The Effect of Unbalanced Dietary Methionine fed to Pregnant Rats on Maternal and Fetal One-Carbon Metabolism

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Protein restriction during rodent pregnancy is a well-established model of developmental programming. Although the Southampton low-protein diet model of developmental programming has been accepted to produce hypertensive offspring, the mechanism of this programming remains unclear. Currently the effects of protein restriction in the Southampton low-protein diet are confounded by a relative elevation of the amino acid methionine. The aim of this project was thus to clarify the roles of protein reduction and methionine elevation within this model, especially within the context of amino acid and one-carbon metabolism. Pregnant Wistar rats were fed casein-based diets *ad libitum* varying in casein (18% or 9%) and methionine content (0.5% or 1.0%) from day 0 through 20 of pregnancy. Two diets exactly replicated the Southampton control and low-protein diets (Con: 18% casein, 0.5% methionine; LP: 9% casein, 0.5% methionine), while a third low-protein high-methionine diet (LP-MET: 9% casein, 1.0% methionine) was employed as a positive control for methionine stress. On day 20 of gestation dams were sacrificed and the feto-placental unit was excised; maternal and fetal blood was collected for HPLC analysis of free amino acids. Maternal plasma was also analyzed for homocysteine content using a spectrophotometric-based enzyme assay. Diet did not affect maternal weight gain, food consumption, litter size or fetal weight. In dams and fetuses, methionine was significantly elevated in both low-protein groups. Maternal homocysteine was significantly elevated only in dams fed the low-protein high-methionine diet. Reductions in maternal serine, proline and glycine levels also occurred in dams fed the low-protein high-methionine diet; fetuses of these dams had significantly reduced levels of all three branch chain amino acids (leucine, isoleucine and valine). Both low-protein diets resulted in drastic reductions

in circulating threonine levels in dams and fetuses. Thus, ingestion of low-protein diets with a relative (0.5%) or overt excess (1.0%) of methionine appears to disrupt one-carbon metabolism at the level of homocysteine remethylation to methionine. This may place strain on the folate cycle, as may be indicated by the reduced levels of threonine, serine and glycine. Further testing is necessary to clarify the extent to which folate stores are being utilized for homocysteine remethylation. Increased competition for placental amino acid transport may explain the alternations in circulating free fetal amino acids. Further investigations into levels of other one-carbon metabolites in dams and fetuses are necessary to fully characterize the effect of low-protein high-methionine diets, particularly within the context of the Southampton model of developmental programming.

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Dedication

For Jamie:

"Keep on beginning and failing. Each time you fail, start all over again, and you will grow stronger until you have accomplished a purpose - not the one you began with perhaps, but one you'll be glad to remember. ~ Anne Sullivan Macy

This may not be the end we foresaw, but it is an end. I love you.

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List of Abbreviations

AdoHcy……..*S*-Adenosylhomocysteine

AdoMet……..*S*-Adenosylmethionine

Ala……Alanine

Arg……...Arginine

Asn……...Asparagine

Asp……..Aspartate

BCAA……..Branched Chain Amino Acid

CBS……...Cystathionine β-Synthase

CON……..Control

Cth……....Cystathionine

Gln……Glutamine

Gly……Glycine

Hcy……. Homocysteine

HPLC……... High Performance Liquid Chromatography

Hyp…….. Hydroxyproline

Ile…….. Isoleucine

LC-MS……..Liquid Chromatography Tandem Mass Spectrometry

Leu……... Leucine

LP……..Low-Protein

LPD……....Low Protein Diet

LP-MET……...Low-Protein High-Methionine

Lys……...Lysine

Met……...Methionine

Orn……..Ornithine

Phe……...Phenylalanine

Pro…… Proline

SDH…….. Serine-Threonine Dehydratase

Ser……Serine

Tau…….Taurine

TDH……Threonine Dehydrogenase

THF……Tetrahydrofolate

Thr…….Threonine

Trp……...Tryptophan

Tyr……Tyrosine

Val……...Valine

Chapter 1
Introduction

1.1 Developmental Origins of Health and Disease Hypothesis (DOHaD)

In human populations associations exist between low-birth weight and an increased susceptibility to the metabolic syndrome- a collection of pathological metabolic disturbances (dyslipidemias, insulin resistance, visceral obesity, elevated blood pressure) that collectively promote development of diabetes and/ or cardiovascular diseases (Gluckman & Hanson 2004). Formulated from these data, the developmental origins of health and disease (DOHaD) hypothesis states a causal relationship exists between metabolic and physiologic adaptations occurring *in utero* and long-term post-natal health. Moreover, it is the uterine environment which induces these adaptations. Termed as 'reprogramming events' these adaptations are permanent, serving adaptive purposes when the uterine and post-natal environments match. In contrast, reprogramming becomes maladaptive, creating an increased predisposition to development of chronic disease(s), when the pre- and post-natal environments differ, as the individual is not optimally equipped metabolically or physiologically to cope with the post-natal environment (Barker 2007, Langley-Evans 2006). For example, adaptation to a nutritionally bereft uterine environment would confer advantages to surviving post-natal nutritional scarcity, but would be maladaptive in a nutritionally plentiful environment (Gluckman & Hanson 2004).

To unravel the mechanism(s) behind fetal reprogramming, numerous experimental approaches (genetic or dietary manipulation, uterine artery ligation, or administration of pharmacological agents) in many animal species (rats, mice, guinea pigs, sheep) have been employed (Langley-Evans *et al.* 2005, Bertram & Hanson 2001). It is interesting that the

divergent insults delivered in these models tend to elicit similar phenotypic outcomes – specifically, hypertension, glucose intolerance and insulin resistance in the offspring (Langley-evans 2004).

1.2 Rat Low-Protein Diet Model of Developmental Reprogramming

Two paradigms of dietary manipulation, global nutrient restriction or iso-caloric protein restriction, also referred to as maternal low protein diet, have been developed (Bertram & Hanson 2001). The rodent maternal low protein paradigm is a well-established model of developmental programming. In this model pregnant rats are fed casein-based iso-caloric low protein diets (LPDs) commencing on day 0 of pregnancy. Two such LPDs, the Southampton and Hope Farms diets, have been shown to result in differential reprogramming; the former diet consistently reprograms for hypertensive offspring (Langley-Evans 2006) while the latter results in offspring with impaired glucose and insulin metabolism (Ozanne & Hales 2002, Hoet *et al.* 2000). These discrepant results hold true even when testing using identical feeding regimens, identical rat strains and methodological techniques (Langley-Evans 2000). Both the Southampton and Hope Farms LPDs contain identical levels of protein (Appendix A and Table 1). Thus dietary protein reduction may not underlie Southampton-LPD mediated hypertension. The Southampton and Hope Farms diets differ markedly in the overall content and source of carbohydrate and fat, as well as in the level of supplemental DL-methionine (Appendix A and Table 1) (Langley-Evans 2000).

Correction of the inherent sulfur-amino acid deficiency (specifically cysteine) of casein is typically achieved by supplementation with methionine, which can be metabolically converted to cysteine (Reeves *et al.* 1993). Indeed, sulfur-supplementation of such casein-based diets is

especially critical during rat pregnancy as casein, containing 0.3% cysteine and 2.3% methionine, supplies less than half the sulfur required by the pregnant rat. However, methionine is the most toxic of all 20 amino acids when present in excess (Muramatsu *et al.* 1971, Sauberlich 1961), affecting parameters such as body growth (Matsueda & Niiyama 1982), methylation reactions (Dong *et al.* 2005) and cardiovascular health (Troen *et al.* 2003) all of which show impairment in the Southampton model of developmental programming (Langley-Evans 2006). To avoid such methionine toxicity in 1993 the American Institute of Nutrition (AIN), a committee formed specifically to address the nutritional needs of laboratory rodents, began recommending supplementation of casein-based diets with cystine rather than methionine (Reeves *et al.* 1993). The Southampton diets do not adhere to this recommendation, instead following AIN 1976 guidelines. It is noteworthy then that the Southampton LPD supplies respectively 2.5 and 6-fold more methionine than the Hope Farms control and LPD, and 2-fold more methionine (relative to total dietary protein) than the Southampton control diet. Thus methionine may be one agent involved in the programming of hypertension observed with the Southampton LPD (Langley-Evans 2000).

Table 1: Critical differences in the Southampton and Hope Farms Diets

	Southampton Diet		Hope Farms Diet	
(g/100 g diet)	Control	LPD	Control	LPD
Casein	18.0	9.0	18.0	9.0
DL-Methionine	0.5	0.5	0.2	0.08

1.3 The Amino Acid Methionine

Methionine, an essential sulfur-amino acid plays a central role in one-carbon (1-C) metabolism (Figure 1), encompassing the folate and methionine cycles, in which a single carbon moiety is transferred between two molecules (Cho *et al.* 2007). 1-C metabolism contributes to the synthesis of purine subunits for the synthesis of DNA, while also governing epigenetic regulation of gene expression, specifically through the 1-C metabolite S-adenosylmethionine (AdoMet) (Friso & Choi 2005). Additionally, AdoMet contributes to the synthesis of the polyamines spermidine and spermine (Chiang *et al.* 1996), molecules which aid in cellular proliferation and in maintenance of structural integrity of DNA (Abraham 1981). Proper 1-C metabolism is thus of critical importance during gestation while long-term methylation patterns are established and organogenesis occurs (Bauchart-Thevret *et al.* 2009, Kim *et al.* 2009). Additionally, 1-C metabolism interconnects with the transsulfuration pathway, through which the sulfur-containing amino acid cysteine is synthesized (Finkelstein 2000). Furthermore, metabolism of betaine (and its upstream metabolite choline) also connects with 1-C metabolism via the methionine cycle. Betaine/ choline metabolism is essential for proper cell signaling, structural integrity of membranes, cholinergic neurotransmission and hepatic lipid transport. Truly the disruption of 1-C metabolism could have profound impacts on physiology and development (Zeisel 2006).

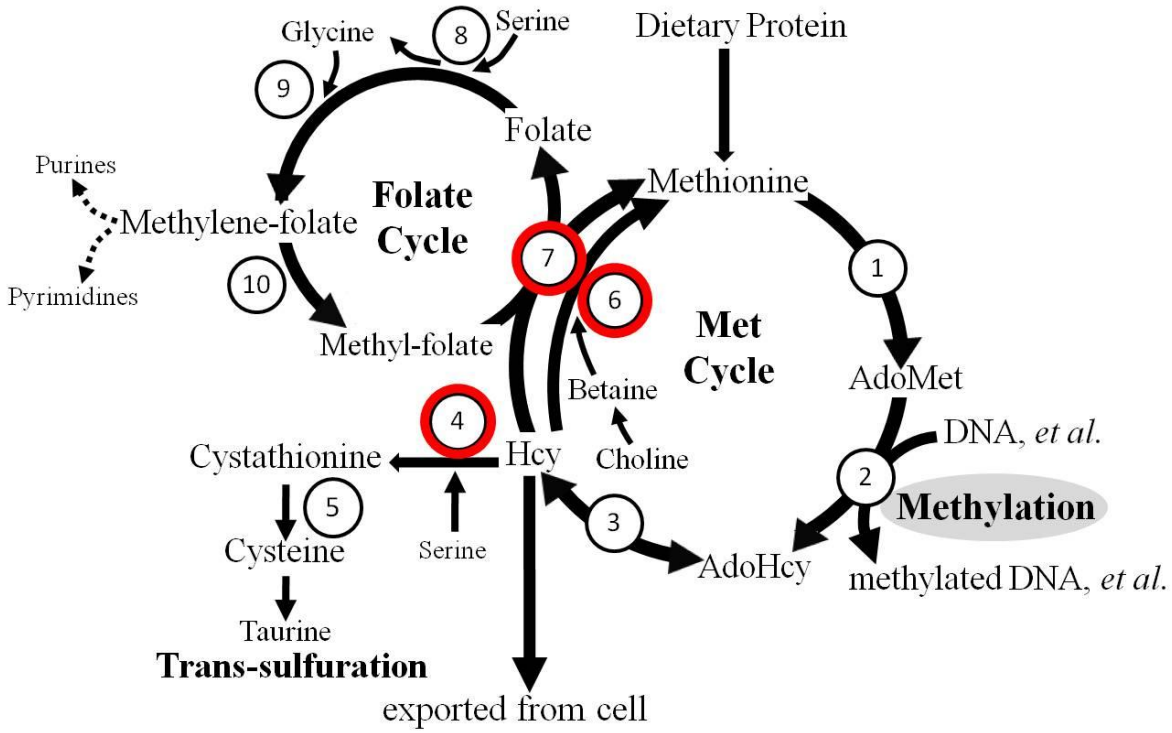

Figure 1: Mammalian Methionine Metabolism

Key reactions that prevent accumulation of homocycteine (Hcy) are represented by numbers 7, 6 and 4. The methionine cycle: reactions 1, 2, 3, 6, 7. AdoMet-dependent transmethylation: reaction 2. Transsulfuration: reactions 4 and 5. Choline/Betaine metabolism: reaction 6. The folate cycle: reactions 7, 8, 9, 10.

Numbers represent enzymes or reactions as follows: **1**, Methionine adenosyltransferase (EC 2.5.1.6). **2**, AdoMet-dependent transmethylation. **3**, S-adenosylhomocysteine hydrolase (EC 3.3.1.1). **4**, Cystathionine β synthase (CBS, EC 4.2.1.22). **5**, γ-Cystathionase (EC 4.4.1.1). **6**, Betaine-homocysteine methyltransferase (EC 2.1.1.5). **7**, Methionine synthase/ Methylfolate-homocysteine methyltransferase (EC 2.1.1.13). **8**, Serine Hydroxymethyltransferase (EC 2.1.2.1). **9**, Glycine cleavage system (EC 2.1.2.10). **10**, Methylenetetrahydofolate reductase (EC 1.7.99.5).

Adapted from (Finkelstein 2000)

1.4 The Methionine Cycle

Upon ingestion, methionine not used for protein synthesis enters the methionine cycle (Figure 1), a cycle which is present in all living cells. The first enzyme of this cycle, methionine adenosyltransferase (MAT), is responsible for the energy-dependent conversion of methionine to AdoMet (Figure 1 Reaction 1). MAT exists in three isoforms, each of which has distinct kinetic properties. Notably, only MATIII, the hepatic MAT isoform, is capable of responding to increased methionine concentrations with increased activity (Finkelstein 1998). AdoMet is a key biological molecule; it serves as the universal methyl donor for cellular transmethylation reactions (such as methylation of DNA and proteins) (Ulrey *et al.* 2005) or may be decarboxylated to form the precursor for spermine and spermidine biosynthesis (Finkelstein 1990). In the case of intracellular transmethylation reactions, after methyl group donation S-adenosylhomocysteine (AdoHcy) is generated from AdoMet (Figure 1 Reaction 2) (Finkelstein 2000).

Because it provides potent allosteric inhibition of intracellular transmethylases, levels of AdoHcy must be tightly regulated within the cell, a feat achieved by three different mechanisms (Hoffman *et al.* 1980, Finkelstein 2000). The first mechanism is the binding of free cytosolic AdoHcy via cytosolic proteins (Svardal & Ueland 1987). The second mechanism involves AdoHcy hydrolysis by S-adenosylhomocysteine hydrolase (SAHH), forming homocysteine (Figure 1 Reaction 3). This reaction however relies upon prompt removal of homocysteine and adenosine from the cell, as SAHH is capable of operating in either the forward (formation of Hcy) or reverse (formation of AdoHcy) direction, and it is the reverse direction which is

favoured thermodynamically (De La Haba & Cantoni 1959). Thirdly, AdoHcy entering the systemic circulation will be cleared by the kidneys (Duerre *et al.* 1969, Walker & Duerre 1975).

Homocysteine, formed exclusively from AdoHcy, may be re-methylated by either of two methyltransferases, betaine homocysteine methyltransferase (BHMT) (Figure 1 Reaction 6) or methylfolate-homocysteine methyltransferase (MFMT) (Figure 1 Reaction 7), with MFMT representing the intersection of the methionine and folate cycles (Figure 1) (Tibbetts & Appling 2010). BHMT relies on betaine, derived from choline metabolism, as a methyl donor while MFMT utilizes methyl-tetrahydrofolate (methyl-THF). While MFMT is ubiquitously expressed in all the cells of the body, expression of BHMT is restricted to the mammalian liver, and also, the kidneys of primates and pigs (Finkelstein 2000). Remethylation of Hcy leads to the reconstitution of methionine, and thus does not serve to eliminate methionine/ Hcy from the cell. Permanent removal of Hcy/ methionine from the cell is accomplished via a process called transsulfuration (Figure 1 Reactions 4,5). During transsulfuration, the heme-containing pyridoxal phosphate-dependent enzyme cystathionine beta synthase (CBS) irreversibly, via a serine-dependent condensation reaction, converts Hcy to cystathionine (Figure 1 Reaction 4) (Oliveriusová *et al.* 2002), which can be used for the eventual synthesis of cysteine, taurine or glutathione (GSH) (Rees *et al.* 2006c). Transsulfuration is limited to occurrence in specific mammalian tissues, the small intestine, kidney, pancreas and liver, owing to the tissue specific expression of the principle enzyme in the pathway, CBS (Finkelstein 2000). Importantly, methionine/ Hcy may be cleared from all cells of the body despite the tissue-specific expression of CBS, as Hcy can be transported across the plasma membrane into the systemic circulation to one of the sites of transsulfuration (Svardal *et al.* 1986).

1.5 Disruption of the Methionine Cycle and One-Carbon Metabolism

1.5.1 Methionine and DNA Methylation

Epigenetics encompasses the study of heritable changes in gene expression that do not stem from alterations in the nucleotide sequence, but rather occur via reversible covalent modifications of the DNA itself, or of the histone proteins which bind DNA (Egger *et al.* 2004). Cytosine residues located in CpG-islands within the DNA may undergo methylation or demethylation, respectively repressing or activating transcription, and thus gene expression (Feinberg & Tycko 2004). Such epigenetic modifications allow for tissue and developmentally specific expression of genes. Thus during the highly plastic stage of development *in utero* during which cellular differentiation and organogenesis are occurring, an exceptionally high frequency of epigenetic events necessarily occur (Lillycrop *et al.* 2005). Impairment of proper methylation, resulting in hyper- or hypo-methylated DNA has been implicated in various disease states ranging from cancers and cardiovascular diseases to neuropsychiatric disorders (Rodenhiser & Mann 2006).

Formed by the adenylation of methionine (Figure 1 Reaction 1), AdoMet is the ultimate intracellular methyl donor. Various intracellular transmethylases perform methyl transfer from AdoMet to co-substrates such as protein, phospholipids, neurotransmitters, RNA and DNA (Mato *et al.* 1997). Following methyl transfer AdoHcy is formed from AdoMet (Figure 1 Reaction 2). AdoHcy potently inhibits most methylation reactions as AdoHcy binds to the active site of most methyltransferases with a much higher affinity than that of AdoMet (Hoffman *et al.* 1979). Thus the ratio of AdoMet: AdoHcy is regarded as an indicator of a tissue's methylation capacity, with increased and decreased ratios reflecting probable hyper- and hypo-methylation

respectively (Waterland 2006). Studies have shown an association between a skewed AdoMet: AdoHcy ratio to aberrant methylation patterns, however, it is noteworthy that only a rough indication of the methylation capacity of any given tissue is provided by this ratio (Yi *et al.* 2000, Caudill *et al.* 2001, Tremolizzo *et al.* 2002). Furthermore, although either an increase in AdoHcy or a decrease in AdoMet create the potential of hypomethylation, it is thought that the former rather than the latter is more likely to result in aberrant methylation patterns (Caudill *et al.* 2001).

1.5.2 Homocysteine

Unavailable from dietary sources, Hcy is a sulfur-containing amino acid formed via the reversible removal of adenosine from AdoHcy by SAHH (Figure 1 Reaction 3). Irreversible methylation of Hcy to methionine by MFMT represents the intersection of the folate and methionine cycles, as the necessary methyl group is provided by methyl-tetrahydrofolate (methyl-THF) (Figure 1 Reaction 7) (Finkelstein 2000). Increases in Hcy levels (either via increased methionine intake or an increased frequency of transmethylation reactions) can precipitate a functional deficiency of cellular folates, a group of coenzyme factors essential for the transfer of 1-C units. Within this context, although dietary folate intake is 'adequate' by recommended standards, a deficiency is created by the metabolic status of the individual – more folate is needed to cope with an increased metabolic demand for Hcy remethylation than what is being ingested. Thus the organism becomes 'functionally' deficient. Additionally, the organism may become hyperhomocysteinemic, due to insufficient methyl-THF to re-methylate Hcy; excess Hcy may thus enter the systemic circulation resulting in hyperhomocysteinemia (Eskes 1998, Brouwer *et al.* 1998).

Hyperhomocysteinemia, defined as elevated plasma or serum Hcy levels, has been associated with numerous adverse health conditions and events ranging from vascular diseases and acute vascular events to neurodegenerative disease and osteoporosis (Maron & Loscalzo 2009). Additionally, hyperhomocysteinemia has been associated with increased risk of neural tube defects, pre-eclampsia and spontaneous abortions during pregnancy (Aubard *et al.* 2000).

1.5.3 Methionine and Pregnancy

1.5.3.1 Developmental Programming and DNA Methylation

Provision of a diet that is unbalanced with respect to methionine has been shown to create epigenetic abnormalities (Pogribny *et al.* 2006, Pogribny *et al.* 2008, Jiang *et al.* 2007, Pogribny *et al.* 1995, Caudill *et al*. 2001). Such abnormalities would be especially detrimental if they were to occur during embryonic/ fetal development, potentially altering organ development. Aberrant methylation may be one of the mechanisms through which developmental programming of adult disease could occur during gestation (Langley-Evans 2006, Lillycrop *et al.* 2005). Indeed, a study performed by Rees *et al.* showed that feeding of a LPD containing 0.5% methionine to pregnant rats elicited global hypermethylation in fetal livers (Rees *et al.* 2000). In contrast in post-weaning offspring, Lillycrop *et al.* observed hepatic hypomethylation of the genes encoding the glucocorticoid receptor (GR) as well as the peroxisome proliferator receptor alpha (PPARα) (Lillycrop *et al.* 2005). Both the GR and PPARα genes have been posited to play a role in fetal programming of hypertension, cardiovascular disease and metabolic disorders (Rees *et al.* 2006c). Additionally, changes in the transcript levels of DNA methyltransferase 1 (DNMT1), a methyltransferase responsible for epigenetic maintenance, were observed in what appeared to be

an age-diet and sex specific manner (Langley-Evans *et al.* 2006). Indeed, studies have shown differential programming of male and female offspring in response to an altered intra-uterine milieu. Amongst the results observed are gender-specific programming of post-natal cardiovascular physiology, metabolism and hypertension (Alexander 2006, Sugden & Holness 2002). Notably, differential expression in a gender-specific manner of mRNA for many genes involved in mediating vascular function and glucose/insulin metabolism have been reported (Chamson-Reig *et al.* 2006, Kwong *et al.* 2007, O'Regan *et al.* 2004). Aberrant methylation patterns are known to alter gene transcription, and altered levels of AdoMet and AdoHcy are known to disrupt proper methylation (Feinberg & Tycko 2004, Waterland 2006). Thus the results of these studies strongly indicate possible increases in the levels of AdoHcy and subsequent disruption of 1-C metabolism.

1.5.3.2 Developmental Programming and One-Carbon Metabolism

Presently, disagreement exists in the literature as to whether dietary protein reduction or methionine elevation causes programming of hypertension in the Southampton model (Rees et al. 2006c, Langley-Evans et al. 2006). Indeed, while some studies have shown altered plasma Hcy levels in LPD-fed dams (Petrie *et al.* 2002), others have not (Langley-Evans *et al.* 2006, Engeham *et al.* 2010). Furthermore, direct measurement of the methyl-donors used for Hcy remethylation (folate, as well as the precursors to betaine: choline and phosphocholine) do not indicate increases in Hcy remethylation (Engeham *et al.* 2010). mRNA of MFMT and methylene-tetrahydrofolate reductase (MTHFR), key enzymes of the folate cycle respectively required for methyl-THF-dependent Hcy remethylation and regeneration of methyl-THF did not

differ from controls in LPD-exposed fetuses or dams (Langley-Evans *et al.* 2006, Engeham *et al.* 2010).

Table 2: Sulfur Amino Acids in the Southampton and Hope Farms Diets

		Methionine		Cystine	
	% Casein	g/kg diet (after supplementation)	% Estimated Requirement*	g/kg diet	% Estimated Requirement*
Southampton	18	9.14	199	0.54	15
	9	7.07	155	0.27	7
Hope Farms	22	7.06	153	0.54	15
	9	2.87	62	0.27	7

*Values based on data from 1993 AIN recommendations for growth, pregnancy and lactation.

However supplementation of the Southampton LPD with folate prevents programming (Lillycrop *et al.* 2005, Torrens *et al.* 2006, Engeham *et al.* 2010). Folate supplementation prevents LPD-associated promoter hypomethylation and increased expression of the GR and PPARα genes in LPD-exposed offspring (Lillycrop *et al.* 2005). Furthermore, LPD supplementation with folate or glycine, necessary co-substrates for the regeneration of methylene-THF (and thus methyl-THF) (Figure 1) prevents programming of hypertension (Jackson *et al.* 2002, Torrens *et al.* 2006) and improves LPD-associated vascular dysfunction (Brawley *et al.* 2004). Interestingly, programming of behaviour (a preference for fatty foods) results from folate supplementation of the Southampton control diet (Engeham *et al.* 2010). Thus the role of disrupted 1-C metabolism in Southampton LPD-mediated programming remains unclear.

Patterns of amino acids also suggest disruption of 1-C metabolism within the Southampton model. Indeed, plasma threonine concentrations show consistent and drastic reductions in both Southampton LPD-exposed dams and fetuses (Rees *et al.* 2006a, Petrie *et al.* 2002, Rees *et al.* 1999, Rees *et al.* 2000). Interestingly, threonine levels rise in non-pregnant animals fed the Southampton LPD. Thus it seems pregnancy may increase threonine requirements (Rees *et al.* 2006a). Two major routes of threonine catabolism exist in mammals: catabolism via serine-threonine dehydratase (SDH) (top of Figure 2) or via threonine dehydrogenase (TDH) (bottom of Figure 2). Although the main activity of SDH is deamination of serine or threonine to their respective keto-acids (House *et al.* 2001), it may also catalyze the condensation of homocysteine and serine, yielding cystathionine (Selim & Greenberg 1959, Selim & Greenberg 1960, Goldstein *et al.* 1962). This CBS-like activity would link methionine and threonine metabolism, possibly explaining the threonine reduction observed with feeding high methionine diets (Daniel & Waisman 1969, Girard-Globa *et al.* 1972, Sanchez & Swendseid 1969).

An alternate explanation for the threonine reduction could be increased catabolism of threonine via the enzyme TDH, to indirectly replenish methyl-THF (Rees *et al.* 2006a). TDH catabolizes threonine to glycine (House *et al.* 2001), one of the necessary co-substrates to produce methylene-THF (Friesen *et al.* 2007). It is commonly accepted that folate requirements increase during pregnancy (Antony 2007), and could further increase with ingestion of excess methionine. Direct measurement of TDH activity revealed no dietary effect (Rees *et al.* 2006a), however, changes in amino acids (glycine, serine) used to regenerate methylene-THF (the

precursor to methyl-THF) have been observed in dams (Petrie *et al*. 2002, Rees *et al.* 1999, Rees *et al.* 2000).

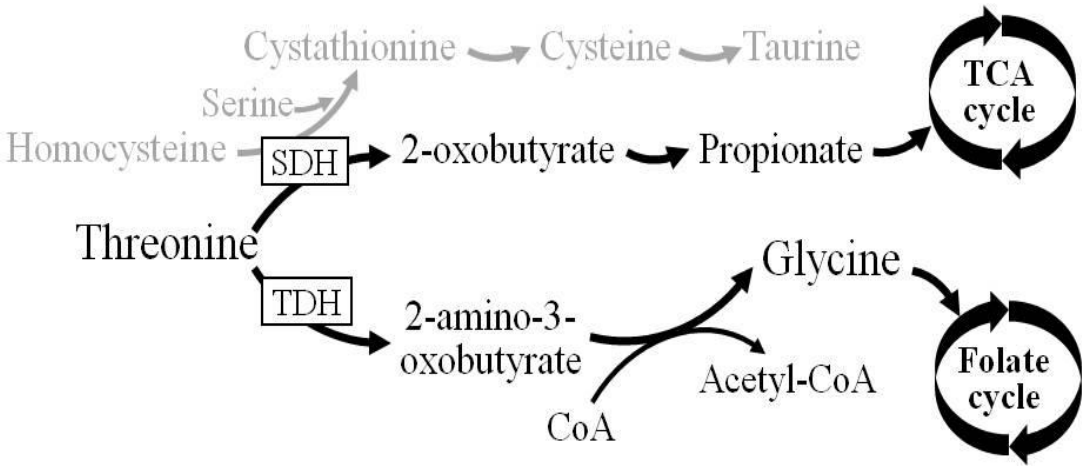

Figure 2: Major pathways of threonine catabolism in mammals

TDH: Threonine dehydrogenase (EC 1.1.1.103). SDH: Serine-threonine dehydratase (EC 4.3.1.19). Note that controversy exists regarding the substrate specificity of the SDH enzyme. Some studies report deamination of serine and threonine to their corresponding keto acids to be the only reactions catalyzed by SDH; other studies have shown SDH capable of forming cystathionine via the condensation of serine and homocysteine. The possible cystathionine synthetase activity of SDH would link threonine catabolism to the methionine cycle and transsulfuration. Likewise, TDH activity is linked into one-carbon metabolism through generation of glycine, a necessary co-substrate used for regeneration of methylene THF in the folate cycle.

While inconsistent effects on circulating Hcy have been reported (Petrie *et al.* 2002, Langley-Evans *et al*. 2006, Engeham *et al*. 2010), the current literature cites no dietary effect on maternal or fetal methionine levels (Petrie *et al.* 2002, Rees *et al.* 2006a, Rees *et al.* 2006b, Rees

et al. 1999). This apparent lack of change however may stem from adaptation during the two week dietary habituation period via increased hepatic methionine adenosyltransferase (MAT) activity, as has been shown to occur in other studies (Finkelstein & Martin 1986). Certainly, the signs of methionine toxicity (decreased birth weights and post-natal growth (Rees *et al.* 2006b, Rees *et al*. 2000), decreased fetal viability (Rees *et al*. 2006b)) as well as changes in gene expression and DNA methylation of fetuses (Rees *et al.* 2000, Langley-Evans *et al.* 2006) and pups (Lillycrop *et al*. 2005) point to the possibility of disrupted 1-C metabolism within this model.

1.6 Research Objectives and Approach

A primary research objective of this thesis was to determine whether the Southampton LPD alters one-carbon and amino acid metabolism in exposed dams and fetuses. Furthermore, clarification of the contribution of protein reduction and methionine elevation in this phenomenon was investigated through the use of an additional low-protein high-methionine diet (Table 3 and Appendix B). Replication of the Southampton control diet and LPD was achieved using a casein-based control and a casein-based methionine-elevated LPD.

The impact of Southampton feeding on one-carbon metabolism, and the relative contributions of reduced protein and elevated methionine within this model was assessed through

1) Analysis of free plasma amino acids in dams and fetuses via HPLC
2) Measurement of maternal plasma Hcy via a spectrophotometric assay.

1.6.1 Diets

Following mating, females were weighed, transferred to a single cage and assigned to one of the three dietary treatment groups. Three purified, isocaloric diets using casein as the protein source (prepared by Teklad, see Appendix B) varied in casein content, methionine content and the protein to methionine ratio (Table 3 and Appendix B). Two diets were designed to replicate the Southampton control and LPD (CON/ 18-0.5 and LP/ 9-0.5 respectively). A second LPD (LP-MET/ 9-1.0) containing 1% methionine, an amount that has been shown to alter levels of key one-carbon metabolites and enzyme activities in other studies (Regina *et al.* 1993, Rowling *et al.* 2002, Finkelstein & Martin 1986), was employed to serve as a positive control for methionine stress. Diets were made iso-caloric by addition of sucrose and corn starch to the 9% LPDs (Appendix B).

Table 3: Composition of Experimental Diets

Diet	% protein*	% methionine	protein : met	our code
Casein Control (CON)	18	0.5	36	18-0.5
Low protein (LP)	9	0.5	18	9-0.5
Low protein / high methionine (LP-MET)	9	1.0	9	9-1.0

*All diets utilize casein as the sole protein source.

Females were maintained on the experimental diets until euthanasia or delivery of pups. Food and water was available on an *ad libitum* basis; thus food consumption was monitored by weighing of all dispensed and remaining food.

Chapter 2

Effects of Low-Protein High-Methionine Diets during Pregnancy on Amino Acid Profiles in Maternal and Fetal Plasma

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2.1 Overview

Objective: Maternal protein restriction during pregnancy is a well established model of developmental programming. Disruption of one-carbon metabolism via excess dietary methionine may underlie this model. The aim was to assess whether low-protein high-methionine diets fed throughout pregnancy altered maternal and fetal amino acid profiles. **Methods:** Female Wistar rats were fed casein-based diets varying in protein and methionine content (control: 18% casein, 0.5% methionine; low protein: 9% casein, 0.5% methionine; low protein/ high methionine: 9% casein, 1% methionine) beginning day zero of pregnancy. Amino acid concentrations were determined using HPLC in maternal and fetal plasma on day 20 of gestation, with the exception of homocysteine, which was measured using an enzymatic method in maternal samples only. **Results:** In dams, both low protein diets were associated with elevated methionine and reductions in threonine. Maternal homocysteine was elevated only by the low protein diet with the higher level of methionine. Also in response to the low protein/ high methionine diet, maternal glycine, serine and proline were modestly but significantly decreased. Dietary effects on methionine and threonine were also seen in fetuses, except that methionine increases were more dramatic, and absolute concentrations of methionine were ~5-fold higher than in dams, even in controls. Branched chain amino acids were decreased in fetuses despite unaltered maternal levels. C**onclusions:** Protein-restricted, methionine-imbalanced diets fed during pregnancy affect both maternal and fetal amino acid levels. Diet effects on fetal amino acid profiles are not necessarily reflected in maternal plasma, but need to be considered with respect to the mechanisms of developmental programming.

2.2 Introduction

The developmental origins of health and disease hypothesis states that adaptive responses, also termed reprogramming events, occur in developing organisms in response to stimuli encountered during critical periods of development (Barker 2007). Critical periods are those during which a high degree of developmental plasticity occurs, such as during rapid cell division or the establishment of tissue-specific patterns of DNA methylation (Langley-Evans 2006). When the uterine environment is predictive of the post-natal environment, the reprogramming event is adaptive. When the two environments differ, the reprogramming event becomes maladaptive, and the individual is predisposed to development of an array of chronic diseases collectively referred to as Metabolic Syndrome (Gluckman & Hanson 2004).

Protein restriction of rats during pregnancy has been used extensively to study developmental programming. In these models, isocaloric diets with casein as the protein source are fed for all or part of pregnancy, beginning the day after mating. Two such models, based on the Southampton and Hope Farms diet formulations, elicit different reprogramming effects despite identical feeding and mating regimes: the Southampton diet produces hypertensive offspring (Langley-Evans 2006) while the Hope Farms diet results in altered glucose metabolism (Hoet *et al.* 2000, Ozanne & Hales 2002) with inconsistent effects on blood pressure (Lucas *et al.* 1996, Petry *et al.* 1997). Even when these two regimes were compared within the same study, with low protein diets, each containing 9% casein, the Hope Farms formulation did not elicit hypertension (Langley-Evans 2000). These findings suggest that the programming effect of the Southampton diet involves more than protein reduction. The Southampton and Hope Farms diets differ in overall content and composition of carbohydrates and fatty acids, as well as in the level

of supplemental DL-methionine (Langley-Evans 2000). Because casein is deficient in sulfur amino acids, supplementation with cystine is currently recommended (Reeves *et al.* 1993). Many laboratories continue to follow earlier recommendations and supplement with methionine, which can be endogenously converted to cysteine. However, it is well established that excess levels of methionine are toxic (Muramatsu *et al*. 1971, Sauberlich 1961). Within the Southampton model, methionine is added to both control and low protein diets at 0.5% (Supplementary Data Table 5), such that the low protein diet contains twice as much methionine relative to total protein as the corresponding control diet. With respect to the differential programming effects, the Southampton low protein diet (LPD) contains 2.5-fold and 6-fold more supplemental methionine than Hope Farms control (22% casein, 0.2% methionine) and low protein diets (9% casein, 0.08% methionine), respectively. Thus, disruption of methionine metabolism may play a role in developmental reprogramming (Langley-Evans 2000).

Methionine is an essential sulfur amino acid that plays a key role in one-carbon metabolism as well as in protein synthesis (Fig. 1). One-carbon metabolism, encompassing the methionine and folate cycles, is the transfer of single-carbon moieties between different molecules, such as during the methylation of DNA (Cho *et al.* 2007). Altered methionine levels could easily disturb the delicate balance of such one-carbon transfers leading to reprogrammed offspring (Cooney *et al.* 2002, Lillycrop *et al.* 2005, MacLennan *et al.* 2004). Ingested methionine enters the methionine cycle and is adenylated to S-adenosylmethionine (AdoMet) (Reaction 1 Fig. 1), the ultimate methyl donor for cellular methylation reactions (transmethylation). After donation of its methyl group during transmethylation, AdoMet is converted to S-adenosylhomocysteine (AdoHcy) (Reaction 2 Fig. 1), a potent inhibitor of

transmethylation. The ratio of AdoMet: AdoHcy is used as an indicator of methylation capacity (Finkelstein 2000, Finkelstein 2007). Studies have shown that altered methionine levels change this ratio and alter gene methylation with resultant changes in gene expression (Pogribny *et al.* 2006, Pogribny *et al.* 2008, Jiang *et al.* 2007). Thus increased methionine in the Southampton LPD could alter gene methylation and expression leading to the observed hypertensive phenotype in LPD offspring (Rees *et al.* 2000, Lillycrop *et al.* 2005).

Alterations in dietary methionine content may also elicit hypertensive offspring by increasing levels of the non-protein amino acid homocysteine (Rees *et al.* 2000, Petrie *et al.* 2002). Homocysteine is formed from the hydrolysis of AdoHcy within the methionine cycle (Reaction 3 Fig. 1). However, the reverse reaction, formation of AdoHcy from homocysteine, is more favourable thermodynamically. To ensure continued AdoHcy hydrolysis and thus continued transmethylation, homocysteine must be removed from the cell. One route achieving this is a folate-dependent remethylation of homocysteine to methionine (Reaction 7 Fig. 1), thus completing the methionine cycle. This reaction is contingent upon an adequate folate supply. Alternatively homocysteine can be converted to cysteine and eventually taurine and glutathione, via an irreversible serine-dependent condensation forming cystathionine in a process termed transsulfuration (Reaction 4 Fig. 1) (Finkelstein 1998, Eskes 1998). Excess methionine has been shown to increase circulating homocysteine levels, thus implying that cellular clearance capacity has been surpassed (Hoffer 2004, Zhang *et al.* 2004). Increases in circulating homocysteine have been associated with a number of adverse health conditions including cardiovascular disease (Ueland *et al.* 2000). On day 4 and 20 of gestation, dams fed the Southampton LPD have been reported to have increased circulating homocysteine levels. Thus, absolute or relative excesses

of methionine leading to elevations in homocysteine may contribute to programming via direct effects on developing vasculature (Petrie *et al.* 2002, Brawley *et al.* 2004).

The objective of this study was to assess maternal and fetal plasma amino acid profiles in the context of the Southampton model: pregnant rats were fed casein-based diets with normal versus low protein levels (18% and 9% casein, respectively), supplemented with the same level of methionine (0.5%). A third diet (9% casein, 1% methionine) was included to produce a definitive methionine excess. We hypothesized that the combination of low-protein and high-methionine would increase maternal plasma levels of homocysteine, which would result in increased transsulfuration and elevations of downstream sulfur amino acid metabolites in dams and fetuses.

2.3 Materials and methods

2.3.1 Animals and Diets

All experimental procedures were approved by the University of Waterloo Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care. Prior to mating, all rats were maintained on grain-based standard rodent chow (Harlan Teklad, Global 2018, 18.6% protein). At 12-20 weeks of age, virgin female Wistar rats were housed overnight with a male of the same strain. Upon detection of a vaginal plug, denoted as day zero of pregnancy, females were weighed and allocated to one of the three dietary treatments. The dams were maintained on their experimental diets, consumed *ad libitum*, until they were killed on day 20 of gestation. Food consumption was monitored.

Two of the experimental diets (CON, LP) were designed to replicate those described previously by Langley-Evans *et al.* (2000). The second low-protein high-methionine diet (LP-

MET), which was supplemented with higher methionine, was intended to produce a methionine excess that would disrupt the methionine cycle while avoiding overt toxicity and pregnancy loss. The control diet contained 180 g casein/kg diet and 5 g/kg supplemental DL-methionine. The low-protein diets (LP and LP-MET) both contained 90 g casein/kg diet, with supplemental DL-methionine provided at 5 and 10 g/kg diet respectively. Experimental low protein diets were held iso-caloric to the control by increasing sucrose and corn starch (Supplementary Data Table 5). All comparisons were relative to the 18% casein diet (CON).

2.3.2 Sample Collection

Dams were killed by carbon dioxide asphyxiation and weighed. Maternal blood was collected into EDTA-coated tubes by cardiac puncture, and stored on ice. The feto-placental unit was rapidly removed and the five fetuses in each horn closest to the cervix were removed for sampling. Fetuses were gently blotted dry on filter paper, sexed via measurement of ano-genital distance, then exsanguinated via decapitation. The blood was placed on ice until being centrifuged at 2500 x g for 20 min at 4ºC. Plasma was collected and stored at -20ºC until analysis.

2.3.3 Amino Acid Analysis

Quantification of free amino acids in maternal and fetal plasma was performed by HPLC, with the exception of homocysteine. Briefly, plasma and the internal standard (6 mM norleucine) were combined and the mixtures deproteinized by centrifugation at 12,000 x g for 15 min in centrifugal filters (Microcon Rose-YM 50, Fisher Scientific, Ottawa, ON, Canada). Filtrates were collected and evaporated to dryness. Pre-column derivatization with phenylisothiocyanate was carried out according to the manufacturer's protocol (PicoTag, Waters, Milford, MA, USA),

after which derivatized samples were stored at -20ºC until analysis. Chromatographic separation was performed using sample injection volumes of 40 µL, a PicoTag reversed-phase column (30 cm by 3.9 mm i.d.), and a gradient of sodium acetate-acetonitrile buffer (as described in the PicoTag protocol) with UV detection at 254 nm. Chromatograms were analyzed with the Millenium 32 software (Waters).

Homocysteine concentrations were determined using a kit (Diazyme, Poway, California) based on an enzyme cycling approach, in which homocysteine quantification was achieved via spectrophotometric monitoring of NAD^+ levels. Volumes of fetal plasma remaining after HPLC analysis were insufficient to measure homocysteine.

2.3.4 Statistical Analysis

All statistical analyses were performed using SAS for Windows, version 9 (SAS Canada, Toronto, ON). The number of litters per diet ranged from 8 to 9, with 3 to 19 pups per litter.

Effects of diet on maternal endpoints (weight gain, feed intake, litter size, plasma amino acids) were analyzed by one-way ANOVA, and analyses significant at $p<0.05$ were followed by posthoc comparisons using the Tukey adjustment for multiple comparisons.

Because fetuses from the same litter share both genetics and a common intrauterine environment, it would be incorrect to consider littermates independent observations. For this reason, fetal endpoints (fetal weight, plasma amino acid concentrations) were analyzed using a mixed model ANOVA, with diet and fetal sex as fixed effects and litter as a random effect. Because fetal sex had no effect on plasma amino acids it was dropped from the model for those analyses. As before, posthoc comparisons using the Tukey adjustment were performed when main effects were significant at $P<0.05$. To reflect the fact that litter was nested within diet, the

litter(diet) mean square was used as the error term for these comparisons, and results are presented as least squares means.

2.4 Results

2.4.1 Effects of Diet on Pregnancy Outcome

Four animals (control, two; LP, one; LP-MET, one) found not to be pregnant on day 20 were dropped from the study. One of them showed signs of fetal resorption; it was presumed that the other three dams were never pregnant. Abnormalities were noted in two of eight CON litters, two of eight LP litters and three of nine LP-MET litters. In the CON group, these consisted of blood clots in three fetuses and three hemorrhagic placentas (one of which corresponded to one of the aforementioned fetuses). In the LP group one enlarged, conjoined placenta and one fetus with abdominal hemorrhaging were observed. In the LP-MET group, three dams displayed asymmetric implantation patterns, favouring implantation in the left uterine horn with litter sizes of 10, 3 and 7 fetuses for these dams. However, the resulting decrease in average litter size for this group (Table 1) did not reach statistical significance.

Maternal weight gain, food consumption and fetal data for the three dietary groups are shown in Table 4. Food consumption of dams (482 ± 101 g, over all three diets) was not affected by diet. Although not reaching statistical significance, there was a tendency for dams consuming the LP-MET diet to gain less weight relative to those on CON or LP diets (Table 4, $P = 0.0992$).

Table 4: Maternal weight gain, food intake, litter sizes and fetal weights on day 20 of gestation following consumption of diets varying in methionine and protein content*.

Diet	CON (n=8)	LP (n=8)	LP-MET (n=9)
Maternal food intake (g)	511 ± 19	463 ± 35	470 ± 19
Maternal weight gain (g)	158 ± 9	158 ± 10	123 ± 10
Litter size	14 ± 1	15 ± 1	12 ± 2
Fetal weight (g)†	3.44 ± 0.37	3.45 ± 0.39	3.36 ± 0.33
Fetal weight, male (g)	3.55 ± 0.06	3.48 ± 0.06	3.44 ± 0.10
Fetal weight, female (g)	3.22 ± 0.09	3.49 ± 0.07	3.29 ± 0.07

*Key differences in diets: CON (control) - 18% casein / 0.5% Met; LP (low protein) - 9% casein/ 0.5% Met; LP-MET - 9% casein / 1% Met

†Values are means ± SEM, except for fetal weights, which are presented as least squares means to reflect the fact that 'litter' was included as a random effect in the analysis of variance.

There were no significant diet effects on any of these endpoints at $P < 0.05$.

The slightly reduced litter size observed in the LP-MET group (attributable to the two aforementioned litters of 3 and 7 fetuses) was not enough to reach statistical significance (overall average of 14 ± 0.7 fetuses/ dam). Fetal weight showed no dietary effect (average 3.4 g ± 0.2). Regardless of diet, male fetuses were heavier than females (Table 4, $p<0.01$). Female and male fetuses were respectively found to have typical ano-genital distances of 1.2 mm (or less) or of 1.8 mm (or greater).

2.4.2 Effects of Diet on Plasma Amino Acid Concentrations

Data for selected sulfur amino acids are presented in Fig. 3. Regardless of diet, methionine and taurine were the most abundant sulfur amino acids in both maternal and fetal

plasma. This was particularly marked in fetal samples, in which concentrations of methionine and taurine were, respectively, 3-5 and 8-9 fold higher than in maternal plasma. Methionine and homocysteine were the only sulfur amino acids affected by diet. Interestingly, the two low-protein diets increased methionine concentrations in maternal plasma to the same extent despite the two-fold difference in their methionine contents (P<0.01; Fig. 3A). In contrast, maternal

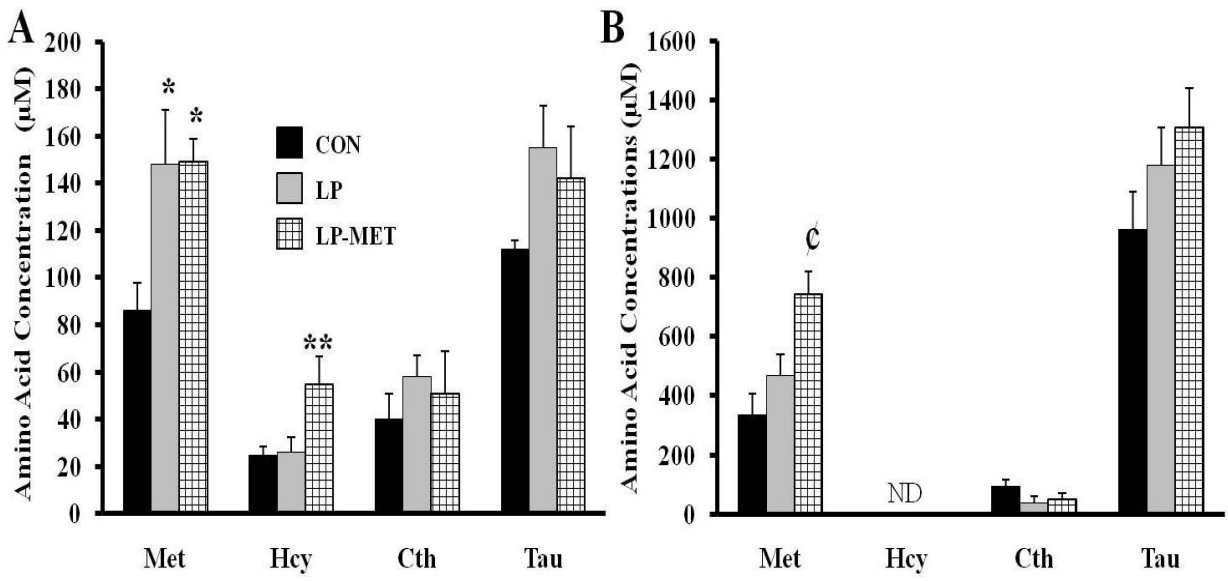

Figure 3: Concentrations of selected sulfur amino acids in (A) maternal and (B) fetal plasma on day 20 of gestation following consumption of diets varying in methionine and protein content.

Key differences in diet composition are noted below Table 1. Maternal data are presented as arithmetic means ± SEM, while fetal values are least squares means ± SEM to reflect the fact that 'litter' was included as a random effect in the analysis of variance. Note the difference in scales that was required to accommodate the high concentrations of methionine and taurine in fetal plasma. For dams: control n 6, LP n 5, LP-MET n 6. For fetuses: control n 44, LP n 47, LP-MET n 49. *Significantly different from CON (p<0.01). **Significantly different from CON (p<0.001). ¢Significantly different from both CON and LP (p<0.05). ND = not determined due to depletion of fetal sample.

homocysteine levels were increased by the LP-MET diet but not by the LP diet formulated according to the Southampton model (P<0.001; Fig. 2A). In fetal plasma, methionine concentrations were not affected by the LP diet, but were increased 2.2-fold by the LP-MET diet (P<0.05; Fig. 3B). Homocysteine levels in fetal plasma were not determined due to insufficient sample volume.

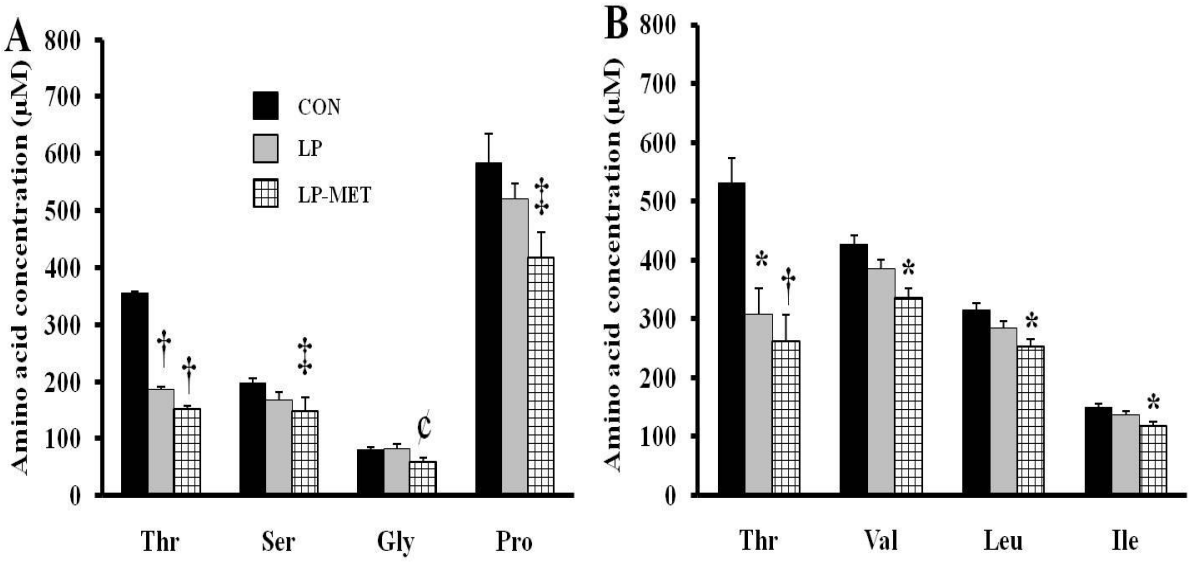

Figure 4: Concentrations of selected non-sulfur amino acids in (A) maternal and (B) fetal plasma on day 20 of gestation following maternal consumption of diets varying in methionine and protein content.

Key differences in diet composition are noted below Table 1. Maternal data are presented as arithmetic means ± SEM, while fetal values are least squares means to reflect the fact that 'litter' was included as a random effect in the analysis of variance. For dams: control n 6, LP n 5, LP-MET n 6. For fetuses: control n 44, LP n 47, LP-MET n 49. *Significantly different from CON (p<0.01). †Significantly different from CON (p<0.0001). ‡Significantly different from CON (p<0.05). ¢Significantly different from both CON and LP (p=0.05).

Data for non-sulfur amino acids affected by diet are shown in Fig. 4. The most dramatic dietary effect on non-sulfur amino acids was a decrease in plasma threonine in both maternal (Fig. 4A) and fetal plasma (Fig. 4B) in response to both LP and LP-MET diets. Other non-sulfur amino acids were differentially affected in dams and fetuses. In maternal plasma, reductions in serine and proline were observed as dietary protein decreased and methionine increased.

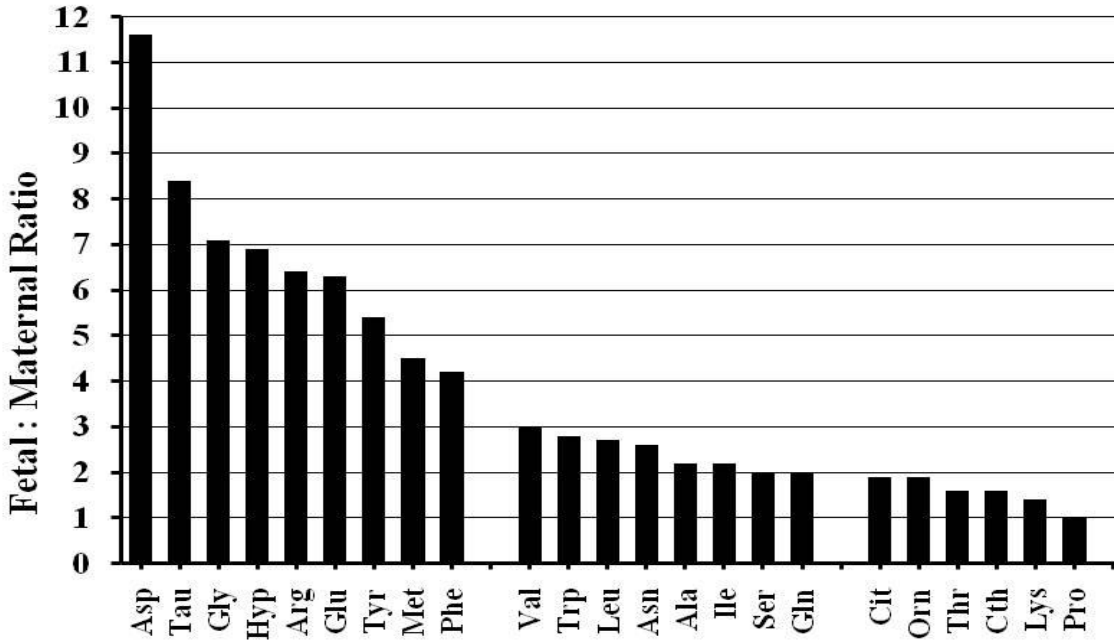

Figure 5: Ratios of mean fetal : maternal plasma amino acid concentrations at day 20 of gestation across all diets (least squares means to reflect adjustment for litter effects).

Amino acids were enriched in fetal plasma, with the exceptions of proline (equal to maternal concentrations). Fetal : maternal ratios were not affected by diet ($P>0.05$).

Ratios of fetal to maternal amino acid concentrations are shown in Fig. 5. Because these ratios were not affected by diet, a ratio was calculated for each fetus relative to its dam, and a

single average was presented across all three diets for each amino acid. With the exceptions of proline (similar in fetal and maternal plasma), all amino acids were more highly concentrated in fetal plasma. Nine amino acids, including methionine, were highly enriched (4 to 11-fold higher in fetal plasma), eight amino acids, including the branched chain amino acids, were moderately (2 to 4-fold) enriched, and five amino acids were slightly (less than 2-fold) higher in fetal plasma.

2.5 Discussion

Although the Southampton model of developmental programming is referred to as 'protein restriction' or 'low protein diet', evidence from several studies has suggested that disruption of one-carbon metabolism may lie at the heart of the programming phenomenon (Rees et al. 2006c). Specifically, the elevated ratio of supplemental methionine to dietary protein has been called into question. A relative excess of methionine leading to increases in circulating homocysteine could disrupt cellular methylation reactions, resulting in an altered epi-genotype and developmental reprogramming. This study sought to characterize the effects of low protein diets with elevated supplemental methionine on plasma amino acid profiles in dams and fetuses late in gestation. In agreement with most studies using the Southampton protocol, pregnant rats consuming the protein-restricted diets displayed a remarkable ability to produce offspring of normal weight and number, without alterations in weight gain or feed intake (Brawley *et al.* 2004, Langley-Evans *et al.* 2006, Torrens *et al.* 2006, Engeham *et al.* 2010). In dams, both low protein diets were associated with elevated methionine and marked reductions in threonine. In response to the higher level of methionine supplementation, maternal homocysteine levels doubled while glycine, serine and proline were modestly but significantly decreased. In fetuses,

effects of the low protein diets on methionine and threonine were also evident. Methionine was among the amino acids highly enriched in fetal plasma, such that fetal levels were nearly 5-fold higher than maternal levels, even in controls.

Both low-protein diets elicited significant increases in maternal plasma methionine levels. To our knowledge, this is the first study to assess plasma methionine using the Southampton protocol, wherein experimental diets are fed beginning day zero of pregnancy. However, several studies in which these same diets are initiated two weeks prior to mating have reported no effect on maternal methionine (Petrie *et al.* 2002, Rees *et al.* 1999, Rees *et al.* 2006a). This two week period prior to pregnancy may permit adaptation via the hepatic enzyme methionine adenosyl transferase (Finkelstein 2000), which has been shown to be upregulated by excess dietary methionine (Finkelstein & Martin 1986). Interestingly, the magnitude of methionine elevation in dams fed low-protein diets was the same despite a 2-fold difference in dietary methionine supplementation. This observation suggests that these dietary effects cannot be explained by an accumulation of un-metabolized diet-derived methionine in maternal plasma.

Maternal plasma homocysteine levels were substantially elevated by the low protein, 1% methionine diet, but not within the context of the Southampton model (control versus low protein, both at 0.5% methionine). Unaltered maternal homocysteine by day 20 of pregnancy, which is consistent with previous reports (Langley-Evans *et al*. 2006, Petrie *et al*. 2002), does not negate the possibility of altered one-carbon metabolism as a programming mechanism. Firstly, programming effects may be mediated by early gestation rises in homocysteine, such as that reported by Petrie and coworkers (day 4, (Petrie *et al.* 2002). The normal levels of maternal homocysteine by day 20 ((Petrie *et al*. 2002), current study) may reflect increased catabolism and

clearance. To this point, cystathionine and taurine levels were unaffected, suggesting that homocysteine catabolism via transsulfuration was not altered by diet. An increased reliance on homocysteine remethylation, at least in dams, is a plausible explanation for the maintenance of normal homocysteine levels. Furthermore, while low-protein fed dams ingesting 0.5% supplemental methionine (Southampton model) appear to have sufficient methyl donors to maintain control homocysteine values, feeding of 1% supplemental methionine may exhaust the available methyl donor supply. Thus, the feeding of low-protein, methionine-unbalanced diets such as the Southampton LPD may perturb folate and/ or betaine (choline) metabolism via an increased demand for methyl donors for homocysteine remethylation.

Diet-associated alterations in fetal amino acid profiles may have been indirect effects of increased maternal methionine levels on competition for placental transport. Maternal consumption of low-protein diets containing 0.5% and 1% methionine caused the already high levels of methionine in fetal plasma to rise 1.4 and 2.2 fold, respectively. The greater increase in fetal levels with 1% versus 0.5% maternal methionine supplementation was particularly interesting given that maternal plasma methionine was not different in these two groups. Decreases in fetal levels of all three BCAA (leucine, isoleucine, valine) and threonine were also observed. Competition amongst several of the neutral amino acids for placental transport has been suggested (Tsitsiou *et al.* 2009). Expressed on the maternal and fetal trophoblast surfaces, System L transports a wide range of neutral amino acids, including the BCAA and methionine (reviewed in (Regnault *et al.* 2005)). Methionine, homocysteine and leucine have been shown to compete with near equal affinity for placental System L transport *in vitro.* Studies in sheep have shown that maternal infusion of a mixture of nine essential amino acids did not increase

circulating fetal threonine levels, but did result in a 3.5 fold increase in fetal plasma methionine (Jóźwik *et al.* 2004). Furthermore, eight amino acids *not* in the infusion mix actually decreased in fetal blood (Jóźwik *et al.* 2004). Infusion of only threonine elicited increased fetal threonine seemingly at the expense of fetal BCAAs, which decreased (Paolini *et al.* 2003). If competition for transport can occur under those conditions, it is possible that the nearly 2-fold increase in maternal methionine levels in the low-protein groups could have contributed to the reductions of BCAA in fetal plasma. This possibility is supported by our finding that decreases in BCAA were only significant in the fetuses from dams receiving the higher methionine supplement (LP-MET diet). This is the first study reporting fetal plasma amino acid levels for the Southampton diet model. Although more in-depth investigation is required to bring clarity to these findings, it is clear that alterations in maternal nutrient consumption could have unexpected effects on fetal nutrient supply.

In rats there are surprisingly few reports of amino acid profiles in fetal blood, and particularly fetal : maternal amino acid ratios in matched samples. In agreement with two previous reports in late gestation rats (Palou *et al.* 1977, Malandro *et al.* 1996), the three most abundant amino acids in rat fetal plasma in the present study were glutamine, alanine and lysine. The same three amino acids are also the most abundant in human fetal blood at 12-17 weeks (cardiac puncture (Jauniaux *et al.* 1999)) and term (umbilical venous samples (Jauniaux *et al.* 2001)). Of the nine amino acids in the present study that were highly enriched in fetal blood relative to maternal blood, seven were also the most enriched in the rat study of Malandro and coworkers ((Malandro *et al.* 1996), the other two amino acids were not quantified). However, our ratios were generally higher. For example, we observed a fetal: maternal ratio of 4.9 for

methionine versus 2.4 (Malandro *et al.* 1996). However, we calculated their ratios posthoc, such that dams and fetuses were not matched and fetal blood samples may have even been pooled. Our ratios were less similar to those reported in humans. In agreement with the present study, aspartate, taurine, arginine, tyrosine, methionine and phenylalanine were among the highly enriched amino acids in human fetal blood at 12-17 weeks gestation, with ratios ranging from 2.1 to 3 (Jauniaux *et al.* 1999). However, ornithine and lysine, which were the most enriched in the human, were only slightly enriched in rat fetal blood.

Modest reductions in glycine, serine and proline, and a marked reduction in threonine occurred in dams fed the low-protein high-methionine test diets. Decreases in maternal threonine concentrations have also been reported in studies using the Southampton diet formulations beginning two weeks prior to mating (Rees *et al.* 1999, Rees *et al.* 2000, Petrie *et al.* 2002, Rees *et al.* 2006a). Because the magnitude of the threonine decrease was proportional to the reduction in dietary protein content, it is tempting to attribute this to a simple deficiency of this essential amino acid. Indeed, once the sulfur amino acid (specifically cysteine) deficiency is rectified by the addition of methionine (Harper 1959, Reeves et al. 1993), threonine becomes the next limiting amino acid in casein-based diets. Inclusion of another group (18% casein with 1% methionine) would have resolved the role of reduced protein versus elevated methionine. Support, however, for increased dietary methionine as the etiology for threonine reduction lies in the a fact that tryptophan, the third most limiting amino acid in casein (Harper 1959), did not differ according to diet. Increased utilization of threonine, glycine and serine for gluconeogenesis is unlikely, since control and test diets were isocaloric. Additionally, changes in other key gluconeogenic amino acids such as alanine and glutamine were not observed. Thus

another explanation must underlie the observed decreases in threonine, glycine, serine and proline.

The reductions in threonine could be rooted in increased enzymatic catabolism via serine-threonine dehydratase (SDH) or threonine dehydrogenase (TDH). Although the main activity of SDH is oxidation of threonine and serine to their respective α-ketoacids ((House *et al.* 2001); Fig. 2), it may also catalyze the condensation of homocysteine and serine to form cystathionine (Selim & Greenberg 1959, Selim & Greenberg 1960, Goldstein *et al.* 1962). This CBS-activity of SDH would link methionine and threonine metabolism, possibly explaining the increased SDH activity and reduced threonine levels observed in response to high-methionine diets (Daniel & Waisman 1969, Girard-Globa *et al.* 1972, Sanchez & Swendseid 1969). Increased SDH activity however seems improbable in the current study, as cystathionine and taurine levels were unaffected. The changes in our maternal amino acid profiles do raise the possibility of increased TDH activity in response to low-protein high-methionine diets. Threonine oxidation by TDH yields glycine ((House *et al.* 2001); Fig. 2). Glycine can be further used for synthesis of structural molecules, and like serine, can be used to regenerate methylene-tetrahydrofolate, and by extension methyl-THF (Friesen *et al.* 2007). Increased folate requirements during pregnancy are commonly accepted, owing to increased nucleotide synthesis and cellular replication (Antony 2007). It thus becomes interesting that threonine reduction in response to Southampton low-protein diet feeding occurs only in pregnant rats, and not their non-pregnant counterparts (Rees *et al.* 2006a). Indeed, the equivalently increased methionine in dams fed the low-protein diets with 0.5% or 1% methionine in the present study, along with elevations in homocysteine in the latter group raise questions regarding folate flux in these animals, questions furthered by the

decreases in threonine, serine and glycine observed. Moreover, impairment of folate cycling in this model is supported by reports showing that supplementation of the Southampton low-protein diet with folate prevents programming effects on blood pressure (Torrens *et al.* 2006), epigenetic alterations in hepatic gene expression (Lillycrop *et al.* 2005), and preferences for fatty foods (Engeham *et al.* 2010).

The notion that the reprogramming effects of the Southampton model involve disrupted one-carbon metabolism arising from excess dietary methionine has been debated for some time (Rees *et al.* 2006c, Langley-Evans *et al.* 2006). Lack of dietary effects on a number of metabolic endpoints, including plasma homocysteine late in gestation and expression of methionine synthase mRNA in maternal liver has led one group to conclude that disruption of methionine-homocysteine metabolism is not involved (Langley-Evans *et al.* 2006, Engeham *et al.* 2010). This issue may prove to be surprisingly difficult to resolve given that maternal liver, fetal liver and placenta may play varying roles depending on gestational stage, and many of the key metabolites are not readily measureable in extracellular fluid. Moreover, reprogramming may have been already set in motion by the transient perturbations in maternal homocysteine levels, and possibly one-carbon metabolism, during the pre-implantation period (Petrie *et al*. 2002, Kwong *et al.* 2000), well before the development of the placenta or the fetal liver. From mid-gestation onwards, the placenta may be a more active participant in the methionine and folate cycles than previously believed. It was recently shown that term human placenta expressed mRNA for methionine synthase and methylene-THF reductase at levels comparable to those in adult liver (Solanky *et al.* 2010). Expression of the enzymes catalyzing other pathways of

homocysteine catabolism was very low to undetectable, suggesting folate-dependent reactions may be particularly important in the placenta.

The Southampton diet model has shed much light on mechanisms underpinning many aspects of developmental programming. This study confirms the changes in maternal amino acids reported by Rees and coworkers (Rees *et al.* 1999, Rees *et al.* 2000, Petrie *et al*. 2002, Rees *et al*. 2006a), and extends these observations by quantifying amino acid levels in individual fetuses. We found effects of the low protein / methionine-unbalanced diets on several fetal amino acids that were not reflected in maternal plasma. Concentrations of methionine in fetal plasma were 4-fold higher than maternal levels even in animals fed the control diet. The fact that fetal methionine concentrations rose still higher in response to the methionine-unbalanced diets raises the concern that the fetus's ability to metabolize excess methionine and maintain normal one-carbon metabolism could be overwhelmed. Altered fetal amino acid profiles are likely due to a combination of competitive interactions among amino acids for placental transport and shifts in the activities of the methionine and folate cycles in response to excess methionine. Clearly, effects at the fetal level are developmentally relevant and need to be considered with respect to the mechanisms of this model of developmental programming.

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2.7 Supplemental Data

Table 5: Composition of Experimental Diets

Component (g/kg)	CON (18% casein, 0.5% Met)	LP (9% casein, 0.5% Met)	LP-MET (9% casein, 1% Met)
Casein	180.0	90.0	90.0
DL-Methionine	5.0	5.0	10.0
Sucrose	198.0	228.0	228.0
Corn Starch	325.0	385.0	380.0
Maltodextrin	95.0	95.0	95.0
Corn Oil	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0
Mineral Mix, AIN76	35.0	35.0	35.0
Vitamin Mix, AIN76A	10.0	10.0	10.0
Choline Bitartrate	2.0	2.0	2.0

Table 6: Maternal plasma amino acid concentrations (μM) on day 20 of gestation.

Diet	CON (n= 6)	LP (n= 5)	LP-MET (n= 5)	P
Ala	732 ± 20	722 ± 56	723 ± 137	NS
Asn	198 ± 7	167 ± 15	147 ± 26	NS
Asp	18 ± 1	15 ± 2	13 ± 2	NS
Gln	1964 ± 107	2008 ± 74	1852 ± 374	NS
Glu	163 ± 19	137 ± 11	148 ± 27	NS
Gly	80 ± 5[a]	82 ± 8[ab]	59 ± 8[c]	=0.05
Ser	198 ± 7[a]	167 ± 15[ab]	147 ± 26[b]	<0.05
Thr	356 ± 24[a]	187 ± 24[b]	151 ± 19[b]	<0.0001
Pro	584 ± 51[a]	521 ± 27[ab]	418 ± 45[b]	<0.05
Hyp	195 ± 4	168 ± 16	130 ± 26	NS
Arg	101 ± 6	95 ± 7	88 ± 14	NS
Lys	914 ± 115	864 ± 91	869 ± 209	NS
Orn	24 ± 3	21 ± 2	25 ± 6	NS
Ile	72 ± 8	62 ± 4	62 ± 11	NS
Leu	127 ± 14	103 ± 8	104 ± 16	NS
Val	154 ± 15	130 ± 10	115 ± 17	NS
Cth	40 ± 11	58 ± 9	51 ± 18	NS
Hcy	25 ± 4[a]	26 ± 6[a]	55 ± 11[b]	<0.001
Met	86 ± 12[a]	148 ± 23[b]	149 ± 10[b]	<0.01
Tau	112 ± 4	155 ± 18	142 ± 22	NS
Phe	58 ± 6	56 ± 3	54 ± 10	NS
Trp	68 ± 6	70 ± 19	82 ± 23	NS
Tyr	52 ± 2	40 ± 3	41 ± 7	NS

Values shown are means ± SEM.

[a,b]Different superscripts for mean values within a row denote significant differences.

NS, not significant. Cth = cystathionine; Hyp = hydroxyproline

Table 7: Fetal plasma amino acid concentrations (μM) on day 20 of gestation.

Diet	CON (n=44)	LP (n=47)	LP-MET (n=49)	P
Ala	1605 ± 68	1714 ± 75	1609 ± 57	NS
Asn	458 ± 28	461 ± 21	488 ± 27	NS
Asp	59 ± 5	68 ± 5	73 ± 8	NS
Gln	3659 ± 179	4047 ± 197	4777 ± 274	NS
Glu	858 ± 52	927 ± 48	977 ± 72	NS
Gly	547 ± 20	548 ± 27	549 ± 28	NS
Ser	358 ± 19	329 ± 16	323 ± 16	NS
Thr	529 ± 31^{a}	310 ± 16^{b}	239 ± 13^{b}	<0.01
Pro	584 ± 35	504 ± 31	410 ± 21	NS
Hyp	116 ± 6	108 ± 6	110 ± 6	NS
Arg	171 ± 8	178 ± 8	194 ± 8	NS
Lys	1183 ± 40	1042 ± 47	1052 ±36	NS
Orn	37s ± 2	46 ± 3	33 ± 1	NS
Ile	149 ± 5^{a}	136 ± 5^{ab}	118 ± 4^{b}	<0.01
Leu	315 ± 10^{a}	282 ± 10^{ab}	252 ± 7^{b}	<0.01
Val	428 ± 16^{a}	385 ± 14^{ab}	338 ± 9^{b}	<0.01
Cth	94 ± 18	38 ± 4	48 ± 9	NS
Met	337 ± 16^{a}	469 ± 18^{ab}	749 ± 39^{c}	<0.05
Tau	968 ± 52	1173 ± 62	1350 ± 82	NS
Phe	232 ± 8	250 ± 10	237 ± 7	NS
Trp	194 ± 16	167 ± 11	166 ± 22	NS
Tyr	256 ± 10	253 ± 11	222 ± 7	NS

Values shown are least squares means ± SEM to reflect the fact that 'litter' was included as a random effect in the analysis of variance.

[a,b,c]Different superscripts for mean values within a row denote significant differences.

NS, not significant. Cth = cystathionine; Hyp = hydroxyproline

Chapter 3
Conclusion

Feeding pregnant rats protein-restricted diets has been established to elicit fetal reprogramming and post-natal disease (Langley-Evans 2000). The Southampton low-protein diet leads to offspring that develop hypertension by weaning. Presently, disagreement exists regarding the mechanism of programming, specifically the level of methionine provided in the low-protein diet, and the impact it may have on disrupting one-carbon metabolism *(Rees et al.* 2006c).

Through the use of three iso-caloric casein-based diets, this study sought to clarify whether low-protein methionine-imbalanced diets affect amino acid and one-carbon metabolism in both dams and fetuses. Two diets (Control and Low-Protein) were designed to exactly replicate the Southampton model diets, containing 18% and 9% casein respectively, with a constant supplement of 0.5% methionine. A second protein-restricted diet (Low-Protein High-Methionine) containing 9% casein and 1.0% supplemental methionine, was employed to serve as a positive control for methionine stress.

Picotag HPLC analysis was employed to quantitate free plasma amino acids from both dams and fetuses, including the sulfur-based amino acids methionine, cystathionine and taurine. This analysis allowed an assessment of certain aspects of one-carbon metabolism, specifically the transsulfuration pathway (via cystathionine and taurine measurement) and the methionine cycle (via methionine measurement). Disturbances of both non-sulfur and sulfur-based amino acid metabolism were revealed, with marked elevations occurring in circulating methionine levels in dams and fetuses. Transsulfuration flux was not altered by diet in either dams or fetuses.

Circulating homocysteine was measured in dams via a spectrophotometric-based assay. Although the Picotag methodology of free amino acid analysis allows superior sensitivity (quantification of picomolar amounts vs. nanomolar) over other methods (Anders 2002), it is not the best method for quantification of amino acids containing free sulfhydryl groups (*i.e.* cysteine and homocysteine) (Tyler 2000). For this reason, a well-established commercially available kit (Diazyme, Poway, California) was employed for homocysteine measurement.

Evidence of disruption of one-carbon metabolism was found in dams ingesting either low-protein diet. Dams of both groups had significant and equal elevations of circulating methionine, despite the higher methionine supplement in the low-protein high-methionine group. Interestingly, circulating homocysteine levels were also significantly elevated within dams of the low-protein high-methionine group. In combination, these results indicate an increased reliance on methyl-donors (either from the folate cycle or choline metabolism) to support increased remethylation of homocysteine to methionine. While the Southampton low-protein diet appears capable of maintaining control levels of homocysteine via this route, the elevations in both methionine and homocysteine indicate that the low-protein high-methionine diet cannot. Although several metabolites of folate and choline metabolism were not found to be affected in fetuses of Southampton-fed dams (Engeham *et al.* 2010), this does not negate the possibility that flux through either of these routes could increase in response to the low-protein Southampton diet. Measurement of methionine synthase and BHMT activity could help resolve this issue.

This project sought to measure hepatic AdoMet and AdoHcy levels in both dams and fetuses, however this was not feasible due to technical difficulties and instrument limitations. As of yet, AdoMet and AdoHcy levels remain unmeasured in this model. The increased levels of

plasma methionine observed in the present study indicate a strong possibility of disruptions to the AdoMet: AdoHcy ratio, a key ratio for control of gene expression. Indeed, in response to high methionine levels activity of the enzyme hepatic methionine adenosyltransferase has been reported to increase, converting methionine to AdoMet (Finkelstein & Martin 1986). Alterations in the levels of AdoMet and AdoHcy could explain the changes in gene expression observed with Southampton diet feeding and could be the mechanism behind fetal re-programming (Lillycrop *et al.* 2005, Rees *et al.* 2000, Lillycrop *et al.* 2007).

Changes of free circulating fetal amino acids in the present study, particularly methionine and the three branched chain amino acids (leucine, valine and isoleucine) suggest the possibility of disrupted placental amino acid transport in response to low-protein methionine-imbalanced diets. Presumably, altered maternal amino acid levels could change competition amongst amino acids for key transport proteins in the placenta, such as System L (Tsitsiou *et al.* 2009). Alterations in nutrient delivery could have important consequences on growth and organogenesis, possibly explaining the abnormal weights of truncal organs observed in fetuses (Rees *et al.* 1999, Rees *et al*. 2000) and pups of Southampton-fed dams (Rees et al. 1999, Rees et al. 2000, Kwong et al. 2000).

The Southampton rodent low-protein diet model is commonly used to study developmental programming, despite a lack of clarity regarding its underlying mechanism. To resolve this question, it will be important for future studies to adhere to the same experimental protocols – to use the same strain of rats and to commence feeding of the low-protein diet on day 0 of pregnancy. Only once the mechanism of programming is understood can true progress be

made in this field, allowing questions with direct relevance to human populations to be addressed.

Appendix A: Composition of Southampton and Hope Farms Diets

	Southampton Diet		Hope Farms Diet	
g/ 100g diet	18% Casein	9% Casein	22% Casein	9% Casein
Casein	18.0	9.0	22.0	9.0
Corn oil	10.0	10.0		
Soybean oil			4.3	4.3
Starch	42.5	48.5	8.0	8.0
Glucose			53.7	66.7
Sucrose	21.3	24.3		
DL-Methionine	0.5	0.5	0.2	0.08
Choline	0.2	0.2		
Cellulose	5.0	5.0	5.0	5.0
Minerals and vitamins	2.5	2.5	6.85	6.95

Appendix B: Composition of Casein-Based Diets

g/kg diet	18-0.5	9-0.5	9-1.0
Casein	180.0	90.0	90.0
DL-Methionine	5.0	5.0	10.0
Sucrose	198.0	228.0	228.0
Corn Starch	325.0	385.0	380.0
Maltodextrin	95.0	95.0	95.0
Corn Oil	100.0	100.0	100.0
Cellulose	50.0	50.0	50.0
Mineral Mix AIN-76	35.0	35.0	35.0
Mineral Mix AIN-76A	10.0	10.0	10.0
Choline Bitartrate	2.0	2.0	2.0

Appendix C: Composition of Standard Rodent Chow (Teklad)

	g/kg diet
Protein	186.0
Fat	62.0
Carbohydrate	442.0
Crude fiber	35.0
DL-Methionine	4.0
Choline	1200 mg/kg
Folate	4 mg/kg
Cobalamin	0.08mg/kg

*Ingredients: Ground wheat, ground corn, wheat middlings, dehulled soybean mean, corn gluten meal, soybean oil, calcium carbonate, dicalcium phosphate, breweres dried yeast, iodized salt, L-lysine, DL-methionine, choline chloride, kaolin, magnesium oxide, Vitamin E, acetate, menadione sodium bisulfite complex, manganous oxide, ferrous sulphate, zinc oxide, niacin, calcium pantothenate, copper sulphate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, calcium iodate, vitamin B12 supplement, folic acid, biotin, vitamin D3 supplement, cobalt carbonate

Appendix D: Composition of Extra Casein-Based Diets

g/ kg diet	18-1.0	9-0.25
Casein	180.0	90.0
DL-Methionine	10.0	2.5
Sucrose	198.0	228.0
Corn Starch	320.0	387.5
Maltodextrin	95.0	95.0
Corn Oil	100.0	100.0
Cellulose	50.0	50.0
Mineral Mix AIN-76	35.0	35.0
Mineral Mix AIN-76A	10.0	10.0
Choline Bitartrate	2.0	2.0

Appendix E: LC-MS Method Development

Attempts to develop a LC-MS method for AdoMet and AdoHcy measurement have been ongoing within the Servos laboratory (University of Waterloo, Department of Biology) under the direction of Leslie Bragg and Dr. Mark Servos. Separation and detection of AdoMet and AdoHcy was achieved, using an Agilent 1200 high performance liquid chromatography instrument with a tandem API 3200 triple quadrupole ion trap mass spectrometer.

Reverse-phase HPLC was initially tested using a C18 column, achieving poor retention and co-elution of AdoMet and AdoHcy. Successful separation of peaks for AdoMet and AdoHcy was achieved through use of a cyano-column. Several mobile phases were tested, run in both isocratic and gradient formats (Table 8). The best peak shapes for AdoMet and AdoHcy were obtained when using water: methanol: acetic acid (95:5:0.1) (mobile phase A) and methanol: water: acetic acid (95:5:0.1) (mobile phase B) with gradient elution. Elution involving simultaneous transition of A from 70% to 30% and B from 30% to 70% over a total run time of 5 minutes produced the cleanest peaks for AdoMet and AdoHcy of several elution schemes tested. Despite these successes, the sensitivity for detection of AdoMet remained poor. Attempts to increase sensitivity (manipulation of instrument settings, different mobile phases, different elution protocols (isocratic, gradient, various gradient combinations of mobile phases), and various preparation methods of standards) were unsuccessful.

Additionally, all commercially available AdoMet is contaminated with AdoHcy. Purification was attempted through solid phase extraction (SPE). Briefly, cartridges (C18, HLB, MCX, NH_2) were pre-conditioned with methanol and water, following which AdoMet standard (1 g/L) was added. Cartridges were washed with 2 mL water prior to extraction. A variety of

extraction solutions were tested, differing only in the ratio of methanol: water. Samples were evaporated to dryness overnight, and then reconstituted in methanol prior to analysis by LC-MS. The highest levels of AdoMet purity were achieved using C18 columns and elution with a 5:95 methanol: water solution; however this method failed to completely purify the AdoMet.

Table 8: Mobile phases tested during LC-MS method development for measurement of AdoMet and AdoHcy

Mobile phases tested	Elution type
85:15 water: methanol	Isocratic
85:15 water: methanol with 0.1% acetic acid	Isocratic
95:5:0.1 water: methanol: formic acid 95:5:0.1 methanol: water: formic acid	Both gradient and isocratic
95:5 water: methanol 95:5 acetonitrile: water	Both gradient and isocratic
40% methanol with 4mM ammonium acetate and 0.1% formic acid and 0.1% heptafuorobutyric acid	Isocratic
95:5:0.1 water: methanol: acetic acid 100% acetonitrile	Both gradient and isocratic
95:5:0.1 water: methanol: acetic acid 95:5:0.1 methanol: water: acetic acid	Both gradient and isocratic

Appendix F: Weanling Sampling Issues

Three novel diets were to be used in this project (9-1.0 – Appendix B, 18-1.0 & 9-0.25-Appendix D) – there are no reports in the literature on the effects of these diets; There have however been reports published on the toxicity of high-methionine diets , and the impact this may have on pregnancy and development (Matsueda & Niiyama 1982, Muramatsu *et al.* 1971). To determine whether any of the diets had such toxic effects a subset of dams from each dietary treatment group were allowed to go to term and deliver. At delivery the dams were switched back to standard (non-casein based methionine-balanced) rodent chow (Teklad 2018 – Appendix C) fed on an *ad libitum* basis. New-born pups were to be marked, weighed and sexed within 24 hours of delivery. Litters were culled to 10 pups of 5 males and 5 females. At weaning (21 days post-natal ± 2 days) pups were to be killed via carbon dioxide asphyxiation, weighed and sampled for plasma, livers and kidneys.

Originally a number between one and ten was assigned to each pup, being marked on the back and abdomen of the pup with a sharpie. Within 24 hours these numbers were no longer visible, having been removed via maternal grooming. Marking of each pup on the back and abdomen with henna dye was also unsuccessful- maternal grooming once again removed the assigned numbers within 24 hours of marking. Working with henna dye also presented additional logistical challenges – a period of time was necessary for the dye to set and dry. Pups had to be kept relatively immobile during this period (rolling caused smudging of the ink) and could not be placed back with the mother. This proved tedious and time-consuming for the researcher, and imposed stress on the dam and pups. After the purchase of a commercially available micro-tattoo

device (Ketchum ™), micro-tattooing of newborn toes was settled on as the method to permanently mark pups in a timely manner.

Very few pups however were able to be sampled at weaning. Independent of the marking method used (sharpie, henna, micro-tattoo) the dams displayed a surprising tendency to cannibalize the young, usually within the first week after birth. Olfactory cues play a critical role during rat reproduction and parenting (Burn 2008), thus fresh gloves were always worn when handling animals to minimize problems with scent contamination/ scent disruption. Other theories were tested, but none met with success. Amongst the tested methods were tattooing of and handling of pups by one person only at birth, acclimating the dam to the tattoo ink by smearing a tiny bit on her nose and fur, not tattooing the pups, and not weighing the pups, and not wearing scented fragrances (e.g. deodorant, cologne). After the failure of all these approaches an article was found showing that cage cleaning two times per week during weaning is enough to stress dams and elicit maternal cannibalism of pups (Burn 2008). At this point, the most plausible explanation for maternal cannibalism of pups seems to be frequent cage cleaning (every day) by the animal care technician. At the time the frequency of cage cleaning was discovered, no animals remained to test this theory. The average number of live pups at birth and weaning for each diet are presented in table 9. Also shown in the table is the average litter size after culling of large litters to 10 pups.

Table 9: Summary of Maternal Cannibalism of Pups

Diet	Live pups at birth	Litter size (after culling)	Live pups at weaning
18-0.5	10 ± 1.8	9 ± 1.0	7 ± 2.0
9-0.5	11 ± 2.0	9 ± 1.3	3 ± 0.3
9-1.0*	15 ± 1.5	10 ± 0	3 ± 2.2
18-1.0	7 ±1.6	7 ± n/a	1 ± 0.4
9-0.25	10 ± 1.7	9 ± 2.2	7 ± 1.9
Standard*	11 ± 3.0	8 ± 1.6	5 ± 2.0

18-0.5, n=4; 9-0.5, n=4; 9-1.0, n=4; 18-1.0, n=4; 9-0.25, n=3; STD, n=3

*One dam from each of these groups was acclimated to the micro-tattoo ink as described above. Pups from these litters were still eaten by the mother.

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