor})}$$

The FPF expresses the relative contribution of [^{2}H-methyl] methionine to choline and betaine flux. We present both FNC and FPF values for the conversion of methionine to choline and betaine.

The relationship of FPF to $Q_{Precursor \rightarrow Product}$ was evaluated by calculating FPF using previously published data by Urschel *et al.* (Urschel et al., 2007). In that study,

researchers used a multi-tracer approach to quantify rates of arginine synthesis from proline, ornithine and citrulline, under conditions of high (+Arg) and low dietary arginine (-Arg) in piglets. We calculated the FPF using the reported means of enrichment, flux, and infusion rate, as well as through substitution of the published $Q_{Precursor \rightarrow Product}$ and $Q_{Precursor}$ values into our FPF equation (Urschel et al., 2007). As expected the two methods of calculating FPF correlated strongly ($R^2 = 0.96$; $p < 0.001$), and moreover, FPF values correlated with reported (Urschel et al., 2007) rates of $Q_{Precursor \rightarrow Product}$ ($R^2 = 0.3$; $p < 0.02$).

5.2.5.3 Calculation of enzyme activity

Enzyme activity levels are expressed as nmol product/min•mg protein. In the case of the MSyn and BHMT assays, the product was methionine and the activity was determined by the following equation:

$$Activity = \frac{(DPM - DPM_{blank})(v_{inc})}{(SA)(v_{add})(t)(c_s)(v_s)}.$$

In the case of CBS, the product was cystathionine, and its activity was calculated by:

$$Activity = \frac{(DPM - DPM_{blank})(v_{inc})(v_{elu})}{(SA)(v_{add})(t)(c_s)(v_s)(v_{count})}.$$

Collectively, DPM is the radioactivity of the eluted sample; DPM_{blank} is the radioactivity of the blank; v_{inc} is the final volume (mL) of the incubation mixture

after stopping the reaction; SA is the specific activity (DPM/nmol) of the radioactive substrate; t is the time in minutes; v_{add} is the volume of incubation mixture that was applied to the column for elution; v_{elu} is the total elution volume that was collected into scintillation vials; v_{count} is the volume of the eluent counted for radioactivity; c_s is the concentration in the supernatant in mg/mL, and v_s is the volume of liver supernatant used to catalyze the reaction. All samples were analyzed in triplicate and repeated if the coefficient of variation was >20%.

5.2.6 Statistics

An unpaired t-test was used to compare values between the **MD-** and **MS+** animals. Published $Q_{Precursor \rightarrow Product}$ values were correlated to FPF values using linear regression. In all cases $p < 0.05$ was considered significant. Statistics were calculated using Prism software 5.0b (La Jolla, CA).

5.3 Results

5.3.1. Animals

The effects of **MD-** feeding were previously described in 3.3. Piglet weight gain was not different between groups on day 7 (381.6 ± 63.5 g/kg **MD-** *vs.* 327.0 ± 77.1 g/kg **MS+**; $p = 0.3$). Prior to the infusion animal weights were not different between groups (pooled: 2.3 ± 0.3 kg).

5.3.2 Methionine kinetics

Prior to calculating tracer flux, steady state was confirmed during the [^{13}C] methionine and [^{2}H-methyl] methionine infusions (the two separate isotopes hereafter referred to as [^{13}C, ^{2}H-methyl] methionine). A plateau was achieved for both methionine tracers by 6 hours in the breath and plasma of all pigs (Figure 5.1a). Furthermore, the plasma appearance of the [^{2}H-methyl] moiety into choline and betaine [M+3] was also at plateau by 6 hours. As expected, the MPE steady state enrichment was lower for betaine than for choline. Steady state could not be achieved for [M+3] DMG.

The rates of methionine flux were calculated for the [^{13}C, ^{2}H-methyl] methionine infusions (Table 5.1). There were no effects of **MD-** feeding on methionine flux, and there were no statistical differences between Q_M and Q_C (Table 5.1).

The major metabolic pathways of the methionine cycle are presented in Table 5.1. Whole body RM was reduced by 65% in the absence of methyl donors ($p = 0.02$), while the rate of TS was low in both groups of animals and did not differ ($p = 0.3$). Whole body TM was 63% lower during methyl restriction ($p = 0.01$).

The rates of whole body protein synthesis (PS), protein breakdown (PB) and protein deposition (PD) were unchanged using this tracer approach (Table 5.1). Methionine oxidation was highly variable in the **MD-** piglets, which was also reflected in whole body TS.

To estimate the relative metabolic fate of methionine among TS, TM and PS, those values were expressed relative to the methionine flux (Q_M), *ie.* methionine

metabolism. The fraction of methionine flux metabolized through TM (TM/Q_M) was reduced by 67% during methyl deficiency ($p = 0.01$) which was partially accounted for by a 7% increase in the fraction of methionine flux diverted to PS (*ie.* PS/Q_M) in the **MD-** piglets ($p = 0.003$). Furthermore, gross methionine partitioning (*ie.* TM/PS) was 70% lower in the **MD-** animals ($p = 0.003$). The fraction of methionine flux oxidized to TS (TS/Q_M) and the rates of TM relative to TS (TS/TM) or RM (RM/TM) were unaffected by methyl restriction.

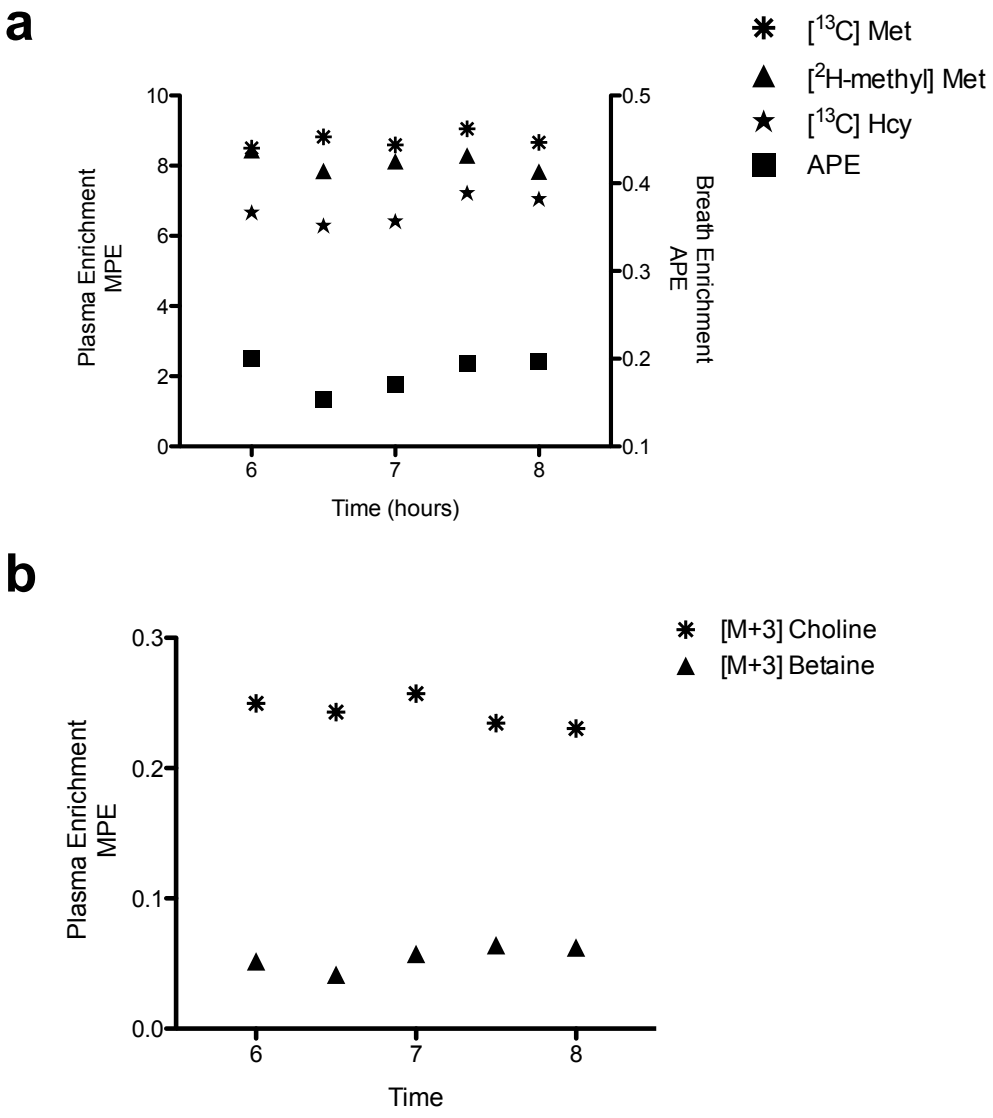

Figure 5.1 Typical time course data for plasma and breath enrichments for infusions of (a) [^{13}C, ^{2}H-methyl] methionine and (b) [M+3] choline and betaine during infusion of [^{2}H-methyl] methionine. Plasma enrichment is expressed as MPE and breath is expressed as APE.

Table 5.1 Whole body methionine kinetics[1,2]

	MD-	**MS+**	p-value
		µmol met/(kg•h)	
Q_M	170.4 ± 65.8	175.1 ± 45.6	0.8
Q_C	166.1 ± 62.4	163.0 ± 43.2	0.9
RM	4.2 ± 5.4	12.1 ± 6.0	0.01
TS	0.9 ± 0.9	1.4 ± 1.0	0.3
TM	5.4 ± 5.4	14.7 ± 5.9	0.01
PS	171.8 ± 65.0	149.0 ± 27.2	0.4
PB	85.2 ± 60.0	63.1 ± 27.1	0.4
PD	86.5 ± 1.0	86.0 ± 1.0	0.3
		%	
TM/Q_M	3.2 ± 3.0	8.9 ± 3.2	0.007
PS/Q_M	97.2 ± 2.9	91.0 ± 3.3	0.003
TS/Q_M	0.49 ± 0.69	0.95 ± 0.62	0.2
TM/PS	3.0 ± 3.1	10.0 ± 4.0	0.004
RM/TM	77.2 ± 23.5	88.0 ± 9.9	0.3
TS/TM	22.8 ± 23.6	12.0 ± 9.9	0.3

[1]Values are means +/- SD; $p < 0.05$ indicates significance; N = 8
[2]Kinetic parameters are flux (Q), remethylation (RM), transsulfuration (TS), protein synthesis (PS), protein breakdown (PB) and protein deposition (PD).

5.3.3 Fraction of product flux and fractional net conversion

The comparison of $FPF_{Met \rightarrow Choline}$ and $FPF_{Met \rightarrow Betaine}$ between methyl restricted (**MD-**) and methyl sufficient (**MS+**) piglets are presented in Table 5.2. The $FPF_{Met \rightarrow Choline}$ and $FNC_{Met \rightarrow Choline}$ calculations gave similar results, but neither were different between dietary conditions. The $FPF_{Met \rightarrow Betaine}$ of **MD-** piglets was significantly higher than **MS+** pigs ($p = 0.0003$), consistent with the FNC calculation ($p = 0.03$). The MPE of betaine and choline were also presented in Table 5.2. Choline

MPE was unchanged between groups but in contrast, the steady state MPE for betaine was 14-fold greater in the **MD-** piglets (p = 0.0001).

Table 5.2 Isotope kinetics of methionine to choline and betaine flux by two calculations[1]

	MD-	**MS+**	p-value
		FNC (%)[2]	
$FNC_{Met \rightarrow Choline}$	4.6 ± 2.3	3.7 ± 1.2	0.3
$FNC_{Met \rightarrow Betaine}$	2.8 ± 2.2	0.6 ± 1.5	0.03
		FPF (%)[3]	
$FPF_{Met \rightarrow Choline}$	3.8 ± 1.6	3.0 ± 0.7	0.8
$FPF_{Met \rightarrow Betaine}$	2.3 ± 1.3	0.1 ± 0.05	0.0003
		MPE	
Choline	3.6 ± 2.1	8.2 ± 5.2	0.056
Betaine	0.14 ± 0.09	0.01 ± 0.01	0.0001
		μM[4]	
Choline	2.2 +/- 2.9	7.5 +/- 4.9	0.04
Betaine	0.7 +/- 0.4	162.1 +/- 51.2	0.0001

[1] Values are means ± SD, N = 8
[2] FNC is fractional net conversion
[3] FPF is fraction of product flux
[4] Choline and betaine concentrations presented in 3.0 and methods found in 3.2

5.3.4 Hepatic activities of MSyn, BHMT and CBS

There were no effects of **MD-** feeding on *in vitro* RM enzyme activities. The MSyn activity was measured by the rate of [^{14}C] MTHF conversion to [^{14}C] methionine and was unchanged between the two dietary conditions (p = 0.8) (Figure 5.2a). The mean MSyn activity for the **MD-** piglets was 0.31 ± 0.09 nmol methionine/min•mg protein which was similar to the **MS+** piglets (0.30 ± 0.08 nmol methionine/min•mg protein). BHMT activity was measured by the *in vitro* conversion of [^{14}C] betaine to [^{14}C] methionine and was also unaffected by methyl restriction (0.25 ± 0.24 nmol methionine/min•mg protein **MD-** *vs.* 0.29 ± 0.21 nmol methionine/min•mg protein **MS+**; p=0.7) (Figure 5.2b).

As opposed to the RM enzymes, the *in vitro* CBS activity was sensitive to methyl restriction (Figure 4.2c). The assay measured [^{14}C] cystathionine formation from [^{14}C] serine, and the CBS activity was reduced by 35% in the **MD-** piglets (1.05 ± 0.36 nmol cystathionine/min•mg protein) as compared to **MS+** (1.61 ± 0.52 nmol cystathionine/min•mg protein) ($p = 0.04$).

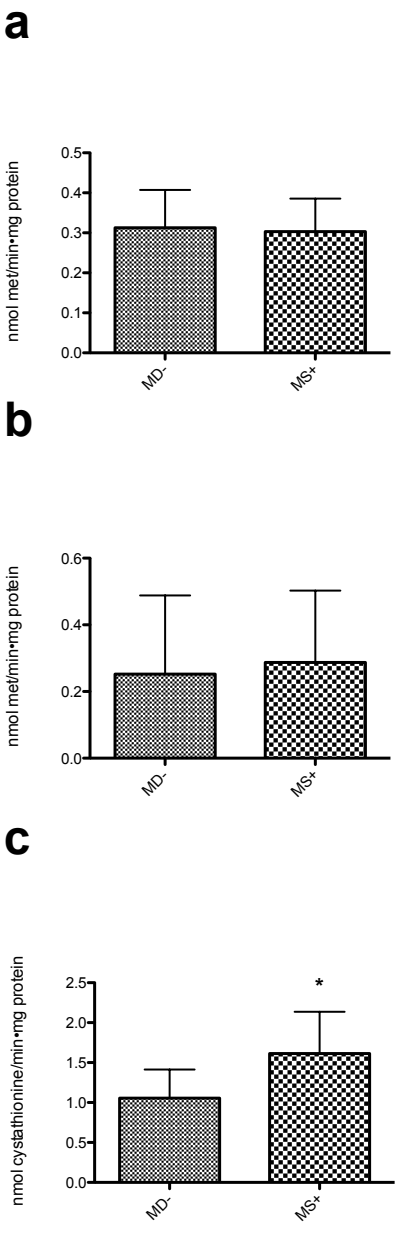

Figure 5.2 Hepatic activities of (a) MSyn, (b) BHMT and (c) CBS in MD- and MS+ piglets on study day 8. Rates are expressed as nmol product/min•mg liver protein ± SD, N = 7; * denotes a significant difference between groups ($p<0.05$).

5.4 Discussion

The objectives of this study were to measure the effects of omitting dietary methyl donors on *in vivo* and *in vitro* methionine cycle activity. Those objectives were achieved by infusing dietary [^{13}C, ^{2}H-methyl] methionine to piglets that were either replete, or deficient in dietary methyl donors (*ie.* choline, betaine and folate). Dietary methyl donors had a significant impact on *in vivo* TM and RM flux, but only on TS enzyme capacity *in vitro*. Furthermore, methyl restriction had no effect on the incorporation of methyl groups from [^{2}H-methyl] methionine (*via* PC) to free choline, but the conversion of *de novo* choline to betaine was greater. This study demonstrated that at the whole body level, *in vivo* RM contributes a significant amount of methionine for the neonate, mostly by providing more substrate for TM.

The *in vivo* flux of whole body RM, TS and TM were measured by an isotope dilution technique as described extensively elsewhere (MacCoss et al., 2001; Riedijk et al., 2007; Storch et al., 1988). As was hypothesized, the rate of RM was reduced by 65% during methyl restriction (**MD-**), and the provision of dietary methyl donors contributed 7.9 μmol methionine/(kg•h), or 9% more methionine than was provided in the diet. The sum of *de novo* and dietary methionine was 98 μmol methionine/(kg•h) in our model, and thus was still 7% lower than the estimated requirement for the piglet (*ie.* 105.2 μmol/(kg•h)) (Shoveller et al., 2003b). We had expected dietary methyl donors to restore methionine to its requirement, as others have reported that RM could maintain PS in piglets fed a sulfur amino acid free diet (Bauchart-Thevret et al., 2009). Because the piglets used here were a slower growing miniature breed, it is possible that the methionine requirement is slightly

less than that estimated in domestic strain piglets (Shoveller et al., 2003a). However, it should be noted that even in our model, protein synthesis was also maintained (or even increased when expressed as PS/Q_M) when methyl donors were omitted from the diet. Regardless, the dietary methyl donors contributed a significant amount of *de novo* methionine to the methionine-restricted piglet.

Dietary methyl donors contributed 9.3 μmol methyl/(kg•h) for TM in these piglets. Indeed, whole body TM was 63% lower during methyl restriction (**MD-**), and thus a 63% reduction in the synthesis of the >50 TM products is also expected. We are unable to determine the sensitivity of all TM reactions to dietary methyl supply, but we did show that hepatic methyl group partitioning towards creatine was diminished with methyl depletion, while the synthesis of PC was enhanced (Figure 3.2). It is not likely that these were the only two reactions sensitive to dietary methyl supply. Further analysis is required to determine the impact of a reduction in TM on the multitude of other methylation reactions.

While methionine flux was unchanged between dietary conditions, TM, as a percentage of methionine metabolism (Q_M), was reduced while PS was enhanced during methyl restriction (Table 5.1). Therefore, during methyl restriction, dietary methionine was preferentially partitioned towards PS at the expense of TM, and indeed, the relative partitioning of dietary methionine (*ie.* TM/PS) was 70% lower due to a reduction in TM, as well as a tendency for greater protein incorporation during **MD-** feeding. Because the primary site of methionine partitioning is likely liver (Bertolo and McBreairty, 2013; da Silva et al., 2009; Schneider and Vance, 1979; Stead et al., 2006; Vance et al., 2007; 1997), this shift in partitioning supports

our previous hypothesis that hepatic and whole body TM were sacrificed to maintain protein synthesis during methyl restriction (see 4.4), and is agreement with data in sulfur amino acid deficient piglets (Bauchart-Thevret et al., 2009).

One of the intricacies of methionine metabolism is that the TM product, PC, can be hydrolyzed to free choline, and theoretically fulfill choline and betaine demands. In order to gain insight into these conversions, the incorporation of [^{2}H-methyl] was traced in choline, betaine and DMG. Interestingly, the percent of choline flux derived from methionine (*via* PC) was unchanged during methyl deficiency. Because the FSR of hepatic PC was greater during methyl restriction (Figure 3.3), it was expected that *de novo* choline synthesis would also be enhanced. However, because circulating choline concentrations were significantly lower during methyl restriction (Table 5.2), PEMT was clearly unable to contribute an appreciable amount of free choline during this metabolic situation. Indeed, the synthesized PC from PEMT was likely needed for its other functions so was not readily hydrolyzed to choline, especially given PC synthesis from choline *via* the Kennedy pathway was likely diminished in the absence of dietary choline. We are not the first group to attempt to understand the significance of choline production as a catabolic product of PC. Indeed, feeding a choline deficient diet is lethal in mice lacking the PEMT gene (Walkey et al., 1998), whereas in men, dietary-induced choline deficiency affects only the liver, the primary site of PC metabolism (Zeisel, 1990). And indeed, a 7 day fast only moderately reduced plasma choline concentrations and had no effect on plasma PC (Savendahl et al., 1997). Those findings were presumably due to enhanced PEMT activity (Schneider and Vance, 1978). As a result, the action of

hepatic PEMT is considered to be an adaptive mechanism for PC synthesis during acute choline deficiency (Walkey et al., 1998). It is difficult to interpret our results compared to others as our **MD-** model is essentially the opposite of a PEMT knockout; but in this case, the fraction of choline from methionine, *via* PEMT and PC hydrolysis, was unchanged from piglets that were choline-replete (**MS+**), likely because PC via PEMT was not available for choline biosynthesis. Future studies should quantify the *in vivo* contribution of PEMT to free choline during various nutritional states.

The contribution of dietary methionine to betaine flux was enhanced during methyl restriction. Because methionine to choline flux was not different, this change is likely due to enhanced flux from choline to betaine. This finding is in contrast to our hypothesis that depleted choline would not be available for betaine synthesis, similar to data in hepatocytes where the mitochondrial conversion of choline to betaine was progressively reduced with prolonged incubation in a choline deficient medium (Schneider and Vance, 1978). This unexpected result could be explained by the plasma betaine concentrations in our piglets. The **MS+** piglets were fed ample betaine resulting in high plasma betaine concentrations (see 3.0) and thus *de novo* betaine synthesis was also reduced in those piglets, as reflected by the MPE of betaine (Table 5.2). With methyl deficiency, betaine synthesis from choline was enhanced, but this higher synthesis was obviously inadequate as plasma betaine concentration in these pigs was <1% of that in **MS+** pigs (Table 5.2). Nevertheless, these data provide unequivocal evidence that a fraction of choline is converted to betaine, and that there was potential for the complete recycling of [^{2}H-methyl].

However, DMG enrichment was undetectable, either because little tracer reached this metabolite *via* BHMT, or because betaine was primarily synthesized to be used as an osmolyte during methyl restriction.

The rates of hepatic TS were very low and unaffected by the presence of dietary methyl donors. This low TS flux is not surprising as dietary methionine was restricted and dietary cysteine was provided in excess to all piglets; therefore, TS demand was low in both groups of animals. This diet design was intentional to ensure that demand for methionine was for TM and PS – and not for cysteine synthesis. However, *in vitro* activity of CBS, the rate-limiting enzyme of TS, was reduced during methyl restriction, which is in agreement with rats fed low and adequate methionine diets (Finkelstein and Mudd, 1967). However, enzyme specific activity can be considered enzyme 'capacity', because activity is measured using optimal conditions and substrate concentrations. So CBS capacity was reduced with methyl deficiency, but because TS flux was very low, it is possible flux through CBS was well below its capacity and so no difference in whole body TS was observed. These data suggest that caution is warranted when using enzyme specific activity to interpret metabolic fluxes through the enzymes as these data are disconnected in this model.

The *in vitro* activities of hepatic RM enzymes were unchanged in these piglets, in spite of clear changes to *in vivo* RM. These enzyme and flux data are similarly disconnected as with TS. It is possible that the control of RM was not *via* enhanced hepatic enzyme capacity, or that enzyme capacity is more relevant with excess rather than depleted substrates. However, other studies have reported that

in vitro BHMT activity was sensitive to IUGR in piglets (MacKay et al., 2012) and to methionine-restriction in rats fed adequate choline (Slow and Garrow, 2006). Our data demonstrate that *in vitro* RM enzyme capacities are not governed by methyl group availability and that such *in vitro* data are disconnected from *in vivo* RM flux data. Furthermore, these data suggest that enhanced *in vivo* RM is due to both BHMT and MSyn in our model.

The usefulness of FPF as an indication of $Q_{Precursor \rightarrow Product}$ was further investigated by comparing computed FPF values during +Arg and –Arg to published $Q_{Precursor \rightarrow Product}$ values (during +Arg and –Arg) (Urschel et al., 2007). The $FPF_{(-Arg)}/FPF_{(+Arg)}$ correlated significantly with the published $Q_{Precursor \rightarrow Product\ (-Arg)}/Q_{Precursor \rightarrow Product\ (+Arg)}$ values ($R^2=0.5$; $p<0.004$). However, this correlation was lost when relating the percent change in FPF and $Q_{Precursor \rightarrow Product}$ between –Arg and +Arg. Therefore, FPF is related to $Q_{Precursor \rightarrow Product}$, but its application in this regard should be with caution. Regardless, the FPF calculation is a thrifty means of exploring the contribution of a precursor to a product, without performing multiple infusions.

As a closing remark, the authors would like to acknowledge the discrepancy between whole body PS rates presented here *versus* rates presented in Section 4.3.3. We consider the methionine infusion and the infusion of an indicating amino acid (*ie.* [^{13}C] phenylalanine; 424.6 ± 44.2 μmol Phe/(kg•h) during **MD-** *vs.* 480.7 ± 37.6 μmol Phe/(kg•h) during **MS+**) to provide different information. While we maintain that the measure of PS and PB using the methionine tracer is valuable, it is not as straightforward as using the non-oxidative disposal of an indicating amino acid. We consider the oxidation of labeled methionine to describe the partitioning of dietary

methionine between PS and oxidative disposal (*ie.* TS), and not a calculation of whole body PS *per se*. Indeed, methionine is considered the poorest tracer for quantifying whole body protein synthesis by oxidation, due to the methionine cycle (Zello et al., 1995).

5.5 General Conclusions

This study demonstrated that dietary methyl donors contributed significantly to whole body TM and RM rates in the neonate. Moreover, during methyl restriction, dietary methionine is preferentially partitioned to PS at the expense of methionine cycle metabolism (*ie.* TM). As a number of other alterations of methionine partitioning have been described in these piglets, the capacity of dietary methyl groups to adjust to methionine restriction is great. However, to this point we have been unable to ascertain the individual contribution of either MSyn or BHMT to *in vivo* metabolism, and moreover, whether betaine or folate can support RM demands alone.

6.0 Betaine is as effective as folate at facilitating remethylation and sparing methionine for transmethylation in the neonatal piglet

Robinson JL, McBreairty LE, Randell EW, Harding SV, Bartlett RK, Brunton JA, Bertolo RF[4]

6.1 Introduction

The ability of dietary methyl donors to affect methionine partitioning and to spare the dietary methionine requirement has been demonstrated in our methionine-restricted neonatal model. However, it is unclear whether dietary methyl donors must act in synergy, or whether betaine/choline or folate can facilitate remethylation (RM) independantly. Indeed, an interesting feature of RM is that flux by either MSyn or BHMT appear to be both interrelated and adaptable. For example, a low-choline intake in rats leads to depleted hepatic folate concentrations (Varela-Moreiras et al., 1992), whereas a low-folate intake depletes hepatic choline concentrations. However, it is unknown whether choline depletion was due to increased betaine conversion or insufficient *de novo* choline synthesis (Kim et al., 1994). This dual metabolic effect further demonstrates the complexity of choline metabolism as it can be considered a transmethylation (TM) product, as well as a substrate for RM. For example, choline deficiency could not only enhance TM demand for methyl groups to synthesize PC, but could at the same time reduce TM

[4] JLR wrote manuscript and performed animal procedures, infusions, diet formulation, data analysis, statistics and contributed to study design. LEM assisted with animal procedures, infusions, diet formulations, diet confirmation and contributed to study design. RKB measured hepatic enzyme activities under the supervision of JLR. SVH measured enrichment of breath samples by IRMS. EWR assisted with the measure of choline, betaine and DMG. JAB and RFB contributed to study design, analysis and project funding as JLRs co-supervisors.

by limiting betaine-mediated RM. It is for this reason that choline will not be considered as a labile methyl donor here, as it is not a substrate for RM *per se*, and it complicates the understanding of whether betaine or folate makes a greater contribution to *in vivo* RM in the neonate, and moreover, whether BHMT or MSyn flux can support RM equally.

Studies that have compared supplementation with either methyl donor have provided conflicting, and sometimes confusing results. For example, in broiler hens that were fed various levels of methionine, choline and betaine, the majority of *in vitro* homocysteine (Hcy) elimination was attributed to folate (Pillai et al., 2006). However, in young women, folate-restriction reduced the percent of serine flux for RM (*ie. via* the folate cycle) but had no effect on whole body RM *in vivo*, which demonstrates that betaine can compensate for a reduced flux *via* MSyn (S. R. Davis et al., 2005a). Moreover, betaine supplementation did not increase RM in folate replete young men, but TM and methionine oxidation were both greater, and it was suggested that excess betaine may *increase* the methionine requirement (Storch et al., 1991). And finally, betaine and folate supplementation were both effective at reducing circulating Hcy, however betaine supplementation was considered more effective at mitigating a Hcy rise during a methionine load (Steenge et al., 2003). What these studies collectively demonstrate is that both betaine and folate are responsive to increased RM demand, but whole body RM is tightly regulated under conditions of adequate dietary methyl supply. However, none of these studies measured the effects of labile methyl donors when RM was limited in neonates, and

it is not clear whether folate or betaine can fulfill *in vivo* RM alone, and by extension spare the neonatal methionine requirement.

The objective of this study was to measure the effects of folate, betaine or both (*ie.* folate + betaine) on methionine partitioning in the methionine-restricted piglet (**MD-**). This was achieved by measuring methionine partitioning during the omission of dietary methyl donors (*ie.* folate, choline and betaine) and then after the provision of one, or both of the labile methyl donors (*ie.* betaine and/or folate). The subsequent provision of labile methyl donors was termed "rescue", and is used hereafter to refer to the period after the repletion of a labile methyl donor.

6.2 Materials and Methods

6.2.1 Chemical reagents and isotopes

All chemicals and reagents were of the highest available purity and were obtained from Sigma (St. Louis, MO), Fisher Scientific (Fair Lawn, NJ) or Alfa Aesar (Ward Hill, MA). Amino acids were from Ajinomoto, Co (Tokyo, Japan). [^{14}C] methyl-tetrahydrofolic acid ([^{14}C] MTHF), barium salt was from Amersham Biosciences, UK Limited (Buckinghamshire, UK). L-[3-^{14}C] Serine ([^{14}C] serine) and [^{14}C-methyl] N,N,N-trimethyl glycine, ([^{14}C] betaine) were acquired from Moravek Biochemicals (Brea, CA). [3,3,3',3',4,4,4',4'-^{2}H] homocystine ([^{2}H] homocystine), [$^{13}C_1$] methionine ([^{13}C] methionine) and [^{2}H-methyl] methionine were obtained from Cambridge isotopes (Tewksbury, MA).

6.2.2 Animals and biochemical analysis

6.2.2.1 Surgical procedures and study protocol

The animal care committee at Memorial University of Newfoundland approved all animal protocols performed herein. On study day 0, 3-8 day old Yucatan miniature piglets were transported to the animal care facility from the University vivarium. Piglets were anesthetized and gastric and venous catheters were surgically implanted as previously described (3.2.2.1).

6.2.2.2 Dietary Regimen

The initial dietary regimen was identical to that of the **MD-** piglets described in 3.2.2.2. Briefly, all piglets (N = 18) were fed diets that were devoid of dietary folate, betaine and choline but were otherwise nutritionally replete for the first 5 days. On the evening of study day 5, the dietary methionine supply was restricted to 80% of the requirement for a piglet based on (Shoveller et al., 2003b). After an 8-hour [^{13}C, ^{2}H-methyl] methionine infusion on day 7, animals were treated with the same diets either supplemented with folate (**MD+F**) (N = 6), betaine (**MD+B**) (N = 6) or both folate and betaine (**MD+FB**) (N = 6). Animals remained on their respective 'rescue' diets until study day 10, when the [^{13}C, ^{2}H-methyl] methionine infusion was repeated. Animals were kept on rescue diets and within a few hours after the second methionine infusion, animals were anesthetized and their livers were rapidly extracted and flash frozen. Tissues were stored at -80 °C until used to measure hepatic activities of CBS, MSyn and BHMT. All of the infusion protocols, calculations and analytical procedures required for this study have been previously described in

3.2.3, 3.2.4, 4.2.3 and 5.2.4. Briefly, we report the effects of **MD-** and methyl group rescue on methionine cycle intermediates, *in vivo* RM, TM, TS and the fraction of choline flux derived from methionine. Furthermore, the *in vitro* activities of hepatic MSyn, BHMT and CBS after methyl group rescue are presented.

6.2.3 Statistics

Plasma concentrations of folate, choline, betaine and DMG between baseline (day 0) and study day 7 were compared with an unpaired t-test. The plasma concentrations of cysteine and Hcy were compared between baseline (day 1) and day 7 with a paired t-test. Piglet weights were compared between study day 7 and 10 with a paired t-test. Methionine partitioning was compared between study day 7 and 10 using a two-way ANOVA to determine if there was an effect of time (*ie.* rescue), and if there was a statistical interaction among the rescue groups. When no interaction was present, the rescue groups were pooled (**MD+Rescue**) (day 10) and compared to **MD-** (day 7) with a paired t-test. The hepatic enzyme activities were compared with a one-way ANOVA and a Tukey's post-hoc test to identify effects of labile methyl group rescue. All statistics were calculated using Prism software 5.0b (La Jolla CA). A p-value of < 0.05 was considered significant in all cases.

6.3 Results

6.3.1 Animals

All groups were balanced for weight and sex. The animals grew during methyl restriction (Table 6.1), and there was no effect of methyl rescue on weight

gain, or final weight between the **MD+B**, **MD+F** and **MD+FB** groups (Table 6.2). Combined weights were 2.4 ± 0.3 kg on day 7, and 2.8 ± 0.3 kg at necropsy (day 10) ($p < 0.0001$).

6.3.2 Progression of methyl restriction

The effect of methyl restriction was described in these piglets by comparing plasma concentrations of methionine cycle intermediates at baseline (day 0 or 1), and immediately prior to methyl group rescue (day 7). The values are displayed in Table 6.1. There was a significant effect of **MD-** feeding on plasma concentrations of choline, folate and DMG. From day 0 to day 7 the circulating concentrations of choline, folate and DMG decreased by 70%, 65% and 99%, respectively ($p < 0.05$). Plasma betaine concentrations dropped below the limit of detection by day 7 in these piglets, and thus could not be compared statistically. The concentration of plasma Hcy was unchanged after 6 days of **MD-** feeding compared to baseline (day 1); however, cysteine concentrations dropped 45% between day 1 and day 7 ($p < 0.0001$).

Table 6.1 Plasma concentrations of metabolites from baseline[1] to day 7 in methyl restricted piglets[2]

	Baseline	**Day 7**	p-value
Weight (kg)	1.6 ± 0.3	2.4 ± 0.3	< 0.0001
Folate (ng/mL)	45.7 ± 22.0	16.1 ± 7.0	< 0.0001
Betaine (μM)	48.7 ± 27.0	<0.55	*
Choline (μM)	23.18 ± 14.0	6.86 ± 6.8	0.0001
DMG (μM)	9.73 ± 6.4	0.10 ± 0.16	< 0.0001
Hcy (μM)	28.8 ± 14.0	30.0 ±14.0	ns
Cysteine (μM)	58.8 ± 12.0	31.8 ± 7.0	< 0.0001

[1] Baseline was study day 0 for folate, betaine, choline and DMG and day 1 for Hcy and cysteine
[2] N = 18

6.3.4 Effects of methyl group supplementation on methionine cycle intermediates

The effects of methyl donor supplementation to **MD-** piglets (*ie.* rescue) on methionine cycle intermediates are summarized in Table 6.2 and were as predicted. Overall, there was no statistical interaction between any of the rescue groups and metabolite concentrations. There was a general effect of rescue to lower plasma Hcy and raise DMG concentrations ($p < 0.05$). Although effects of rescue on plasma betaine and folate concentrations were as predicted, the statistical analyses were complicated by detection issues. Betaine concentrations were at the lower limit of detection for all piglets on day 7, and in the **MD+F** group on day 10. This prohibited statistical comparison using our model, but we can conclude that the dietary

supplementation of betaine led to higher betaine concentrations. Similarly, folate concentrations were at the upper limit of detection when folate was supplemented to these piglets (*ie.* **MD+F** and **MD+FB**). All plasma folate results above the upper analytical range for the folate assay were reported as 80 ng/mL. As expected, the choline and cysteine concentrations were unaffected by the provision of methyl groups after methyl restriction.

Table 6.2 Plasma metabolites before and after the provision with dietary methyl donors[1,2]

	MD+F		**MD+B**		**MD+FB**		p-value
	D7	D10	D7	D10	D7	D10	Rescue
Weight (g/kg•d)	91.0 ± 27.4	93.9 ± 69.9	77.5 ± 20.6	85.6 ± 48.1	71.1 ± 16.2	66.9 ± 36.5	ns
Hcy (μM)	32.1 ± 22.0	19.9 ± 12.0	26.6 ± 6.0	23.5 ± 12.0	31.4 ± 11.0	19.4 ± 10.0	0.009
Cys (μM)	31.1 ± 9.0	34.9 ± 9.0	32.8 ± 5.0	32.6 ± 9.0	31.3 ± 7.0	37.3 ± 7.0	ns
Folate (ng/mL)	14.6 ± 9.0	>80	15.3 ± 10.0	39.8 ± 24.0	18.4 ± 3.0	>80	*
Betaine (μM)	<0.55	<0.55	<0.55	146.0 ± 58.0	<0.55	103.0 ± 64.0	*
Choline (μM)	5.5 ± 4.7	1.9 ± 2.1	9.5 ± 9.7	5.8 ± 9.3	5.5 ± 5.2	7.2 ± 6.3	ns
DMG (μM)	0.13 ± 0.26	0.07 ± 0.07	0.11 ± 0.08	8.4 ± 10.2	0.08 ± 0.11	5.9 ± 7.7	0.02

[1] N = 6 for all groups and values are means ± SD
[2] ns' indicates p > 0.05; * indicates that some values were out of the detectable range

6.3.2 Methionine kinetics

A plateau in [^{13}C, ^{2}H-methyl] methionine enrichment was achieved in plasma and breath after 6 hours. Furthermore, the appearance of the [^{2}H-methyl] moiety into choline was also at plateau after 6 hours. Steady state could not be achieved for [M+3] betaine and DMG in these piglets. Typical examples of steady state during the methionine infusions were presented in Figure 5.1.

The kinetic parameters of the [^{13}C, ^{2}H-methyl] methionine infusions are presented in Table 6.3. The rates of methionine flux during the enteral infusions of the [^{13}C, ^{2}H-methyl] methionine tracers were unaffected by methyl group rescue, and there was no statistical interaction. Overall, the feeding of any methyl donor was sufficient to increase the rate of RM ($p < 0.0001$) and TM ($p < 0.0001$). As there was no statistical interaction between methyl donors, the RM and TM data were pooled for comparison. Overall, RM was 10.3 ± 9.0 μmol met/(kg•h) after 7 days of methyl restriction, which increased by 40% to 17.0 ± 9.3 μmol met/(kg•h) after the provision of a methyl group ($p < 0.0001$). The rate of TM was 37% greater on day 10 (17.7 ± 9.2 μmol met/(kg•h)) after methyl groups were provided compared to day 7 (11.3 ± 9.1 μmol met/(kg•h)) ($p < 0.00001$). The rates of transsulfuration (TS) were low in these piglets and were unaffected by rescue. The low rate of TS was expected since dietary methionine was restricted and adequate dietary cysteine was fed. Whole body protein synthesis (PS) measured using this tracer was unaffected by methyl group rescue; however, the fraction of methionine flux going into protein (*ie.* PS/Qm) was reduced by methyl rescue ($p < 0.05$), as was the gross partitioning between PS and TM (*ie.* PS/TM) ($p < 0.05$). Furthermore, methyl group rescue

significantly enhanced the fraction of methionine flux going to TM (*ie.* TM/Q_M) ($p < 0.05$). Together these data demonstrate that TM reactions were sacrificed during methyl restriction, giving priority to whole body PS. Similarly, the provision of methyl donors also resulted in a reduction of whole body protein breakdown (PB) ($p < 0.05$), and no change in protein deposition (PD). In all cases the paired values matched significantly ($p < 0.0001$).

Table 6.3 Kinetic parameters of a [^{13}C, ^{2}H-methyl] methionine infusion before and after addition of methyl donors[1]

	MD+F		MD+B		MD+FB		p-value
	D7	D10	D7	D10	D7	D10	Rescue
	µmol Met/(kg•h)						
Q_M	133.1 ± 15.2	135.2 ± 28.0	152.0 ± 31.4	150.6 ± 32.1	152.6 ± 31.0	148.5 ± 23.8	ns
Q_C	124.7 ± 16.9	119.2 ± 23.3	137.4 ± 31.0	131.6 ± 32.4	144.7 ± 27.4	132.4 ± 20.6	ns
RM	8.49 ± 5.3	15.9 ± 8.3	14.6 ± 13.4	19.0 ± 13.5	7.9 ± 6.0	16.0 ± 6.0	<0.0001
TS	0.6 ± 0.6	0.6 ± 0.7	1.1 ± 1.7	0.6 ± 1.0	1.1 ± 1.2	0.8 ± 0.7	ns
TM	9.0 ± 5.2	16.5 ± 8.6	15.7 ± 13.3	19.6 ± 13.2	9.0 ± 6.5	16.9 ± 5.9	<0.0001
PS	124.1 ± 16.7	119.1 ± 22.3	136.3 ± 30.6	131.5 ± 22.3	143.6 ± 26.3	131.5 ± 20.2	ns
PB	37.2 ± 16.8	32.2 ± 22.2	62.1 ± 54.8	37.9 ± 25.4	57.2 ± 27.5	45.0 ± 20.6	0.04
PD	86.9 ± 0.7	86.8 ± 0.7	74.2 ± 29.7	74.6 ± 30.1	86.4 ± 1.3	86.6 ±0.7	ns
	%						
RM/TM	86.8 ± 18.0	95.8 ± 4.3	93.0 ± 9.7	95.7 ± 6.8	86.2 ± 21.1	94.4 ± 4.3	ns
TS/TM	13.1 ± 18.0	4.2 ± 4.3	7.0 ± 9.7	4.3 ± 6.7	13.8 ± 21.1	5.6 ± 4.3	ns
TS/Q_M	0.4 ± 0.5	0.4 ± 0.5	0.7 ± 1.1	0.3 ± 0.5	0.6 ± 0.7	0.6 ± 0.4	ns
TM/Q_M	6.9 ± 4.2	11.9 ± 4.8	10.1 ± 8.4	13.0 ± 8.4	5.6 ± 3.2	11.3 ± 3.1	0.0002
PS/Q_M	93.0 ± 4.3	88.6 ± 5.1	89.9 ± 8.4	86.6 ± 15.4	94.3 ± 3.2	88.7 ± 3.1	0.003
TM/PS	7.7 ± 13.7	13.7 ± 6.4	12.2 ± 11.9	17.4 ± 11.1	6.1 ± 3.6	13.0 ± 4.1	<0.0001
$MPE_{Choline}$	0.17 ± 0.07	0.31 ± 0.09	0.15 ± 0.06	0.30 ± 0.12	0.20 ± 0.06	0.31 ± 0.12	<0.0001
$FPF_{Met \rightarrow Choline}$[2]	1.8 ± 0.9	3.5 ± 1.0	2.0 ± 2.5	4.0 ± 3.3	2.7 ± 0.9	3.4 ± 1.1	0.0007
$FNC_{Met \rightarrow Choline}$[3]	2.1 ± 2.4	4.1 ± 3.5	2.1 ± 2.4	4.1 ± 3.5	2.9 ± 0.9	3.7 ± 1.3	0.001

[1] N = 6 for all groups of piglets. Values are means ±SD and $p < 0.05$ indicates a significant effect of providing a methyl group. 'ns' indicates $p > 0.05$.
[2]FPF is fraction of product flux
[3]FNC is fractional net conversion

6.3.3 Conversion of [^{2}H-methyl] methionine to choline

The provision of dietary methyl donors had a profound effect on choline enrichment (MPE), which reflects enhanced PC oxidation to free choline (*via* methionine) when expressed as the fraction of product flux ($FPF_{Methionine \rightarrow Choline}$) ($p = 0.0007$) and fractional net conversion ($FNC_{Methionine \rightarrow Choline}$) ($p = 0.001$) (Table 6.3). There was no interaction with the individual methyl donors and thus all rescue groups had a similar effect on the partitioning of dietary methyl to free choline. The enrichment of [M+3] betaine was below detection, and thus it was not possible to calculate the contribution of methionine to betaine flux.

6.3.4 Hepatic specific activities of MSyn, BHMT and CBS

The *in vitro* activities of hepatic RM and TS enzymes were unaffected by rescue diet (Table 6.4). Because we were only able to take a liver sample at necropsy, it was not possible to determine if there was an effect of methyl group rescue on hepatic enzyme capacity. Regardless, *in vitro* capacity was in partial agreement with the *in vivo* kinetic data, *ie.* the provision of any labile methyl donor had similar effects on RM and TS.

Table 6.4 Hepatic activities of MSyn[1], BHMT and CBS[1] in pigs with one or more labile methyl group[2]

	MSyn	BHMT	CBS
	nmol product[1]/(min•mg liver protein)		
MD+F	0.15 ± 0.02	0.15 ± 0.04	0.96 ± 0.24
MD+B	0.13 ± 0.02	0.17 ± 0.12	1.37 ± 0.50
MD+FB	0.14 ± 0.04	0.22 ± 0.07	0.97 ± 0.21

[1] Product is methionine for MSyn and BHMT, and cystathionine for CBS
[2] N = 6 for all groups, values are means ± SD

6.4 Discussion

The objective of this study was to determine the individual and synergistic effects of betaine and folate on methionine partitioning in the neonate. We accomplished this by restricting the supply of dietary methyl to methionine-restricted piglets, and subsequently 'rescuing' methyl metabolism with the provision of remethylation precursors in the form of dietary betaine, folate or both. We tracked the metabolic effects of methyl restriction, and measured the effect of rescue on methionine kinetics. Methyl restriction resulted in the reduction of plasma folate, betaine, choline and cysteine; moreover, plasma folate and betaine concentrations were responsive to their respective dietary rescue in these piglets. We demonstrated an equal capacity of folate and betaine to *enhance* whole body RM, TM, $FPF_{Methionine \rightarrow Choline}$, and to *lower* PB and plasma Hcy during methionine restriction. There appears to be no metabolic advantage of providing both folate and betaine during methionine restriction.

The effects of methyl restriction in these piglets were generally as expected. The omission of folate, choline and betaine during methionine restriction resulted in their respective decreases in plasma. Furthermore, the provision of betaine and folate as methyl rescue resulted in their respective increases in plasma, and all rescue treatments resulted in a similar reduction in Hcy, consistent with higher RM flux. It is interesting that cysteine concentrations were also lower after 7 days of methyl and methionine restriction, which suggests a decrease in TS flux. Although adequate dietary cysteine was provided to these piglets, the lower plasma cysteine concentrations by Day 7 likely represent lower methionine oxidation (*ie*, TS) since methionine was fed below requirement. Moreover, there was no effect of labile methyl group rescue on plasma cysteine concentrations, which suggests that the increased remethylation did not synthesize enough methionine to exceed the requirement, or we would have seen TS and cysteine concentration increase.

The rates of whole body methionine flux were determined with a constant [^{13}C, ^{2}H-methyl] methionine infusion. Overall, the rescue with labile methyl groups resulted in an overt increase in RM. This was anticipated as both folate (Brouwer et al., 1999) and betaine (Steenge et al., 2003) are effective at reducing plasma Hcy. We further demonstrated that betaine is as effective as folate in facilitating whole body RM *in vivo*, and that supplementation with both folate plus betaine did not result in a further increase in RM. These data demonstrate the fine control of RM in response to methyl group availability (Mudd and Poole, 1975), and is in agreement with others (S. R. Davis et al., 2005a; Storch et al., 1991). Combined, the provision of labile methyl donors led to 40% more *de novo* methionine. Of this additional 6.7

μmol met/(kg•h) formed by RM, a full 82% was partitioned towards TM. However, of the 88 μmol met/(kg•h) fed during this period, the additional methionine from RM amounted to ≈8% more methionine, which is still below requirement.

The provision of either folate or betaine also led to greater rates of *in vivo* whole body TM, but TM was not further enhanced by rescue with both labile methyl donors. The increased TM after rescue corresponded to a higher fraction of methionine flux for TM, and a lower fraction of methionine flux for PS after rescue. These data suggest (as in 5.0) that dietary methionine was prioritized for protein incorporation during methyl restriction. As methyl groups facilitated remethylation after rescue, then more methionine was available for partitioning through TM, and the reliance on PB as a methionine source was reduced. Therefore, labile methyl donors enhanced methionine availability for TM during methionine restriction, which refutes previous evidence that labile methyl donors may increase the methionine requirement (Storch et al., 1991). It seems that once RM demand is fulfilled then methionine is used to maintain levels of TM and PS. Regardless, the provision of labile methyl donors spared an additional 5.5 μmol methyl/(kg•h) for the >50 TM reactions.

While we did not investigate the effects of methyl group provision on a specific TM reaction in this study, we did measure the whole body FPF of methionine to choline *in vivo*. We found that the provision of a methyl group resulted in significantly higher *de novo* choline formation. This is in agreement with our previous finding (see 3.0) that the FSR of PC was greater during **MD-** feeding (Figure 3.2). Assuming that PEMT was also enhanced in these **MD-** piglets, we

hypothesize that the provision of labile methyl donors resulted in a higher flux through PEMT, which is supported by greater [M+3] choline appearance during rescue. The fate of the *de novo* synthesized choline remains unknown, but it is suspected that it was reincorporated into PC by extrahepatic tissues to help overcome acute choline restriction (Walkey et al., 1998). Alternatively, it is possible that betaine synthesis was also enhanced (especially in **MD+F** piglets), however steady state could not be achieved for [M+3] betaine in these piglets so this could not be verified.

There were no effects of the individual methyl donors on the *in vitro* activities of the enzymes that eliminate Hcy. This was contrary to our hypothesis that both BHMT and MSyn capacity would be affected when their respective substrates were eliminated, and suggests that labile methyl donors do not affect the hepatic capacity for RM. While this is not surprising, it leads to interesting discussion, especially with respect to MSyn activity during betaine rescue (**MD+B**). Betaine can provide methyl groups directly for BHMT, as well as indirectly for MSyn via demethylation to DMG and sarcosine. While plasma folate concentrations before and after rescue could not be statistically compared (Table 6.2), there appeared to be higher folate concentrations after the provision of betaine alone. This suggests that betaine supplementation could have facilitated flux of methyl groups via both BHMT and MSyn, which provides a possible explanation as to why MSyn activity was unchanged between rescue groups in these piglets. To test this, we attempted to measure plasma sarcosine concentrations by HPLC-tandem mass spectrometry using the method described in 3.2, but were unsuccessful. It is also feasible that

enhanced remethylation by folate could generate more choline which then provides methyl groups for BHMT. However, rescue with folate alone did not raise betaine concentrations above nadir and DMG tended to be lower with folate rescue, perhaps not surprisingly given the conversion of choline to betaine is thought to be very low in this model (5.4). So this dual remethylation role appears to be unique to betaine *via* demethylation to DMG and sarcosine.

The provision of labile methyl groups also had no effect on *in vitro* CBS activity. This was surprising given that we have demonstrated previously that methyl restriction resulted in lower hepatic capacity for CBS (Figure 5.2). Because in this study, hepatic CBS activity was measured after rescue with betaine, folate or both, it is possible that provision of either labile methyl donor affected hepatic CBS capacity similarly. Furthermore, as dietary choline was not provided to these animals (unlike in the previous study with methyl deficiency, see 5.0), then there may also be some interaction with choline and CBS expression. However these are merely suggestions; and because cysteine was provided, more focused studies are required to understand the effects of methyl restriction on neonatal TS.

6.5 General Conclusions

This study provides unequivocal evidence that both betaine and folate can spare methionine equally. Moreover, we have demonstrated that this spared methionine is used to enhance TM while maintaining whole body protein synthesis. These findings have broad implications not only for pediatric nutrition, but also for the general population. Because both folate and betaine are sometimes

administered to lower circulating Hcy, these data demonstrate that betaine is as effective as folate for this purpose as well as for methionine sparing; indeed, given betaine's ability to remethylate *via* both remethylation pathways, it may be theoretically more effective. Furthermore, due to widespread polymorphisms in folate metabolism, the inclusion of betaine to infant formula, and multivitamin mixtures may be prudent.

7.0 General Discussion

The preceding studies have led to a number of conceptual inroads with respect to the effects of dietary methyl donors on methionine partitioning in neonates. More specifically, we demonstrated that dietary methyl donors act either alone, or in concert, to contribute to the neonatal methionine requirement, RM, TM, and the synthesis of protein, creatine and PC. Furthermore, when limiting, dietary methionine is preferentially partitioned for protein synthesis, and the TM requirement is secondary. The purpose of this chapter is to further integrate some of these findings, and moreover, to propose new questions regarding dietary methyl donors in the neonate.

7.1 Methionine requirement studies

We have successfully characterized an *in vivo* model of neonatal methyl restriction that was consistent between two independent cohorts of piglets. Perhaps the most obvious progression would be to calculate the extent which dietary methyl donors spared the neonatal methionine requirement. The most logical way to approach this calculation is by indicator amino acid oxidation (IAAO), which has been described extensively elsewhere (Ball and Bayley, 1984; House et al., 1997; Shoveller et al., 2003b; 2003a; Zello et al., 1993). Briefly, the oxidation of an indicating amino acid (*eg.* [^{13}C] phenylalanine) would be measured during the feeding of various levels of methionine to piglets with and without dietary methyl donors (*ie.* betaine, choline and folate). A breakpoint analysis would be subsequently used to determine the methionine requirement. The calculation of the

putative sparing effect of dietary methyl donors would complement the studies presented here, and allow for more comprehensive understanding of methionine kinetics. Furthermore, it would be interesting to investigate the potential sparing effect of each dietary methyl donor individually. I would hypothesize that there is a greater sparing capacity of betaine due to its contribution into the folate pool.

Measuring the exact capacity of dietary methyl donors to spare the methionine requirement would also have other advantages, such as to provide a methionine requirement that is specific to our model. We measured RM in the presence of dietary methyl donors (*ie.* choline, betaine and folate) in chapter **5.0**, as well as in the presence of one or both labile methyl donors (*ie.* betaine and folate) in chapter **6.0**; and those studies demonstrated methyl groups provided an additional 7.9 μmol met/(kg•h) and 6.7 μmol met/(kg•h), respectively. Therefore the sum of methionine provided to those piglets was ≈97.5 μmol met/(kg•h), which is below the published requirement of the piglet (105 μmol met/(kg•h)) (Shoveller et al., 2003a). There are two questions that follow from these observations. Is the methionine requirement of Yucatan miniature piglets ≈97.5 μmol met/(kg•h)? Or, is there some maximal value of RM such that methyl donors can only reduce some fixed fraction of the methionine requirement? The determination of the methionine requirements in the Yucatan miniature piglet is considered the most direct route of addressing those questions.

7.2 Choline and betaine kinetics

Studying *in vivo* choline and betaine kinetics would dramatically advance the understanding of how dietary methyl donors are important for neonates. There is nothing known about whole body betaine and choline kinetics in the neonate, which is especially surprising for choline given its critical role during fetal development (Zeisel, 2006). Because we have developed methods of measuring choline and betaine enrichment during various states of methyl restriction, our group is particularly poised to advance understanding this area of pediatric nutrition. By selecting appropriate isotopomers of choline, betaine, methionine, DMG, sarcosine and serine, a number of kinetic relationships surrounding RM can be ascertained. Examples of appropriate labels and the postulated labeling patterns are found in Figure 7.1. Perhaps the most significant outcome, *inter alia*, is the calculation of the capacity of PEMT to synthesize choline. It is understood that PEMT evolved as a mechanism to overcome acute choline restriction (Walkey et al., 1998), but its capacity to spare dietary choline is unknown, and would be invaluable for understanding the pediatric choline requirements, which must also factor the portion of choline flux for *in vivo* betaine conversion (*ie.* $Q_{Choline \rightarrow Betaine}$). Indeed, an additional outcome of this infusion would be to clarify the dynamics of the methionine cycle as methyl groups are traced from methionine into the one carbon pool *via* betaine.

The role of betaine in mammals is poorly understood and there is very little information on betaine intakes within the population (Ross et al., 2014). Moreover, despite the fact that dietary betaine is considered the primary source of this

osmolyte (Lever and Slow, 2010), it is presently unclear if there is a dietary betaine requirement in the neonate. We demonstrated in 5.0 that methionine contributes to betaine flux during methyl restriction (**MD-**), however, because we could not measure betaine flux, it is difficult to interpret those data; and it was assumed that betaine was synthesized as an osmolyte, and not as a RM substrate. Therefore, an additional future aim would be to determine betaine flux and more specifically, the portion of betaine flux for RM (*ie.* $Q_{Betaine \rightarrow DMG}$). This may suggest other roles of betaine in the body, and furthermore, the calculation of $Q_{Betaine \rightarrow Sarcosine}$ would measure the contribution of betaine to the folate pool. We (see chapter **6.0**), and others (S. R. Davis et al., 2005a) have evidence that betaine contributes methyl groups for RM by MSyn during folate restriction, and thus comparison of $Q_{Betaine \rightarrow Sarcosine}$ relative to $Q_{Serine \rightarrow Methionine}$ would focus understanding on how betaine contributes to the one-carbon pool. Furthermore, the infusion of labeled serine permits the calculation of *in vivo* RM *via* MSyn ($Q_{Serine \rightarrow Methionine}$) for comparison to BHMT flux (*ie.* $Q_{Betaine \rightarrow DMG/Methionine}$). Indeed, a significant finding of chapter 6.0 was that RM and TS are not governed strictly by the supply of labile methyl donors, and further suggests that maximal RM capacity is predetermined, likely by the TM requirement. Therefore, while folate and choline are known to be essential, betaine may be a conditionally dispensable nutrient. Focused studies on betaine kinetics would permit calculations of how much betaine is synthesized by the neonate, and moreover, if there is a dietary requirement of betaine.

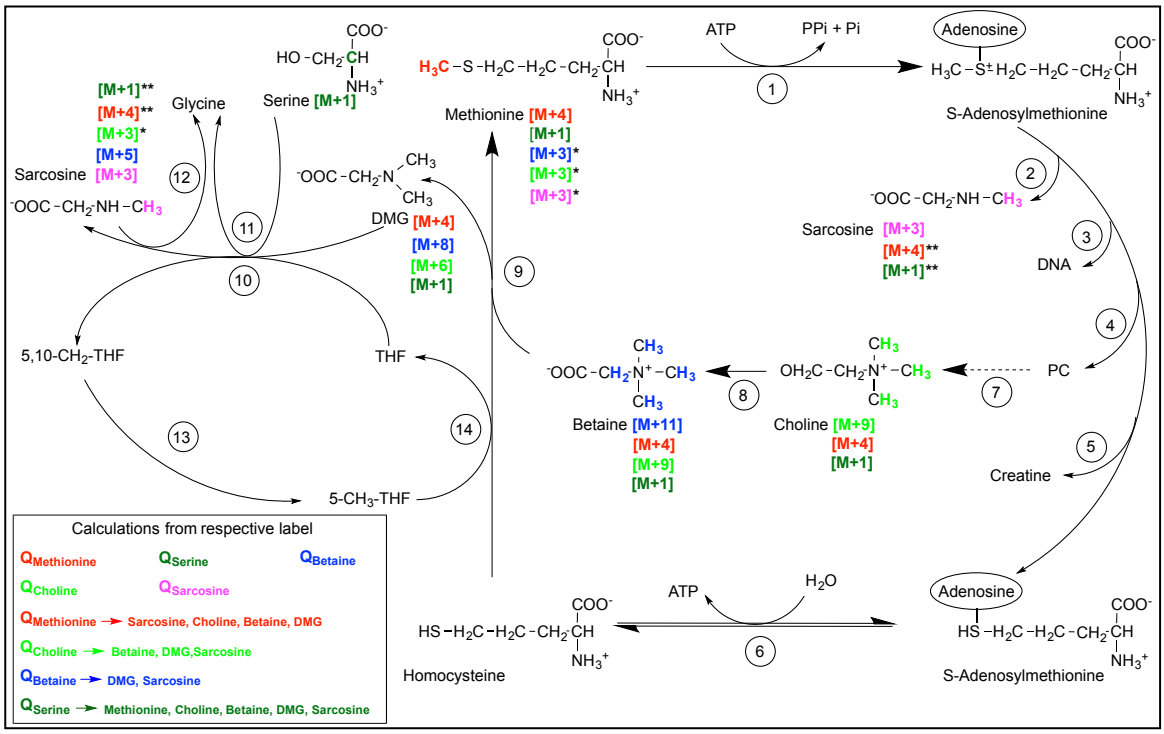

Figure 7.1 Proposed multi-isotope infusions that include hypothetical label patterns and calculable variables based on commercially available isotopomers of methionine, sarcosine, choline, betaine, dimethylglycine (DMG) and serine. Common colours indicate the predicted fates of labels and the calculations that can be made using that label. * indicates a redundant labeling pattern as there is more than one possible precursor. ** indicates that [M+3] and [M+1] sarcosine can arise from both TM and RM (*via* DMG). However, it is expected that the contribution of serine and methionine to sarcosine flux *via* BHMT would be very low, *ie.* $Q_{Methionine \rightarrow Betaine} / Q_{Betaine \rightarrow Sarcosine} \bullet Q_{Methionine \rightarrow Sarcosine}$. Circled numbers correspond to enzymes that are as follows: 1) Methionine adenosyltransferase (MAT; 2.5.1.6), 2) Glycine N-methyltransferase (GNMT; 2.1.1.20), 3) DNA methyltransferase (DNMT; 2.1.1.204/37), 4) Phosphatidylcholine methyltransferase (PEMT; 2.1.1.17), 5) Guanidinoacetic acid methyltransferase (GAMT; 2.1.1.2), 6) S-Adenosylhomocysteine hydrolase (SAHH), 7) various phospholipases (mostly D; 3.1.4.4), 8) Choline oxidase (1.1.3.17), 9) betaine homocysteine methyltransferase (BHMT; 2.1.1.5), 10) Dimethylglycine dehydrogenase (DMDG; 1.5.8.4), 11) Serine hydroxymethyltransferase (SHMT; 2.1.2.1), 12) Sarcosine dehydrogenase (SDH; 1.5.8.3), 13) methylenetetrahydrofolate reductase (MTHFR; 1.5.1.15), 14) Methionine synthase (MSyn; 2.1.1.13).

7.3 Effects of methyl restriction on hepatic bile synthesis

One of the major findings of methyl restriction was the increased rate of PC synthesis from PEMT. PEMT occurs at the interface of amino acid and lipid metabolism, and the observed increase of PEMT action garners interest on the effects of methyl restriction on bile and VLDL synthesis. A regret of the author is that a sample of bile was not removed at necropsy to measure radioactivity after the [^{3}H-

methyl] methionine infusion. This simple analysis would have provided preliminary information on the effects of methyl restriction on PC incorporation into hepatic bile. Due to the high turnover of hepatic PC (Kuipers et al., 1997), it is possible that bile synthesis may have been affected by methyl restriction. PEMT provides a mechanism for PC synthesis when the Kennedy pathway (*ie.* CDP-choline pathway) is diminished by choline restriction (Vance et al., 2007), and thus it makes sense that we observed an increase in PEMT activity during **MD-** feeding. The extent that PEMT can cover the burden of PC synthesis is unknown, but it is possible that **MD-** feeding reduced bile synthesis and VLDL secretion. However, our studies are unique and there are no data concerning bile and VLDL synthesis when dietary methyl donors and methionine were restricted. The PEMT knockout models are essentially the opposite of our piglets, and thus it is difficult to extrapolate those data to the **MD-** piglets to formulate a mechanistic hypothesis. Furthermore, it would be prudent to analyze hepatic VLDL synthesis during the [^{3}H-methyl] methionine infusion, but this is a complicated analysis, especially in liver where it is not known the fraction of dietary methionine that is secreted as hepatic protein. These issues might be investigated further in collaboration with others focused on lipoprotein metabolism and bile synthesis.

7.4 Effects of methyl restriction on hepatic B12

A curious finding of progressive methyl restriction was a rise in plasma B12 concentration. In 3.4, it was postulated that the increase in plasma B12 was the result of hepatic B12 release due to diminished amounts of MSyn. Despite the fact

that there was no evidence of diminished MSyn capacity in any of the livers analyzed here, it is important to recognize that MSyn was measured *in vitro* with ample B12 provided, and moreover it was not indicative of *in vivo* MSyn activity (see chapters 5.0 and 6.0). However, choline was not provided to **MD-** piglets, and choline restriction alone has been shown to cause liver damage (Zeisel, 1990). Indeed, alcoholic liver damage causes hepatic release of B12 into plasma (Baker et al., 1998), and perhaps choline restriction caused liver damage to **MD-** piglets. While it is acknowledged that alcoholic and non-alcoholic liver damage are not the same, they do share similarities (Corbin and Zeisel, 2012), and thus it isn't outside the realm of possibility that choline restriction might also enhance the hepatic release of B12 into plasma. On the contrary, the effect of methyl restriction on plasma B12 remains equivocal as providing labile methyl donors to methyl-restricted piglets (*ie.* **MD+Rescue**) did not seem to lower plasma B12 concentrations (data not presented: pooled plasma B12 concentrations were 1.1 ± 0.3 ng/mL in **MD+Rescue** piglets) compared to **MS+** piglet plasma (0.3 ± 0.2 ng B12/mL, Table 3.3). The measurement of hepatic B12 concentrations in these piglets might have provided a simple explanation for this result. However, it was not possible here due to the radioactivity of liver samples in the **MD-** and **MS+** piglets and restrictions for the equipment used. Additional analyses that could be performed to further this hypothesis would be the measure of methylmalonic acid, which is an indicator of B12 status, or indicators of liver damage such as alanine aminotransferase in the liver.

7.5 Clinical applications

This study demonstrated that betaine was as effective as folate at performing remethylation. The study of betaine metabolism in clinical populations is lagging compared to folate, B12 and choline. For example, a PubMed clinical queries search (as of February 15, 2015) on betaine netted <900 hits, which is minimal compared to folate (~23,000 hits), B12 (~5,000 hits) and choline (~12,000 hits). In particular focus might be drawn to the capacity of betaine to spare folate, B12 and choline as methyl donors, as well as for nucleotide, phosphatidylcholine and acetylcholine synthesis. Indeed, as new genetic variants of choline, folate, B12 and betaine metablism are described, methyl donor supplementation may be prudent for infants to ensure that not only are methyl demands met, but also ensure that individuals with compromised methyl metabolism can devote more nutrients to the non-methyl roles of those metabolites.

An additional importance of this work is the elucidation of the transmethylation priority. Indeed, it was demonstrated that creatine synthesis is sacrificed during methyl restriction, seemingly to accommodate for enhanced PC synthesis and to maintain hepatic protein synthesis. Due to the vast importance of PC and creatine for metabolism, this point of control is interesting. Indeed, the clinical applications of creatine supplementation are mounting (Deminice et al., 2011; 2009; 2015; Gualano et al., 2012), and thus creatine supplementation might be a strategy to ensure methyl availability for PC synthesis *via* PEMT, and to mitigate the effects of choline restriction (Deminice et al., 2015), which frequently occurs

within the population (Zeisel and da Costa, 2009). However, it is not yet clear whether widespread creatine supplementation is warranted.

8.0 Effects of supplemental creatine and guanidinoacetic acid on episodic-like memory and the brain of Yucatan miniature pigs.

Robinson JL, McBreiarty LE, Walsh CA, Martin GE, Ryan RA, Brunton JA, Bertolo RF[5]

8.1 Abstract

Creatine has emerged as a potential supplement to enhance cognitive function in humans, and recently the pig has been shown to exhibit episodic-like memory. We analyzed the effects of supplementing 3-month-old miniature pigs with creatine (**+Cr**; N = 7) and its precursor, guandinoacetic acid (**+GAA**; N = 8), for 2 weeks and measured memory performance. In a 5'x5' pen, pigs explored 2 sets (4As, 4Bs) of objects 50 min apart. Pigs were then tested with both objects (2As+2Bs) with one of each object in the same position as before (A1, B1), and the others moved (Am, Bm); the test was repeated 24 h later with a third object (C). An additional short-term test was performed 24 h later where pigs were explored two objects (C1, C2), and 20 min later were tested with one familiar object (C1) and one novel object (C2). There was no effect of guanidino compound supplementation on memory performance despite correlations between creatine and guanidinoacetic acid (GAA) concentrations in some brain regions ($p < 0.05$). However, pigs did explore older objects more than recent objects ($p < 0.01$), and moved objects more than stationary

[5] JLR wrote the manuscript, contributed to study design, coordinated behavioural testing procedues, measured brain metabolites, and compiled/interpreted behavioral data. LEM contributed to study design and assisted with behavioural testing. CAW and GEM designed/interpreted behavioural tests and provided statistical expertise. RAR helped with behavioural testing and measured brain metabolites in the 2012 pigs. JAB and RFB contributed to study design, analysis and project funding as JLRs co-supervisors.

objects ($p < 0.05$). Supplementation with creatine did not affect concentrations of creatine or GAA in the cerebellum, hippocampus, prefrontal cortex or caudate nucleus; but in general creatine concentration was greater in the hippocampus and cerebellum when compared to the prefrontal cortex only ($p < 0.05$). The **+GAA** pigs had greater GAA concentrations in the cerebellum only ($p < 0.05$), and a greater creatine concentration in the prefrontal cortex compared only to **+Cr** pigs ($p < 0.05$). Furthermore, %/d weight gain was greater in the **+GAA** pigs compared only to the **+Cr** group. We conclude that guanidino compounds in brain were associated with improved memory performance, but dietary supplementation was not effective. A pre-existing deficiency or stressor might need to be present to observe an effect of supplementation, similar to that observed in vegans and sleep-deprived humans.

8.2 Introduction

Creatine (*N*-aminoiminomethyl-*N*-methylglycine) is a guanidino compound that acts primarily as an energy buffer in vertebrates when it is phosphorylated to phosphocreatine by the enzyme creatine kinase (CK; EC 2.7.3.2). Dietary sources of creatine are primarily from the consumption of animal products, or by creatine monohydrate supplementation. Indeed, creatine is among the most popular dietary supplements in the world that is especially prevalent in athletes to increase intramuscular availability of phosphocreatine (Harris et al., 1992). More recently, creatine supplementation has emerged as a potential treatment for numerous pathologies including cancer, lupus, arthritis, type II diabetes and various neuropathies (Gualano et al., 2012). The effectiveness of supplementation of creatine has remained controversial as healthy individuals have the endogenous capacity to synthesize creatine.

Endogenous creatine synthesis is a two-step process that requires arginine, glycine and methionine. Creatine synthesis commences in the pancreas and kidneys when L-arginine: glycine amidinotransferase (AGAT; EC 2.1.4.1) catalyzes the formation of guanidinoacetic acid (GAA) and ornithine from arginine and glycine (Bera et al., 2008; da Silva et al., 2009). GAA is transported into the liver by γ–aminobutyric acid transporter 2 (GAA2) (Tachikawa et al., 2012), and forms creatine when it is methylated by guanidinoacetate N-methyltransferase (GAMT; EC 2.1.1.2). The *de novo* synthesized creatine is released into blood and transported into other tissues *via* an active Na^+/Cl^- dependent membrane protein called creatine transporter-1 (CT1), which is coded by the gene SLC6A8. The CT1 protein is a

member of solute carrier family 6, and it mediates the transport of GAA, creatine, phosphocreatine, gamma-aminobutyric acid, betaine and taurine with co-transport of 2 Na^+ and 1 Cl^- (Béard and Braissant, 2010; Tachikawa and Hosoya, 2011). The CT1 transporter is found in tissues with a high creatine demand such as the central nervous system (CNS), heart and skeletal muscle (Kurosawa et al., 2012). However, there is very little CT1 found in tissues that synthesize creatine such as the liver, kidney and pancreas (Carducci et al., 2002). While the importance of endogenous creatine synthesis has been long established for energy metabolism, it has recently been identified as being critical for nervous function.

Inborn errors of metabolism have underscored the importance of GAA and creatine for proper brain function. Indeed, all patients with inborn errors of creatine metabolism present with phenotypes associated with CNS disturbances such as mental retardation, language disturbances, seizures and movement disorders (Béard and Braissant, 2010; Carducci et al., 2012). Patients with a mutation affecting either creatine synthesis or transport share common clinical presentations, and a lack of creatine in the brain (Béard and Braissant, 2010; Longo et al., 2011). Therefore, it follows that creatine supplementation is an effective treatment for individuals that lack capacity for creatine synthesis, such as in AGAT and GAMT deficient patients, but not in CT1 deficient patients (Andres et al., 2008). However, GAMT and CT1 deficiencies are further complicated by the intracranial accumulation of GAA (Sijens et al., 2005a; 2005b) that may be toxic (Zugno et al., 2006). Indeed, the intrastriatal administration of GAA to rats led to the inhibition of CK and affected motor capacity (Zugno et al., 2006). Therefore, GAA accumulation is

believed to contribute to the neurological complications observed in GAMT deficient patients including epilepsy, seizures (Zugno et al., 2006) and dystonia (Sijens et al., 2005a).

A creatine transporter (CT1) deficiency is the second-most common cause of X-linked mental retardation in humans and cannot be overcome by supplemental creatine (Rosenberg et al., 2004). The SLC6A8 gene is located on chromosome Xq28 and thus mutations at this locus are more severe in males than females (Kurosawa et al., 2012). Because creatine is absent from brains of SLC6A8 deficient patients (Cecil et al., 2001; Kurosawa et al., 2012), it would appear that the brain lacks the biochemical machinery required for creatine synthesis; however, this does not appear to be the case (Andres et al., 2008). To reach the brain, the guanidino compounds must be transported across either the blood-brain-barrier (BBB), or the blood-cerebrospinal fluid barrier (BCSFB) *via* CT1. While it was initially assumed that all brain creatine was transported from the periphery, the permeability of the BBB and BCSFB to guanidino compounds seems limited. For example, SLC6A8 expression is absent in most cells of the BBB, and there seems to be little capacity for creatine and GAA transport across this tissue (Andres et al., 2008). Therefore, it seems that, while limited in permeability, GAA is transported into the brain by CT1 where it is methylated to creatine in healthy individuals. Moreover, it has been reported that GAA accumulates in CT1 deficient patients (Sijens et al., 2005b), and thus there may be some additional mechanism for GAA transport across the BBB or the BCSFB, such as GAA2.

Within the CNS, AGAT and GAMT can be found in almost every cell type (*ie.* neurons, astrocytes and oligodendrocytes), however they are rarely co-expressed in the same cell (Braissant et al., 2010). It seems that the presence of creatine in all brain regions is partially reliant upon GAA synthesis by cells containing AGAT, followed by the transport of GAA to GAMT expressing cells for creatine synthesis (Béard and Braissant, 2010). Therefore, it is expected that SLC6A8 is ubiquitously expressed throughout the brain as GAA and creatine are transported among the various brain regions and cell types. This might explain why there is a lack of creatine in the brain of SLC6A8 deficient patients, as they cannot distribute the creatine once it is synthesized intracranially. As the importance of creatine and GAA for proper brain function has come into focus, this has garnered interest into the potential for supplemental creatine to enhance cognitive function in healthy individuals.

Creatine supplementation has been effective at enhancing cognitive ability during times of stress, such as during sleep-deprivation in younger (McMorris et al., 2006) and older adults (McMorris et al., 2007), as well as when endogenous creatine demand was enhanced (Benton and Donohoe, 2011; Rae et al., 2003). What can be hypothesized from those studies is that creatine supplementation enhances the capacity of the brain to convert ADP → ATP from phosphocreatine during times of stress. However, others have found no cognitive benefit of creatine supplementation under normal conditions (Rawson et al., 2008). Overall creatine supplementation appears most effective at improving memory during times of stress, but few studies have combined biochemical and behavioural outcomes during creatine

supplementation, and there are no such studies after GAA supplementation. Due to the inherent difficulties in measuring creatine and GAA in the human brain, and because the neurotoxicity of GAA has not been established, animal models are necessary to further understand how creatine and GAA supplementation affect the mammalian brain.

The pig has emerged as a behavioural model that is more comparable to humans than rodents (Lind et al., 2007; Pond et al., 2000). Recently, pigs have been shown to exhibit episodic-like memory, which in humans is defined as the recollection of a personally experienced past event. Put more simply, it is the ability to identify what, when and where something occurred in the past (Kouwenberg et al., 2009). The objective of this study was to determine the effects of 18 days of GAA and creatine supplementation on both brain metabolite concentrations and episodic-like memory in weaned pigs. GAA and creatine concentrations were measured in the hippocampus, prefrontal cortex (PFC), cerebellum and caudate nucleus after guanidino compound supplementation. Those particular regions were selected to represent regions with a diversity of functions, and moreover, because CK has been shown to be active in those regions in rats, and in some cases, CK activity was responsive to pharmacological interventions and stress (Assis et al., 2007; Comim et al., 2011).

8.3 Methods

8.3.1 Animals

This study was completed in two distinct parts. In the summer of 2011 fifteen male Yucatan miniature pigs (14-16 weeks old) were obtained from the Memorial University of Newfoundland breeding colony and weight-matched to control (N = 7) or creatine supplemented (**+Cr**) (N = 8) groups. In the fall of 2012, an additional fifteen pigs were obtained from the same institution, and weight-matched to control (N = 7) or GAA supplemented (**+GAA**) (N = 8) groups. Pigs were group housed and fed standard pig grower diet twice daily. The **+Cr** pigs were supplemented with creatine monohydrate (200 mg/(kg•d)) and the **+GAA** pigs were supplemented with an equimolar amount of GAA (157 mg/(kg•d)). The guanidino compound supplementations were based on 20 g creatine/day in humans, which is commonly used during a 'loading phase' in humans to rapidly increase muscle creatine levels. The supplemental creatine and GAA were mixed with an aliquot of grower feed and hand fed to experimental groups at two equal daily feedings for 18-19 days. All animal handling procedures were approved by the Institutional Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

8.3.2 Animal habituation and episodic-like memory testing

The test of episodic-like memory in these pigs required that they first become habituated to the testing apparatus, which was a 152 cm x 152 cm box (90 cm height) with a latchable door for entry and exit and attachment locations for the

fixture of various objects (Figure 8.1). Habituation was initiated immediately by introducing the apparatus into the housing room each day to allow pigs to become comfortable with the apparatus during feeding. As the supplementation progressed, pigs were introduced into the box individually with the door unlatched, and then repeatedly with the door latched for 5 minutes. Food was placed in the box to promote a positive association upon entering the testing apparatus, and objects were latched in the box for pigs to explore. Researchers monitored pig behaviour during box visits and discouraged the pigs from jumping out of the testing apparatus. Between animal visits, the floor and interior of the testing apparatus was washed with a high-pressure water hose. Finally, on day 15, pigs began testing for episodic-like memory. Researchers were assigned to record the time that pigs spent exploring specific objects with a stopwatch. Exploration was defined as the visual or physical orientation towards a spatially controlled object. The majority of exploration was recorded during visual fixation within 10 cm of the object as well as touching or manipulating the object with their nose and front hooves. All memory tests were videotaped to ensure that recorded times were accurate. The objects used for testing were selected at random and all pigs were tested with all objects at least once during the testing phase. For ease of understanding the episodic-like memory tests, the objects are listed arbitrarily as: (A) a wooden coat hanger, (B) a can strainer, (C) a large metal spoon and (D) a hammer holder.

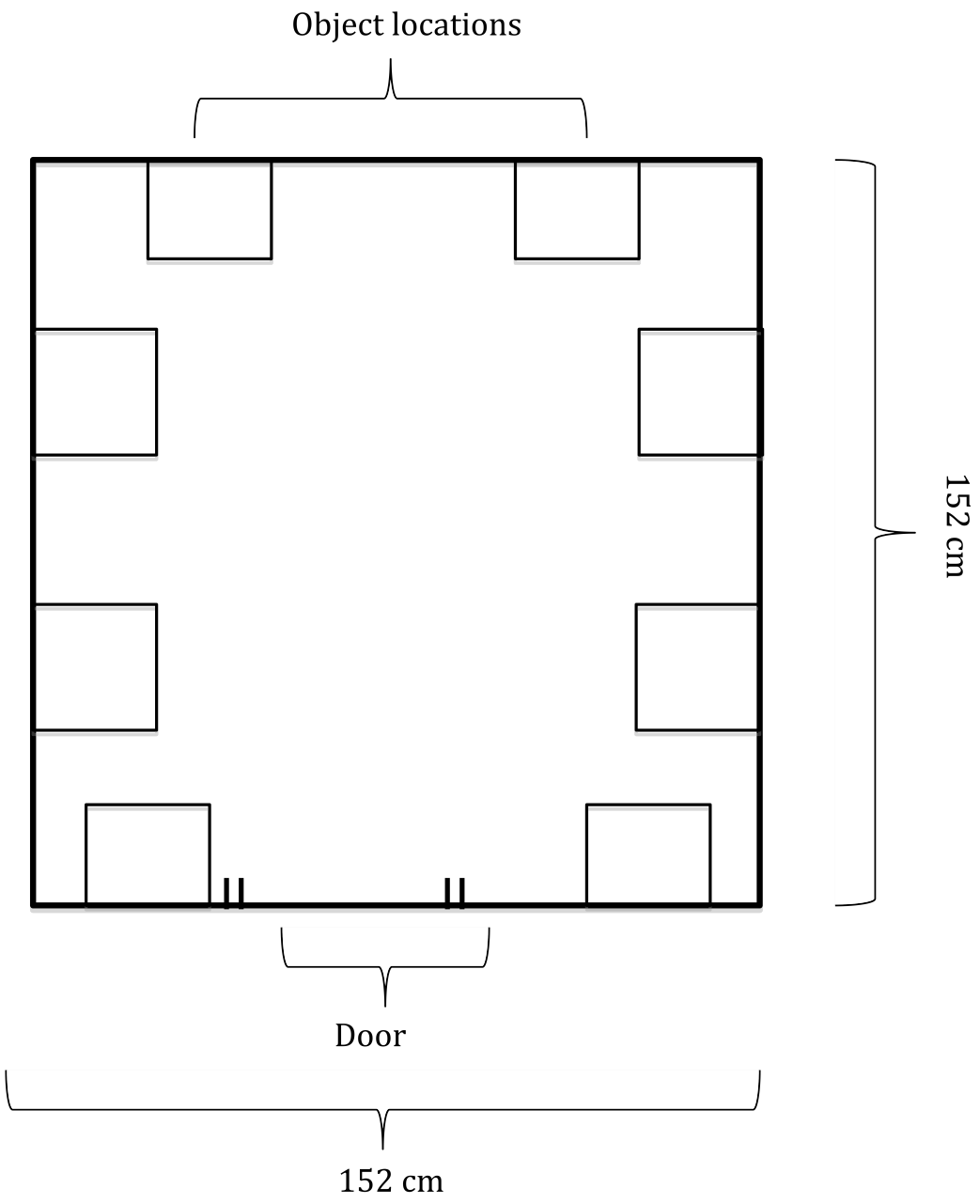

Figure 8.1 A schematic representation of the testing apparatus. The box was 152 x 152 cm with a latchable door. The rectangles within the larger box represent sites which objects could be attached for pig exploration.

4-object test

After the morning feed on study day 15, the pigs entered the testing apparatus individually to complete a 4-object test (Figure 8.2). They were initially free to explore four object A's that were fixed at specific locations within the apparatus for 5 minutes. The time that each pig explored each object was recorded, and after the pigs exited the apparatus, the objects were removed and four object B's were fixed at four novel locations. The pigs entered the box 50 minutes later and they explored these B objects for 5 minutes. Before entering the box 10 minutes later, the researchers manipulated the objects a third time. The final configuration tested the pigs by showing them a combination of "older" object A's and "recent" object B's at either familiar (*ie.* A_1/B_1) or novel locations (*ie.* A_M/B_M). The exploration times were recorded and used to determine if supplemented groups performed this test differently.

24-hour test

After the morning feed on day 16, pigs were introduced into the testing apparatus. In this test the pigs were given 5 minutes to explore two objects (Figure 8.3). One of the objects was a familiar object, in a familiar location (ex. Object A_1 from the 4-object test), and the other was the novel object C. Pigs were free to explore for 5 minutes, and the time spent at each object was recorded.

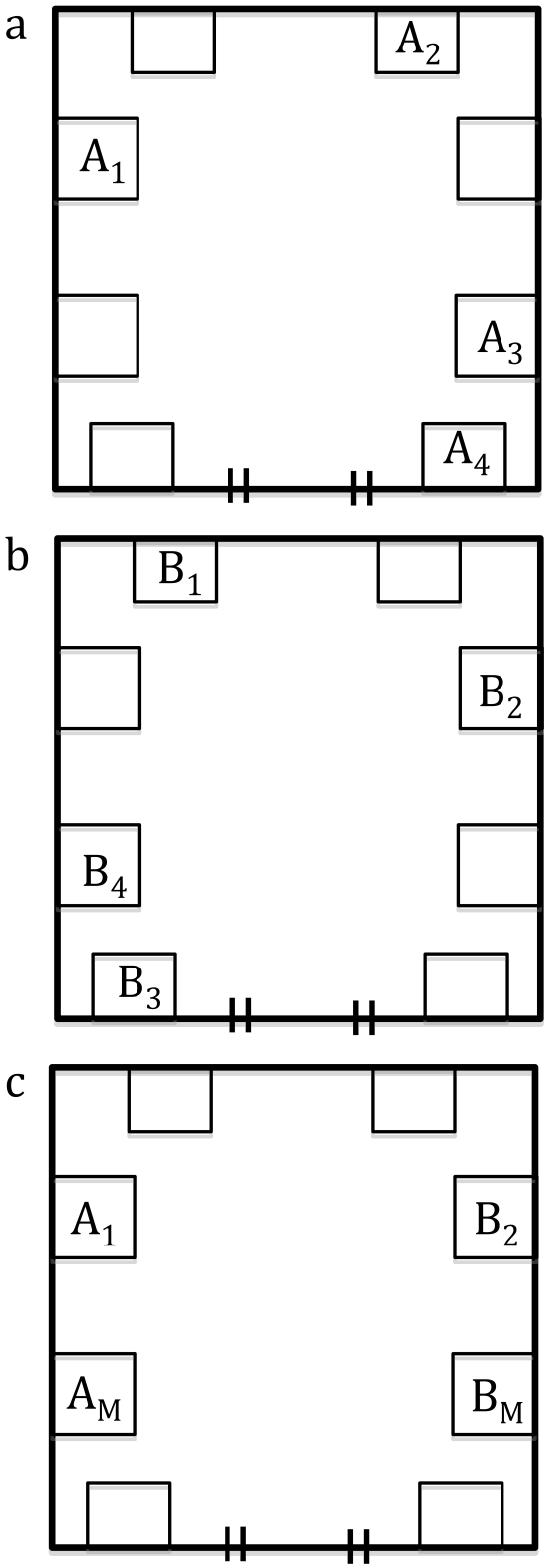

Figure 8.2 A schematic of the 4-object test. The pigs entered the box 3 different times and were free to explore carefully positioned objects. The first box visit (a) showed the pigs 4 identical objects. 50 minutes after the first visit the pigs explored 4 different objects in different locations (b). 10 minutes after the second visit, the pigs entered the box for a third time (c), in which pigs were tested with one old object (*ie.* A_1) and one recent object (*ie.* B_1) in a familiar location, as well one old object (*ie.* A_M) and one recent object (*ie.* B_M) in novel locations.

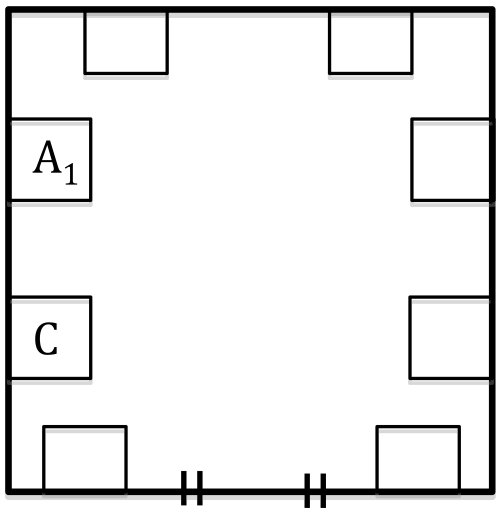

Figure 8.3 A schematic of the 24-hour test. Pigs entered the testing apparatus the following day after the 4-object test (Figure 8.2). There was an object that was consistent from the previous day's testing (A_1) as well as a novel object (C).

Short-term 2-object test

The final test was completed on day 17 (Figure 8.4). After the morning feed, the pigs were introduced into the testing apparatus where there were two identical object C's at fixed locations. Pigs were free to explore objects for 5 minutes, and then they were released. The pigs were tested 20 minutes later when researchers replaced one of the object C's, with a novel object D in the same location. Object exploration times were monitored for 5 minutes.

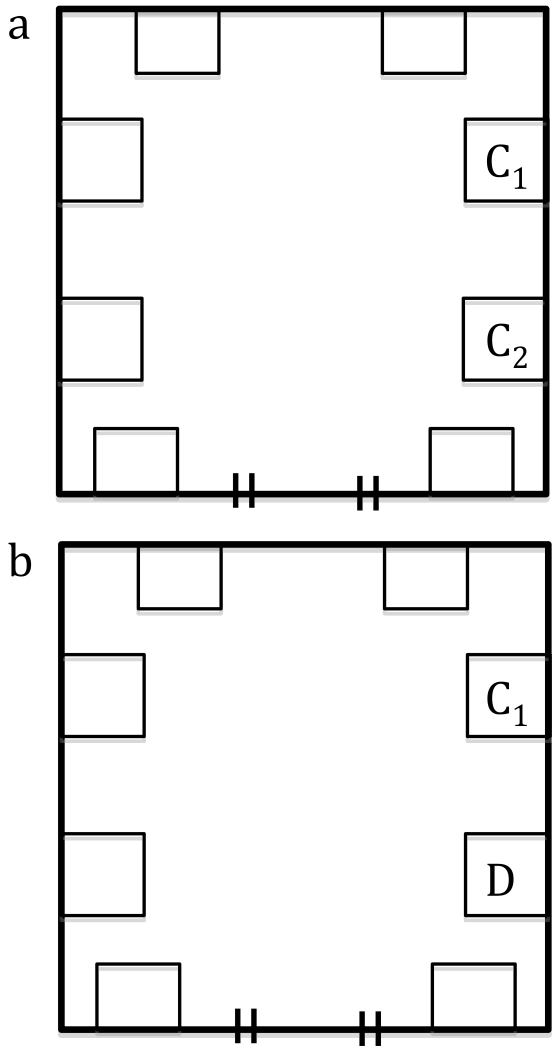

Figure 8.4 A schematic of a short-term 2-object test. Pigs entered the testing apparatus initially and explored for 5 minutes with two identical objects (a). After exiting the apparatus for 20 minutes, pigs were introduced a second time (b), with one consistent object as well as one novel object.

8.2.3 Necropsy and brain sampling

On the final day of supplementation, pigs received their morning feed and were transported by truck to the small animal care facility on the Memorial University campus in carrying crates. Within 1-1.5 hours of their last feed, anesthesia was induced with isoflurane (1-2%) in oxygen (1.5 L/min). The liver was rapidly removed, and the animals died of exsanguination. A scalpel was used to

expose the skull of the pig, and a hacksaw was used to make a saggital cut along the midline of the skull. Two additional cuts were made along the coronal plane of the skull. The top of the skull was removed and the brain was carefully extracted. The brain was separated into right and left hemispheres and then the hippocampus, caudate nucleus, prefrontal cortex (PFC) and cerebellum were carefully excised, and placed into liquid nitrogen. The sampling of brain regions was balanced between left and right hemispheres such that an equal number of structures from both regions were collected. Tissues were stored at -80 °C until analysis.

8.3.4 Tissue analysis

The creatine and GAA concentrations were measured in brain tissues using the ninhydrin method as described in 3.2. Prior to the analysis, the entire structure of each brain region was ground into a powder using a mortar and pestle in liquid nitrogen. Therefore, the reported creatine and GAA concentrations are representative of the entire structure of each brain region.

8.3.5 Statistics

Metabolite concentrations and pig weights were compared by one-way ANOVA with Tukey's post-hoc test to determine statistically significant groupings when permitted. A mixed ANOVA was used to compare the effects of GAA and creatine supplementation on the ability to remember recently experienced objects and the spatial configuration of the objects. Sphericity was not assumed; and the Greenhouse-Geisser adjusted degrees of freedom were used. The one-way and

mixed ANOVA was initially performed between the two control groups to ensure that they could be pooled. Regression analysis was performed to compare creatine and GAA concentrations in brain regions to various behavioural outcomes. The one-way ANOVA and regression analysis were performed with Prism software 5.0b, and the mixed ANOVA was performed using SPSS software (Version 15+). In all cases, presented values are means ± SD, and $p < 0.05$ was considered significant.

8.4 Results

8.4.1 Animals

The supplementation of guanidino compounds had no noticeable effects on pig behaviour or weight at any point in the study (Table 8.1). However, the GAA supplemented (**+GAA**) pigs showed greater % weight gain compared to the creatine supplemented (**+Cr**) pigs from day 0-18 ($F(2, 28) = 4.15$; $p < 0.05$).

Table 8.1 Pig weights on day 0, 5, 10, 15 and 18[1] and weight gain from day 0 to 18[2,3]

	Day 0	Day 5	Day 10	Day 15	Day 18	Day 0 -18
			kg			%
Control	12.8 ± 1.9	13.8 ± 1.7	14.5 ± 1.8	15.4 ± 2.0	16.4 ± 2.1	29.2 ±9.1^{AB}
+GAA	12.3 ± 2.0	13.5 ± 1.9	14.1 ± 2.0	15.5 ± 2.4	16.4 ± 2.2	33.7 ± 7.1^{A}
+Cr	13.2 ± 1.4	13.9 ± 1.3	14.9 ± 1.4	15.3 ± 1.5	16.2 ± 2.2	22.6 ± 3.4^{B}

[1]Weights expressed as kg ± SD
[2]Weight gain is expressed as g/kg ± SD; letters indicate statistical groupings
[3]N = 14 for control, N = 8 for +Cr and N = 8 for +GAA

8.4.2 Concentrations of GAA and creatine in brain regions

The concentrations of GAA and creatine were measured in the PFC, cerebellum, caudate nucleus and hippocampus, and are presented in Table 8.2. In the cerebellum of the **+GAA** group, the GAA concentration was greater than control and **+Cr** groups ($F(2, 26) = 11.62$; $p < 0.05$), whereas in the PFC, the **+GAA** group had a greater concentration of creatine ($F(2, 26)= 3.38$; $p < 0.05$) and a lower concentration of GAA ($F(2, 26)= 2.9$; $p < 0.05$) compared to the **+Cr** pigs only. Among controls, GAA concentrations were consistent across brain regions, and there was an average value of 27.5 ± 15.3 nmol GAA/g tissue. However, the creatine concentration in control pigs varied among brain regions as creatine was lower in the PFC than the cerebellum and hippocampus, but was not significantly different from the caudate nucleus ($F(2,26) = 5.57$; $p < 0.05$) (Figure 8.5).

Table 8.2 GAA and creatine concentrations in brain regions of pigs supplemented with GAA (+GAA) or creatine (+Cr) compared to control[1,2].

	GAA (nmol/g tissue)			Creatine (µmol/g tissue)		
	Control	+CR	+GAA	Control	+CR	+GAA
PFC	28.3 ± 13.4[ab]	38.0 ± 21.1[a]	13.4 ± 3.9[b]	5.6 ± 1.6 [ab]	4.6 ± 1.3 [a]	7.5 ± 3.5 [b]
Cerebellum	24.2 ± 14.0[a]	21.6 ± 8.8[a]	58.6 ± 27.4[b]	9.8 ± 3.0	8.2 ± 2.9	9.5 ± 5.3
Hippocampus	29.5 ± 19.0	22.0 ± 6.8	31.43 ± 12.0	8.9 ± 4.0	7.0 ± 1.8	6.4 ± 1.9
Caudate Nucleus	28.0 ± 17.0	31.7 ± 20.7	22.9 ± 7.8	6.9 ± 2.0	7.9 ± 2.4	7.1 ± 4.6

[1] Values are mean ± SD, N = 8 for supplemented groups and N = 14 for control.
[2] Letters after values indicate statistically significant groupings ($p < 0.05$).

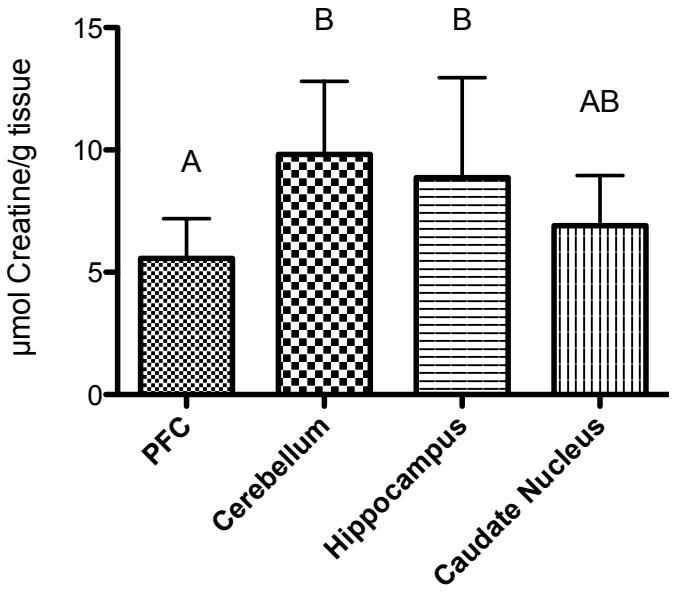

Figure 8.5 Concentrations of creatine in various brain regions in 14-16 week old pigs. Different letters above bars indicate significant groups based on concentrations ($p < 0.05$). Values are mean ± SD. N = 14 for all samples and are found in Table 8.2.

8.4.3 Episodic-like memory tests

4-Object test

The first data that were analyzed from the 4-object test was the total time spent exploring objects by ANOVA. There was no effect of guanidino compound supplementation on the time devoted to object exploration ($F(2,26) = 0.036$, $p > 0.1$; $\varepsilon = -0.074$). The total time spent exploring objects during the 4-object test was 150.6 ± 70.2 seconds in the **+Cr** pigs (N = 8), 150.0 ± 76.6 seconds in the **+GAA** pigs (N = 8) and 156.9 ± 55.7 seconds in the control pigs (N = 13).

A 3 x 2 x 2 ANOVA (diet group x object movement x older *versus* recent) was carried out on absolute exploration times of the various objects during the test phase. One pig was excluded from the **+GAA** group due to a lack of data. The three groups spent similar amounts of time with each of the four objects ($F(2,25) = 0.065$;

p> 0.1). The purpose of this test was to determine whether supplemented groups were better able to discern between an older object (*ie.* Object A) *versus* a recently viewed object (*ie.* Object B), as well as if the pigs could recognize that objects were moved (*ie.* A_M and B_M). There was no differential treatment of recent *versus* old objects as a function of groups ($F(2, 25) = 0.023$; $p > 0.1$). The pigs did, however, explore older objects (46.7 ± 29.2 seconds) more than recently experienced objects (29.9 ± 23.5 seconds) ($F(1,25) = 4.79$, $p < 0.01$; $\varepsilon = 0.161$). The pigs also spent more time exploring the moved (*ie.* A_M/B_M) objects compared to the stationary objects (*ie*. A_1/B_1) (Table 8.4) ($F(1,25) = 7.80$; $p < 0.05$). However, there was no group x moved object interaction ($F(2,25) = 0.552$; $p > 0.1$), which indicated that supplementation did not improve memory that any object was moved. The summary of this analysis is found in Table 8.3. No other differences were significant ($p > 0.3$), and these findings indicate that guanidino compound supplementation had no effect on pig performance during the 4-object test.

Table 8.3 Summary of 3 x 2 x 2 ANOVA (diet group x object movement x older *versus* recent) results of object exploration using the Greenhouse-Gassier adjustment

	Degrees of freedom	F-value	Estimated epsilon (ε)
Older (A) versus recent (B) objects	1	4.79**	0.161
A versus B x Groups	2	0.08	0.006
Error (A versus B)	25	NA	NA
Stationary (A_1, B_1) versus moved (A_M, B_M)	1	7.805**	0.238
Stationary versus moved x Diet groups	2	0.552	0.042
Error (stationary versus moved)	25	NA	NA
A versus B x Stationary versus moved	1	0.662	0.026
A versus B x Stationary versus moved x Diet groups	2	1.30	0.094
Error (A versus B x Stationary versus moved)	25	NA	0.0

**** Indicates a significant effect ($p < 0.01$)**

Table 8.4 Total exploration times of previously viewed objects that were either stationary or moved during the 4-object test ($F(1,25) = 7.805$, $p < 0.01$; $\varepsilon = 0.238$). Pig groups were pooled.

Stationary versus moved	Exploration time (seconds)
Stationary Objects A_1, B_1	29.95 ± 5.5
Moved Objects A_M, B_M	46.75 ± 10.8

24-hour test

A 3 x 3 mixed ANOVA (Diet groups x older (C) *versus* recent (A)) was used to compare performance on the 24-hour test. There was no effect of diet on total object exploration time in this test ($F(2,26) = 0.235$, $p > 0.1$; $\varepsilon = 0.018$). The average time

spent exploring both objects during the 24-hour test was 61.3 ± 44.9 seconds in the **+Cr** pigs (N = 8), 33.1 ± 14.7 seconds in the **+GAA** pigs (N = 8) and 46.3 ± 40.2 seconds in the control pigs (N = 13). The pigs could not identify a novel object after 24 hours as the time spent with recent ($F(2, 26) = 1.19$, $p > 0.1$; $\varepsilon = 0.084$) and older objects ($F(1, 26) = 0.144$, $p > 0.1$; $\varepsilon = 0.006$) was the same.

Short-term 2-object test

A 3 x 2 ANOVA was performed (*ie.* Groups x older (C) *versus* recent (D)) for the short-term 2-object test. The pigs spent more time exploring the novel object in this test ($F(1,24) = 20.782$, $p < 0.01$; $\varepsilon = 0.464$), which is in agreement with others (Kouwenberg et al., 2009). However, there was no effect of guanidino compound supplementation on identifying the recent object during this short-term memory test ($F(2,24) = 1.009$, $p > 0.1$; $\varepsilon = 0.078$). Once again, pigs in the three conditions spent a similar amount of time exploring the objects ($F(2,26) = 1.202$, $p > 0.1$ $\varepsilon = 0.078$).

Correlations

To investigate associations of tissue creatine and GAA concentrations with episodic-like memory, the absolute exploration times of specific objects were correlated to concentrations of GAA and creatine in brain regions. Because there was no effect of supplementation on episodic-like memory, the creatine and GAA concentrations of all pigs were pooled across study groups for each brain region. In the 4-object test, animals that had a higher concentration of GAA in the PFC spent

more time with the older, unmoved object (*ie.* Object A_1 in Figure 8.2c) ($R^2 = 0.2$, $F(1,25) = 5.4$, $p = 0.02$) (Figure 8.6a). And the creatine concentration in the cerebellum correlated with the time spent exploring the recently observed object that was moved (*ie.* Object B_M in Figure 8.2c) ($R^2 = 0.3$, $F(1,25) = 11.2$, $p = 0.0002$) (Figure 8.6b). Lastly, in the 24-hour test the concentration of GAA in the cerebellum correlated with the time spent exploring a new object after 24 hours (*ie.* Object C in Figure 8.3) ($R^2 = 0.2$, $F(1,25) = 5.6$, $p = 0.02$) (Figure 8.6c).

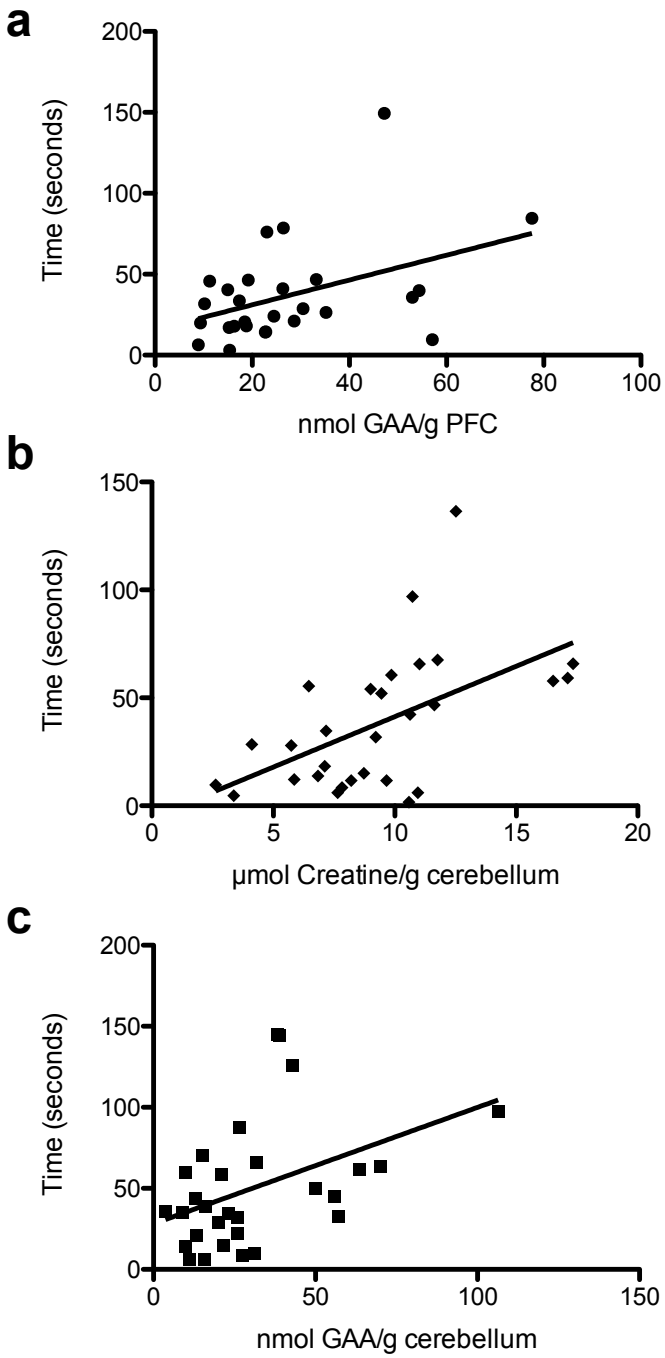

Figure 8.6 The relationship between concentrations of (a) GAA in the PFC and a familiar object during the 4-object test, (b) creatine in the cerebellum and the recently experienced moved object during the 4-object test and (c) GAA in the cerebellum and a novel object during the 24-hour test.

8.5 Discussion

The objective of this study was to determine the effects of guanidino compound supplementation on brain concentrations of GAA and creatine, as well as on episodic-like memory in pigs. Pigs were hand fed supplements of either creatine or GAA for 15 days prior to, and during a battery of memory tests. Overall, GAA supplementation affected GAA concentrations in the cerebellum only. However, the PFC was also sensitive to guanidino compound supplementation but only between supplemental groups. There were no effects of either supplement on pig memory, but GAA and creatine concentrations in the cerebellum as well as the GAA concentration in the PFC were associated with some metrics of episodic-like memory.

This study demonstrated that there were no overt effects of either creatine or GAA supplementation on episodic-like memory in weaned pigs. This was contrary to our hypothesis given the dietary intake of creatine in these animals was thought to be low (Harris et al., 1997), and thus, like studies on vegetarians (Benton and Donohoe, 2011), we hypothesized that creatine supplementation would improve memory. Because episodic-like memory tests rely on hippocampal function (Kouwenberg et al., 2009), one possible explanation for this null result is that there were no observed effects of guanidino compound supplementation on creatine or GAA concentrations in the hippocampus. Therefore it is possible that either the amount or duration of guanidino compound supplementation was insufficient to affect this particular region, so did not improve episodic-like memory in these pigs.

The supplementation of creatine and GAA resulted in some interesting findings within the brain regions. Indeed, the GAA concentration in the cerebellum was higher with GAA supplementation, suggesting that cerebellum is a site of GAA uptake. Furthermore, in the PFC, GAA supplementation led to lower GAA concentrations compared to the creatine supplemented group, while creatine supplementation led to lower creatine concentrations compared to GAA supplemented pigs. These data suggest that the PFC is a regulatory site of creatine synthesis and release in the pig brain that is responsive to dietary creatine and GAA. Further analysis is warranted to elucidate the roles of the various regions in creatine synthesis and transport in brain, including regional measurements of SLC6A8 transcripts as well as CT1, AGAT, GAMT and CK activities.

The concentration of creatine across regions of the unsupplemented pig brain was variable. Indeed, the creatine concentration in the PFC was lower than in cerebellum and hippocampus, where creatine concentrations were 35-40% greater than in 7-11 day old domestic pig brains (J. T. Brosnan et al., 2009). Therefore, the cerebellum and hippocampus appear to be sites of creatine accumulation in pigs as they develop. Because memory recollection relies on cerebellum (Andreasen et al., 1999) and episodic memory relies on hippocampus (Kouwenberg et al., 2009), then it is hypothesized that these regions would require more phosphagen derived energy as pigs get older and these skills are developed. Future measures of creatine in the pig brain should consistently sample a specific brain region, or homogenize the entire organ. Meanwhile, the GAA concentration was uniform across brain

regions, and so randomly sampling brain tissue for GAA analysis should provide consistent results in pigs of this age under normal dietary conditions.

The performance in episodic-like memory tests was in some cases associated with creatine and GAA concentrations in pig cerebellum and PFC. In general, a positive association between object recall and creatine concentration can be postulated as the result of enhanced capacity for ATP synthesis *via* phosphocreatine. The strongest correlation was between creatine concentration in the cerebellum, and the ability to recognize that an object was moved during the 4-object test (Figure 8.6b). Because the cerebellum becomes activated during memory recollection in humans (Andreasen et al., 1999), an association of cerebellar creatine concentration with a short-term memory task is plausible. In particular, this association is interesting because pigs were successful at noticing the moved objects during the 4-object test (Table 8.4), however, cerebellar creatine concentrations correlated only with movement of the recently viewed object (*ie.* B_M). Combined with the fact that the cerebellum had the highest creatine concentrations of the regions, it is clear that this region is a good target to investigate the role of creatine in memory.

Unlike creatine, we did not hypothesize a physiological relationship between GAA and memory, other than with respect to the capacity of GAA to support creatine synthesis. Regardless, the GAA concentration in the cerebellum was associated with 24-hour recall in these pigs (Figure 8.6c), which is interesting because pigs were unable to identify the novel object in that test. Moreover, the cerebellum was the only region to positively respond to GAA supplementation, more than doubling GAA

concentrations with supplementation. There was also an association between GAA concentration in the PFC and the exploration of a previously experienced object during the 4-object test (*ie.* A_1) (Figure 8.5a), in spite of a reduction in GAA concentration with supplementation. While it is difficult to discern whether GAA in the PFC was beneficial or detrimental, the association of the PFC with visual working memory is not ambiguous as PFC contributes to working memory (Courtney et al., 1998) and fear extinction (Milad and Quirk, 2002). However, we do acknowledge that all of the presented associations are statistically weak, and thus further studies are required to confirm associations between guanidino compounds in the brain and behaviour.

An unexpected finding was that GAA supplemented pigs exhibited greater weight gain (%) than creatine supplemented pigs. While we do not know the source of this difference, it would have been expected that if anything, supplemental GAA would consume methionine for creatine synthesis, and potentially reduce protein synthesis and growth; moreover, we expected that supplemental creatine would have potentially spared methionine for protein synthesis and growth. A hypothetical mechanism for this difference in weight gain was that GAA supplementation enhanced creatine accretion in the muscles of the **+GAA** pigs, leading to an increase in intramuscular water (Powers et al., 2003). However, this hypothesis is weak because weight gain in the **+GAA** pigs was not significantly greater than in the control group. Indeed, it could be argued that creatine potentially reduced growth in these pigs. Future studies are warranted to investigate pig growth in response to

GAA and creatine supplementation, with a particular focus on protein accretion and body composition.

The author feels it important to mention a number of unreported observations for others pursuing this type of research. One of the initial aims of this study was to correlate behavioural data with a number of other measures of methyl metabolism (*ie.* hepatic methyl partitioning, protein synthesis, organ creatine/GAA). However, we initially designed the study to test creatine supplementation only and that study was completed in summer 2011; after we completed the initial **+Cr** study, we decided to add a **+GAA** group, and so the entire study protocol was completed in 8 GAA supplemented pigs in summer 2012, without a control group. This proved to be adequate for the biochemical analysis of methyl metabolism; however the observed pig behaviour was drastically different than our **+Cr** and control pigs from the summer 2011. For example, the 2012 pigs were generally apathetic, they spent very little time exploring objects, they were observed to be skittish, and had little interest in the behavioural testing protocols as a whole. Because there was no control group to compare with those **+GAA** pigs, it could not be said with certainty that GAA supplementation affected pig behaviour. It was noted that the housing conditions were also somewhat different (*ie.* more pigs of various ages), which may have had some effect on the pigs' social behaviour. Therefore, those **+GAA** pigs were excluded from this behaviour study. The study was repeated with an additional **+GAA** and control group in the fall of 2012 and those data are presented here. The salient point is that the process of habituation and environment are absolutely

critical in studies of this nature. While this may seem obvious, it was not clear to our group until after this experience.

Other retrospective suggestions include a more careful measurement of weights of the brain regions to allow measurement of the total amount of creatine and GAA found in each region. Furthermore, the extraction of brain tissues takes expertise as well as time to remove the brain from the skull and to separate it into the sampled regions. Indeed, it is assumed that a significant amount of phosphocreatine was converted to creatine during brain extraction. However, because we repeated this process numerous times, the time to freezing was relatively consistent between pigs and samples, and conversion to creatine was likely not relevant to the conclusions. Regardless, we can offer no suggestion of a quicker means of separating the brain into distinct regions for rapid freezing.

8.6 General Conclusions

This study reports that supplemental creatine and GAA was unable to enhance cognition during a series of episodic-like memory tests in pigs. However, creatine and GAA concentrations were associated with some memory outcomes, and there were concentration and growth effects of supplementation. Therefore, future studies of this nature are warranted, not only for pig welfare, but also for humans as the connections between nutrition and behaviour continue to expand.

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