The effect of a protein-restricted diet during pregnancy on the expression of the amino acid transporter System $B^{0,+}$ in early rat embryos.

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

Epidemiological studies have shown that human adults have a higher chance of developing various metabolic disorders, such as type II diabetes and hypertension, due to maternal under nutrition. The concept that conditions encountered in early development can have far reaching implications for an individual's adult life is known as the Developmental Origins of Health and Disease (DOHaD) hypothesis. *In vitro* studies have shown that if the amino acid leucine is not available at a high enough concentration the embryo will not exhibit normal trophectoderm protrusive activity which precedes implantation. The amino acid transport system System $B^{0,+}$ is the main gateway into these cells for leucine but its expression at the transcription level has never been shown in preimplantation blastocysts. We investigated System $B^{0,+}$ expression in preimplantation blastocysts from dams fed a control versus a low-protein isocaloric diet (18% and 9% casein, respectively). Using RT-PCR to detect the System $B^{0,+}$ transcript, $ATB^{0,+}$, we found that indeed there is expression of this amino acid transporter in the pre-implantation rat blastocyst. Due to the gender-specific nature of many DOHaD phenomena, the blastocysts were sexed using gender specific primers and a nested PCR approach. Quantitative real time PCR (qRT-PCR) results show no significant difference in $ATB^{0,+}$ expression in blastocysts taken from dams fed either diet (9% n= 56, 18% n = 52; 7 dams from each diet group). Furthermore, separating the data by gender reveals no significant difference in expression. However, while not significant, there does appear to be a trend in the protein restricted blastocysts towards increased transcription of $ATB^{0,+}$, suggesting System $B^{0,+}$ may be responding at the transcription level to the diet. This could be part of the predictive adaptive response leading to a reprogrammed phenotype as described by the DOHaD hypothesis. Further work is needed to elucidate System $B^{0,+}$'s role in developmental programming.

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List of Abbreviations

4E-**BP1** – Eukaryotic Initiation Factor 4 - binding protein 1. Pg. 21

A^{vy} – Viable yellow agouti mice. Pg. 16

cDNA – Complimentary DNA (reverse transcribed from RNA). Pg. 33

CV – Cardiovascular. Pg. 4

DOHaD - Developmental Origins of Health and Disease. Pg 2

EGA - Embryonic Genome Activation. Pg 12

gDNA – Genomic DNA. Pg. 33

GLUT1, GLUT2, GLUT3, GLUT4 – Glucose transporters 1-4. Pg. 13

H19 – Long Non-coding RNA H19 (imprinted gene). Pg. 18

Hprt – Gene. Hypoxanthine-guanine phosphoribosyltransferase. Pg. 34

ICM – Inner-cellular mass. Pg. 10

Igf2 – Insulin-like growth factor 2 (imprinted gene). Pg. 18

IUGR – Intrauterine Growth Restriction. Pg. 23

IVF – *In vitro* fertilization. Pg. 17

MgCl – Magnesium Chloride. Pg 35

MLP - Maternal Low Protein. Pg. 5

mTOR – mammalian Target of Rapamycin. Pg. 21

P70S6K1 or **S6K1** – p70 Ribosomal S6 Kinase 1. Pg. 21

PBS+**BSA** – Phosphate buffered saline containing bovine serum albumin. Pg. 32

PCR – Polymerase chain reaction. Pg. 33

PVP - Polyvinyl pyrrolidione. Pg. 33

Slc6a14 – Gene. Solute carrier member 6a14 (member 14 of family 6). Codes for System $B^{0,+}$. Sodium and chloride dependant neutral and basic amino acid transporter $B^{0,+}$. (Also known as $ATB^{0,+}$ when referring to mRNA/Protein/Activity of System $B^{0,+}$). Pg. 22

Sry – Gene. Sex determining region of the Y-Chromosome. Pg. 36

qRT-**PCR** – Quantitative Real Time PCR. Pg. 34

TE – Trophectoderm. Pg. 10

Zfy – Gene. Region of the Y-chromosome implicated in male germ cell development. Pg. 36

1. Introduction

Adult late-onset diseases, such as type II diabetes and hypertension, have been linked to sub-optimal growth conditions encountered during early development (i.e. *in utero* or *in vitro*). Low birth weight, an indicator of poor gestational conditions, is a predictor for a variety of late-onset adult diseases. These include: type II diabetes, hypertension, obesity, and many others. These conditions have been grouped into a condition known as "metabolic syndrome" [1-3]. An estimated 25% of the American population has metabolic syndrome [1]. Research indicates that people with metabolic syndrome are three times more likely to die of cardiovascular complications [4].

Epidemiological research by Barker *et al.* in 1986 established the link between maternal under nutrition and increased adult disease rates. When examining epidemiological data from post World War II England, Barker noticed low birth weight correlated with cardiovascular disease rates in the adults born during that same time period [5]. He theorized that poor nutrition in pre-natal life increases susceptibility to the effects of an affluent lifestyle. An affluent lifestyle, in this context, is an abundance of high energy and low cost food. This has come to be known as the thrifty phenotype, and is the most common example of the developmental programming phenomenon.

Developmental programming describes the elevated disease risks encountered by an individual when there is a mismatch between its pre-natal and postnatal nutritional conditions. For example, a thrifty phenotype individual has been developmentally programmed by poor nutrient conditions *in utero* to expect poor nutrient conditions for the duration of its life. This is

a survival advantage if nutrient conditions remain poor after birth. However, if that individual encounters a nutrient rich environment throughout postnatal life the *in utero* programming becomes detrimental and the individual develops obesity and metabolic syndrome. The diagram below summarizes the thrifty phenotype phenomenon.

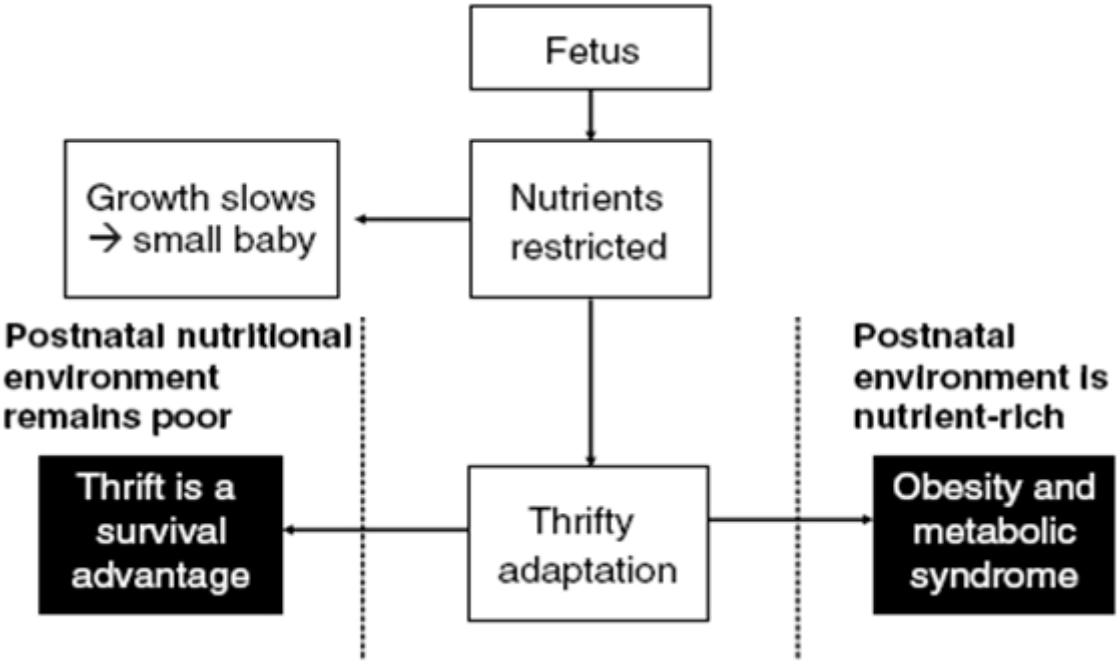


Figure 1. The "Thrifty Phenotype." Offspring developing in nutrient poor environments tend towards metabolic syndrome and obesity when placed in a nutrient rich environment after birth. However if the postnatal nutritional environment remains poor the thrifty phenotype is a survival advantage [6].

Barker's observations opened a new and exciting area of research. If pre-natal nutrition can have such a drastic effect on adult-life disease susceptibility then the implications are tremendous. The Developmental Origins of Health and Disease (DOHaD) hypothesis proposes that sub-optimal intrauterine environment, like that found during maternal under-nutrition, may alter the embryo's development through predictive adaptive responses. These predictive adaptive responses are thought to be physiological adaptations made by the embryo in

response to a given maternal environment to best utilize available resources *in utero* and to prepare itself for postnatal life. If there is a mismatch between the *in utero* diet and postnatal diet then a diseased phenotype will arise.

1.1 Rodent Models for Developmental Programming

There are many species currently being used to examine developmental programming and this topic has been reviewed by Bertram *et al.* [7], Nathanielsz *et al*. [8] and McMullen *et al.* [9]. The species used include rats, mice, guinea pigs, sheep and non-human primates [7]. Rats are the most common animal model for many reasons: their development under normal conditions is well understood and documented, rats are small and economical to house, rats are easier to handle than mice, they have a short gestation period [7] and the rodent placentation style is similar to our own [10]. The disadvantages of using a small-animal model such as the rat are that they are dissimilar to humans in the following ways: they have a large litter size, rats are born physiologically immature, the diets fed to rodents are not completely equivalent to human dietary patterns and rodent disease symptoms and progression differ somewhat from their human counterparts [9].

Most developmental programming models involve dietary manipulation of the mother during pregnancy. Several dietary regimes linked to the metabolic syndrome have been investigated, and they involve deficiencies or overabundances in macronutrient (protein, carbohydrates and fatty acids) concentrations of the diet. Offspring of dams fed a high-fat diet suffered from hypertension when fed a normal diet after birth [11]. Woodall *et al.* fed rats a globally nutrient-restricted diet (50% of normal caloric content) during pregnancy and found the offspring rapidly gained weight during weaning, became overweight and suffered from cardiac hypertrophy and altered CV function in adult-hood [12]. A maternal low protein diet programs for reduced offspring birth weight and hypertension and many other programming effects covered below in section 1.2. Vickers *et al.* showed that global nutrient restriction and

low protein *in utero* programs rat offspring to be sedentary and prone to increased adipose tissue formation, which is exacerbated by high energy diets [13].

In addition to changes in macronutrient concentration there are other programming models. Iron deficiency [14] and calcium deficiency [15] during pregnancy in rats programs the offspring for hypertension. In mice, zinc deficiency during pregnancy programmed the offspring (and two subsequent generations) for immunodeficiency [16]. Exposure to elevated levels of glucocorticoids (from maternal stress or exogenous sources) is associated with reduced birth weight and development of metabolic syndrome in adulthood [7, 9]. Chronic maternal hypoxia during pregnancy programs offspring to be vulnerable to cardiovascular disease, by way of suppressing fetal cardiac function, altered cardiac gene expression, increased myocyte apoptosis, myocyte hypertrophy and cell cycle disruptions of myocytes [17]. Over 50 genes are affected by the maternal hypoxia model, as reviewed by Zhang *et al.* [17], including genes involved in the oxidative stress response pathway, cell signaling/survival genes, metabolism and neurotransmitters. Uterine artery ligation is a model used to simulate impaired nutrient perfusion though the placenta [18], reviewed by Fernandez-Twinn *et al*. [19]. Unilateral or bilateral uterine artery ligation results in: low birth weight offspring, reduced beta cell mass at birth persisting through adulthood leading to type II diabetes, decreased nephron number and renal function and a decrease in glomerular number resulting in compensatory glomerular enlargement [19].

1.1.1 Developmental Programming Model - Maternal Low Protein Diet

One of the most common models used to investigate the DOHaD hypothesis is the maternal low protein diet (MLP) model. It is important to note that there are two low protein

diet regimes in the available literature. Langley-Evans compared them in 2000 [20]. The "Southampton" diet uses a control diet with 18% casein and a 9% casein diet to achieve normal and low protein conditions, respectively. Casein is protein supplement derived from milk.

The MLP model programs offspring for a wide variety of symptoms and at-risk phenotypes. It lowers offspring birth weight, increases postnatal growth rate, causes hypertension, altered organ/bodyweight ratios, reduced cell numbers during the blastocyst phase of development [21], altered imprinted gene expression [22], altered cardiac gene expression [23], altered pancreatic beta-cell gene expression [24], accelerated rates of renal failure in adult life [25] and a shorter offspring lifespan [26] perhaps due to increased susceptibility to oxidative stress [27]. The MLP model also appears to program rat offspring behavior; rats exposed to a low protein diet during gestation show a marked preference for high-fat food over control rats [28]. It is important to note that the models listed above are merely highlights taken from a long list of examples. In every example additional work is required to dissect and understand the mechanisms at work during the observed fetal programming. One particularly interesting phenomenon of the fetal programming story is the gender-specific manner in which offspring are affected.

1.1.2 Gender Specific Programming

The effects seen in the offspring of the MLP model appear to be gender specific. For example, under the MLP conditions, female (but not male) offspring birth weight is reduced [21]. [29]. Female offspring from the same study showed elevated Glut-2 and insulin mRNA levels [29]. An increase in systolic pressure and abnormal organ/body mass ratios in male (but not female) offspring were also observed [21]. Maternal low protein conditions generate male

offspring with a reduced beta-cell mass, reduced insulin content and marked glucose intolerance upon reaching adult-hood Adult male offspring from MLP conditions showed reduced insulin-stimulated glucose uptake in both muscle and adipose tissue and a decrease in both GLUT 4 and PKC-ζ protein levels in muscle [30]. In another MLP study adipocytes isolated from 6 week old male offspring displayed increased insulin receptor expression and at 3 months male offspring adipocytes were found to be more responsive to insulin stimulation of glucose uptake [31].

Other programming models exhibit gender-specific effects. Adult male offspring (but not female offspring) of endotoxic dams were heavier and showed increased adipose tissue formation, increased food intake, elevated circulating leptin, increased insulin resistance and reduced corticosterone in response to stress. In contrast, adult females of endotoxic dams had the following symptoms: increased testosterone levels, higher baseline corticosterone levels, enlarged heart:body mass ratio and enlarged adrenal glands [19]. Glucocorticoid-exposed offspring also demonstrate a gender-specific effect; males exhibit a typical metabolic syndrome phenotype (low birth weight, obese as adults, hypertension, hyperphagia, insulin resistance, etc.) whereas females are merely hypertensive and have a large increase in type-2 angiotensin receptor expression [19]. This might explain the observed renal remodeling and hypertension in these rats [19]. McCormick *et al.* noticed sex-specific effects of prenatal stress provided by electric shock; female offspring had elevated hypothalamic-pituitary-adrenal responses to stress, but males seemed unaffected [32]. High-fat fed dams yield offspring with gender specific effects; adult male offspring are more hyperinsulinemic during an oral glucose challenge than females [33]. Male offspring of high-fat fed dams also had a higher liver

triglyceride content than female offspring and their pattern of insulin signaling protein expression was consistent with reduced liver insulin sensitivity [19]. McMullen *et al.* observed gender-specific effects of prenatal low-protein and carbenoxolone exposure on renal angiotensin receptor expression levels; the female offspring showed a marked upregulation in expression when compared to males [34].

The gender specific effects observed in the various developmental programming models remain difficult to explain. In general, males appear to be more adversely affected by sub-optimal developmental environments than females. Male pre-implantation embryos develop more quickly than female ones [35]. *In vitro* produced male embryos have a higher metabolic rate, grow faster and have differential transcription of genes on the sex chromosomes and the autosomal chromosomes than their female counterparts [36]. In mouse blastocysts over 600 genes are differentially expressed based on gender [37]. It has been proposed that some genes expressed on the Y-chromosome may accelerate growth of preimplantation embryos, while expression of some X-chromosome products retard development [36]. A female embryo, having two X-chromosomes and no Y-chromosomes, would have none of the Y-chromosome genes which accelerate growth. It is possible that male pre-implantation embryos ignore the uterine nutrient conditions (at their own peril) in order to maximize growth rates. Female preimplantation embryos, having a slower growth rate, lower metabolism and different gene expression profile may cause them to be less affected (or unaffected) by some sub-optimal developmental conditions, as their growth rates may remain in step with the lowered nutrient availability. This tentative explanation is consistent with the literature which shows males and

females are differentially affected by the various DOHaD models during development, with males being the more fragile of the two.

1.2 Early Rodent Development

Fertilization occurs in the ampulla region of the oviduct. As the zygote moves down the oviduct and into the uterus, it progresses through serial cleavages (every 12-24 hours) from one cell to the morula stage (~12-30 cells) and then on to the early blastocyst stage [38]. Figure 2, below, illustrates this. Blastocysts have a unique feature, a blastocoel, which is a fluid filled central cavity which morulae lack. A blastocyst also has a distinct inner-cell mass (ICM) and trophectoderm (TE) which give rise to the embryo and extra-embryonic structures respectively. This loss of totipotency is an important milestone in development. Once mature, blastocysts will hatch out of the zona and rapidly expand. This is an important landmark that precedes implantation. If the maternal environment is receptive, the implantation process begins [39].

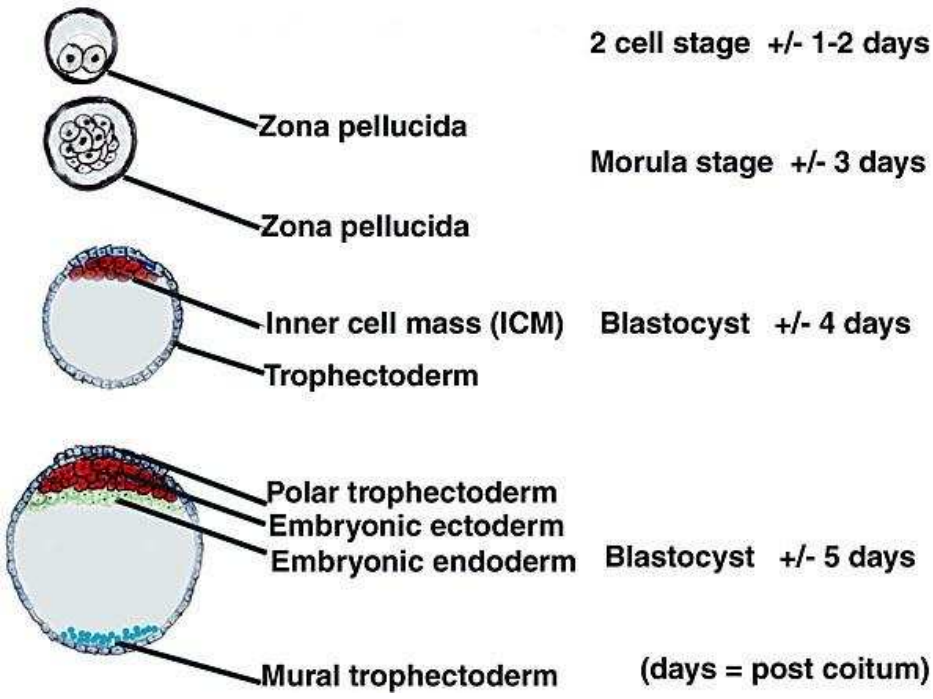


Figure 2. The Early Stages of Rat Embryo Development. [38] The developing embryo at the 2-cell stage (just after the first cleavage), the morula stage (up to 16-cells), the early and late blastocyst stages.

1.2.1 Implantation

1.2.1.1 The Attachment Phase

After a blastocyst has hatched out of its zona it begins the attachment phase of implantation [40]. Implantation in rodents occurs on the antimesometrial side of the uterus [38]. Within a few hours of the trophectoderm cells making contact with the uterine epithelium there is an increase in vascular permeability of the stromal cells underlying the conceptus [41]. Then, the intracellular matrix between uterine stromal cells changes as well as the stromal cell morphology [42]. This is known as the primary decidualization reaction [40]. The secondary decidualization reaction occurs 2-3 days later and involves similar processes over a much larger region of the endometrium as it prepares for placentation [43]. After the attachment phase is complete, the conceptus burrows itself into the endometrium; this process is known as penetration or invasion.

1.2.1.2 The Invasive Phase

The invasive stage of implantation occurs only in primates, rodentia and carnivora (cats, dogs, etc.) [40]. After the attachment phase, uterine epithelial cells are removed as giant trophoblasts engulf and digest them. The decidual tissue and uterine glandular tissue neighbouring this invasion site are also digested, releasing nutrients which are taken up by the conceptus [44, 45]. There is also rapid proliferation of trophectodermal cells into the spaces between uterine epithelial cells. These trophoblast cells will continue to divide and differentiate, becoming the “Träger”, a cluster of cells which are a precursor to the placenta. At the leading edge of the invasion, giant trophoblasts will continue to phagocytize decidual cells until an ingrowth of allantoic vessels and cells develops and becomes the placental labyrinth. As

more and more of the uterine cells are removed, the conceptus buries itself deeper into the uterine wall. Eventually it is almost completely encompassed on all sides. Then, alongside the uterine cells, the trophectoderm cells divide and differentiate into the extra embryonic tissues that will support gestation until birth.

1.2.2 Embryonic Genome Activation

Before implantation and placentation many key developmental milestones must be reached. One major developmental transition after fertilization is embryonic genome activation (EGA). Following fertilization the development of the embryo is directed by proteins and transcripts inherited from the oocyte. EGA gradually transitions the control of development away from maternally inherited proteins and transcripts to zygotic ones. EGA is also known as zygotic genome activation (ZGA) or the maternal to zygotic transition (MZT). This topic has been reviewed extensively by Schultz *et al.* [46, 47] and Minami *et al.* [48]. Most research regarding EGA has been done in mice; where EGA has occurs at the 2-cell stage [47]. Blocking RNA synthesis in mice embryos with the chemical agent α-amanitin results in normal cellular cleavages occur until the 4-cell stage and then development ceases, demonstrating that maternal products are controlling development for the first few cleavages [48]. Newly synthesized transcripts and the resultant proteins do not take full control until the maternal transcripts and proteins are exhausted [47].

There are three components of EGA. First, oocyte-specific mRNAs that are no longer necessary are destroyed [47]. This restricts the period of time in which maternal genes can be expressed and thus prevents expression of proteins which are no longer necessary. Secondly, maternally inherited transcripts that are still necessary for development are replaced with

zygotic transcripts from the newly created zygotic nucleus [46]. Lastly, new transcripts that are not expressed in the oocyte are also created at this time due to changes to the gene expression profile via the creation of the chromatin-based transcriptionally repressive state. This transcriptionally repressive state is set in place to block expression of nonessential genes and to enhance expression of genes important for further development of the embryo [47, 48].

Many thousands of genes are activated during EGA [49]. These include genes involved in: cell cycle signaling, protein synthesis, DNA replication, DNA repair, DNA recombination, control of expression for other genes, cellular assembly and organization, carbohydrate metabolism, protein degradation, lipid metabolism, and many others. Many genes involved in the transport of external substrates and nutrients are also transcribed at this time. Glucose transporters GLUT1, GLUT2 and GLUT3 are all active during this period [50]. The cationic amino acid transporter System Y+ (Slc7a3), the proton/peptide transporter Slc15a2, the monocarboxylic acid transporter Slc16a1, and many others are also actively transcribed at this time [51]. The zinc transporter Znt1 has been proven crucial to pre- and post-implantation development and is transcribed during EGA [52]. The transcript for the amino acid transporter LAT-1, which is responsible for transport of large neutral amino-acids, is present in the zygote (as a maternal transcript) but from EGA onwards LAT-1 transcription increases with the strongest expression during blastocyst hatching [53].

1.2.3 The Epigenetics of Early Development

Setting up the transcriptionally repressive state, mentioned above, is an alternate way of saying setting up the epigenetic control over the genome. The embryonic DNA must be configured such that most genes are silenced by a tight chromatin structure and a few select

genes are not. Prior to fertilization, both the male and female pronuclei are densely packaged for storage and are, for the most part, transcriptionally inert [47, 48]. Following fertilization the pronuclei are unpackaged and combined into a new zygotic genome. The chromatin of this newly created zygotic genome is then tightly configured to silence the majority of the genome. The male pronucleus is packaged with protamines rather than histones [48], which are rapidly degraded and replaced with new histones following fertilization. These histones are more highly acetylated than those in maternal chromatin, allowing transcription of some genes before the pronuclei are combined. Highly acetylated chromatin is transcriptionally active; thus early transcription of paternally inherited genes is greater than maternally inherited ones. However, until the late 1-cell stage, the majority of zygotic genes are silenced. Then, by removing the silencing on a select genes, development proceeds forward in a tightly organized manner, with one gene leading to expression of others, and those genes leading to expression of still others [47].

Genes can be silenced by many different mechanisms. CpG sequences on the genomic DNA itself can be methylated; causing a tighter chromatin structure and excluding the transcription machinery from accessing that gene, preventing transcription [54]. In addition to direct DNA methylation, there are proteins which are associated with genomic DNA, organizing it into bundles called nucleosomes. These proteins are known as histones, and they can be methylated, acetylated and/or phosphorylated to change the chromatin structure and either promote or discourage transcription [55]. Failure and/or improper reprogramming of the epigenetic machinery has been implicated in a broad range of diseases, including many cancers

[56], imprinting diseases [57] (discussed below), and diseases linked to suboptimal developmental conditions [58-60].

During mammalian development the methylation pattern of the genome is laid down in two distinct stages [60]. The first is a major DNA demethylation event which occurs during primordial germ cell differentiation. This event erases DNA methylation-based silencing on all imprinted and single copy genes. Proper methylation of all imprinted genes is then established. Then, after fertilization, the second phase occurs. This phase occurs during preimplantation development and it strips the methylation of all non-imprinted genes. The methylation of those genes is then reestablished [61] and multi-copy genes are silenced (for example those on the second X-chomosome in females) [60]. By the morula stage there is a low methylation status of the entire genome, and subsequent differentiation into one of the two possible cell types (TE or ICM) involves epigenetic reprogramming (including methylation) of the genome resulting in cell type specific expression [58]. For example, in the ICM *de novo* remethylation occurs due to preferential nuclear localization of DNA methyltransferase 3b (Dnmt3b) [58]. Most of the epigenetics research has been on DNA methylation; unfortunately most of the changes occurring at the histone level during these events remain unknown [58, 60].

1.2.3.1 Epigenetics and Fetal Programming

It has been demonstrated that during this burst of epigenetic activity the preimplantation embryo is sensitive to perturbations in its environment. This has been reviewed by Dolinoy *et al.* [62] and Burdge *et al.* [63]. This sensitivity can lead to changes in the epigenetic programming, altering the gene expression pattern during development. This altered

expression has been linked to various diseases and may be causing the phenotypes described in the DOHaD literature.

The epigenetics of a developing embryo can be altered *in vivo* by nutritional conditions. The viable yellow agouti (A^{vy}) mouse is a classic model for the epigenetic effects of nutrition. Normally transiently expressed in hair follicles during a specific stage of hair growth, *agouti* encodes a signaling molecule that causes yellow hair pigmentation when ectopically expressed in A^{vy} mice [64]. Maternal methyl supplementation from extra folic acid, Vitamin B_{12}, choline and betaine causes hypermethylation of the *agouti* gene, silencing the ectopic expression of *agouti* and restoring a normal brown phenotype in offspring [62]. Hypomethylation of the *agouti* gene causes the yellow phenotype.

Manipulation of the macromolecule concentrations in the maternal diet can cause epigenetic programming in developing fetuses. For example, a maternal high-fat diet caused acetylation of fetal hepatic histone 3 in Japanese macaque offspring and a 40% change in transcription of glutamic pyruvate transaminase 2 (GPT2), Hsp70 co-chaperone DNAJA2 and retinol dehydrogenasse 12 (Rdh12) [65]. A protein restricted diet induces hypomethylation of the peroxisomal proliferator-activated receptor-alpha promoter region (PPARα) and glucocorticoid receptor (GR) promoter region causing increased transcription of these genes in rat offspring livers [66] and this shift in the metabolic phenotype of the offspring has been linked to hypertension [63]. In addition, the MLP diet causes hypomethylation of the promoter region of the angiotensin II receptor (AT_{1b}) gene in offspring adrenal glands and suggests a direct link between epigenetic modifications due to *in utero* nutritional conditions and

hypertension [67]. Therefore, changes in the concentrations of either key nutrients or entire macromolecule classes during pregnancy can cause epigenetic changes in developing individuals leading to disease-prone reprogrammed adult phenotypes.

In vitro fertilization (IVF) research has provided more insights into the epigenetic reprogramming of embryos that can occur during early development. IVF studies in sheep and cows suggest the prevalence of abnormal placental growth and perinatal mortality are higher in IVF embryos than in naturally fertilized embryos [58]. This work lead to studies using early bovine and sheep embryos which showed that gene expression is altered due to perturbations in the nutritional content of the media, including addition of fetal calf serum, resulting in disruptions in organ and skeletal development [58]. Culture oxygen concentrations are also important, reducing them during *in vitro* culturing of sheep embryos caused an increased proportion of ICM cells and altered gene expression of GLUT1, a transmembrane glucose transporter [68]. Techniques such as intracytoplasmic sperm injection and round spermatid injection can induce aberrant DNA methylation patterns in rabbit and mouse blastocysts [60]. A study by Li *et al.* has shown that the IVF procedure and certain culture media can cause abnormal DNA methylation and histone methylation, resulting in abnormal imprinting [69]. While highly controversial, epidemiological evidence in humans suggests than some of the artificial reproductive technologies may be associated with an increased risk of epigenetic disorders resulting in low birth weight, imprinting related diseases (more on imprinted related diseases below in 3.3.2) and congenital malformations [58].

1.2.3.2 Imprinted Genes and Fetal Programming

Imprinted genes are a small but very important subset of genes whose alleles have been shown to be preferentially silenced based on their parent of origin. They have only been described in metatherians (marsupials) [70], eutherians (placental mammals) [71] and angiosperms (flowering plants) [72]. Many imprinted genes modulate fetal growth and development and can influence placental function [73]. Paternally expressed imprinted genes increase the transfer of nutrients to the fetus, promoting growth, and maternally expressed imprinted genes decrease nutrient transfer to the fetus, conserving maternal resources for future offspring [74]. Imprinted genes appear to be more sensitive to the environmental conditions encountered *in utero* and have been implicated in disease susceptibility [75, 76]. Genomic imprinting is an important part of the balancing act that is fetal growth regulation. The fetus must be afforded every chance to reach its genetic potential, but the mother's resources must not be depleted beyond her ability to recuperate [74]. A change in imprinted gene expression could affect placental function and could therefore alter fetal growth and cause fetal programming.

There are two well described imprinted genes, *H19* and Insulin-like growth factor 2 (*Igf2*). *H19* is a long non-coding RNA paternally methylated in normal imprinting; meaning the maternal copy of the allele is expressed and the paternal copy is silenced. *Igf2* is a growth factor similar in structure to insulin and is maternally silenced in normal imprinting [77]. *H19* has been linked to the suppression of *Igf2* expression [78] and deletion of the *H19* allele causes offspring to be about 27% heavier than wild-type [78]. Much of the research into imprinted genes centers on pathologies that occur when both alleles of an imprinted gene are active,

known as a “loss-of-imprinting”, wherein both the paternal and maternal allele are expressed [58-60]. Both *Igf2* and *H19* have been implicated in Beckwith-Wiedemann syndrome, an over-growth disorder associated with a high risk of cancer [79].

Maternal protein restriction during early development can cause changes in the expression of imprinted genes. In particular, Kwong *et al.* have identified changes in *H19* and *Igf2* when dams were exposed to a low protein diet during the first 4 days of gestation [22]. They found that male offspring had a 30% reduction in *H19* mRNA at day 4 of gestation. They also found that exposure to the maternal low protein diet for the first 4 days of gestation followed by a normal diet caused male day 20 fetal *H19* and *Igf2* mRNA levels to be 10% lower than normal. To investigate the methylation status of *H19*, fetal liver DNA was examined. No changes at the differentially methylated region, described by the *H19* imprinting literature, were noted, suggesting the imprinting of *H19* was unaltered. However, no other DNA CpG regions and histone modifications were investigated [22]. Changes in expression of these genes is thought to be responsible for the observations by the same group that a maternal low protein diet during preimplantation gestation programs the offspring for hypertension [21, 22]. *In vitro* studies have shown that culture media can also affect changes in imprinted gene expression [58-60].

1.3 Amino Acids, Their Transporters and Amino Acid Signaling

1.3.1 Amino Acids

There are nine essential amino acids required by human adults: histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine [80]. These amino acids cannot be synthesized in the body and must be consumed in the form of protein, which is chemically and enzymatically broken down in the stomach and intestine into individual amino acids and then absorbed by the small intestine. Beyond the essential amino acids required for adults, certain amino acids are conditionally essential, i.e. during fetal development and in prepubescent children. Prepubescent children need cysteine, taurine, tyrosine and arginine in their diets because they have not reached full metabolic capacity to synthesize these amino acids from the essential amino acids [81]. During fetal development cysteine, glutamine, proline and arginine are all required for proper organ development, particularly during neurulation [82].

1.3.2 Amino Acids Regulating Physiological and Developmental Processes

Beyond their function as simple nutrients required to assemble new proteins, amino acids are also known regulators of many physiological processes. For example, when leucine or tryptophan is absorbed they act as a satiety signal to the brain, lowering the desire to find and consume food [83]. Amino acids also act as nutrient availability signaling molecules. Plasma amino acid concentrations, particularly leucine concentrations, regulate global protein synthesis rates and keep those rates in step with amino acid availability [84]. For example, amino acid deprivation increases the phosphorylation of eukaryotic initiation factor 2α, a major

component of the cell's protein synthesis machinery, slowing protein synthesis rates [82]. Conversely, after plasma leucine levels rise (post-feeding), the mammalian target of rapamycin (mTOR) signaling pathway responds and phosphorylates its main downstream targets: eukaryotic initiation factor 4 binding protein 1 (4E-BP1; when unphosphorylated it binds and prevents eukaryotic initiation factor 4 from functioning, slowing protein synthesis) and p70 ribosomal S6 kinase 1 (p70S6K1; a protein kinase that increases protein synthesis rates when it is phosphorylated) [85].

Concentrations of the individual amino acids have been implicated as regulators of preimplantation development and implantation [86]. With glutamine supplementation the percentage of embryos in culture that made it to the blastocyst stage, blastocyst cell number, percentage of successful hatching blastocyst where all significantly higher [87]. Studies using minimal culture media to demonstrate that addition of certain non-essential amino acids (such as glutamine and taurine) can drastically increase blastocyst yield, the yield of hatching blastocysts and successful establishment of pregnancy of embryos upon introducing them into a receptive female [88]. A reduced dead cell count and increased blastocyst cell numbers were observed when both essential and non-essential amino acids were added to culture media, suggesting that they promote development in human embryos [89]. Finally, amino acids have been shown to regulate mouse trophectoderm differentiation, possibly part of the regulation pathway for the blastocyst adhesion phase and/or motility (part of the invasion phase) [86, 90]. Integral to understanding how amino acids can act as regulators is the means by which they are moved into (and out of) cells, a process mediated by membrane spanning amino acid transporters.

1.3.3 Amino Acid Transporter Nomenclature

All membrane spanning substrate transporters, including all amino acid transporters, have been classified into solute carrier families based on their sequence homology. Sequences with > 25% amino acid sequence identity have been grouped into the same transporter family [91]. Currently there are 298 transporters grouped into 43 families [91]. A nomenclature scheme was proposed which uses a superscript addition operator (+) to indicate preference for cationic forms of amino acids, and a superscript subtraction operator (-) to indicate preference of the system for the anionic form of an amino acid, and a superscript 0 to indicate preference for neutral amino acids [92, 93]. Subscript letters ($_{A, G, C}$) are used to indicate preference for individual amino acids. Finally, names are capitalized (i.e. ATB instead of atb) to indicate the transporter is sodium dependent [94]. For example, System $B^{0,+}$ is a sodium-dependent and chloride dependent neutral and cationic amino acid transporter.

System $B^{0,+}$ was originally described by its activity and later the protein responsible for this activity was discovered and named $ATB^{0,+}$. $ATB^{0,+}$ is of a coded for by the gene *Slc6a14*. *Slc6a14* is an abbreviation designating this gene as member 14 of solute carrier family 6, which are the sodium and chloride ion dependent transporters [95].

1.3.4 Expression and Regulation of Amino Acid Transporters

Amino acid transporters are expressed on the plasma membrane of every cell and in the membranes of various organelles within cells (notably the Golgi apparatus, mitochondria and the chloroplasts of plant cells) [91]. They are membrane-spanning proteins in that they have hydrophobic transmembrane alpha helices which connect the inner and extra-membrane

regions of the transporter protein. A large proportion of amino acid transporters are expressed in the small intestine, the main absorptive surface in most species, and they have been reviewed by Broer *et al.* [96]. Most amino acids are moved into the body in the small intestine by sodium-dependent amino acid transporters. Sodium-dependent amino acid transporters bind sodium ions and amino acids and then move them across the basolateral surface of the epithelial cells which line the intestine using the electrochemical gradient of sodium across the epithelium [96]. Therefore, their activity can also be altered by a shift in the extracellular concentration of the co-transported ion (*i.e*. sodium). In addition, their activity can be altered by accessory protein binding. Amino acid transporter expression can also be regulated at many levels, such as the transcription level, post-transcription level, translation level and post-translation level. Furthermore, the transporter protein must be moved to and integrated into the cell membrane before it is active.

1.3.5 Amino Acid Transporter Expression Changes during Development

Intrauterine growth restriction (IUGR) is a condition characterized by the embryo failing to reach its maximum growth potential [97]. IUGR babies are born underweight and can suffer from many peri-natal, post-natal disorders and late onset adult diseases like those described by the DOHaD hypothesis [98]. IUGR can be induced in rats using a maternal low protein diet [99].

Changes in amino acid transporter expression and activity have been linked to IUGR. When rats were fed a low protein diet throughout pregnancy both the activities and expression of System A and System X^-_{ag} fell [100], and in particular, placental uptake of lysine (a preferred substrate of System A) was reduced by about 40% across both sides of the syncitium [101]. In

rats fed a low protein diet down regulation of some amino acid transporters in the placenta actually *precedes* the onset of IUGR. In particular, the System A isoform *Slc38a2* had transcription levels that were only 20% of normal at gestational days 19 and 21, before IUGR was detectable [98]. Chemical inhibition of System A and System X^-_{ag} in pregnant rats also causes IUGR [103]. A human IUGR study found that expression of the amino acid transporter TAUT is reduced in full term IUGR placenta [97]. In contrast, a study using material from human pregnancies complicated by Type I Diabetes (i.e. large for gestational age fetuses) found that System A transport was increased in that tissue [102]. The decrease in expression and activity of these amino acid transporters caused by maternal protein restriction would lead to a lowered intracellular level of their substrate amino acids which may cause developmental reprogramming. Therefore, it is conceivable that a similar shift in amino acid transporter expression rates could be occurring even earlier, in the preimplantation embryo, from dams fed a protein restricted diet, slowing growth and leading to disease-prone adult phenotypes.

Placental amino acid transporters are carefully regulated to balance the maternal supply with the fetal demand for nutrients [104]. When this balance is upset, fetal growth rates respond, leading to increased or decreased fetal weights, and then, much later, adulthood diseases [6]. In mice lacking the placental *Igf2* promoter (*Igf2* $P0^{+/-}$) only placental (not fetal) *Igf2* expression is eliminated. Placental growth in these mice was greatly reduced compared to wild type mice. However, fetal growth is normal until late gestation, suggesting the placenta was adapting at a functional level to increase nutrient transfer and maintain fetal growth rates [105]. A follow up study found that, in these *Igf2* $P0^{+/-}$ mice, fetal nutrient demand exceeded maternal supply and the placenta responded to protect the fetus by upregulating transcription

of the System A isoform *Slc38a4* [106]. When both placental and fetal *Igf2* activity was completely abolished in a knockout model, this upregulation effect vanished [106], suggesting fetal *Igf2* is directing the placenta to upregulate transcription of System A (and likely other nutrient transporters) to maintain or increase fetal growth rates. Therefore, it is logical to look for changes in amino acid transporter expression in response to maternal nutrient restriction in other DOHaD research models.

1.4 System $B^{0,+}$

System $B^{0,+}$ transports 18 of the 20 amino acids used by the body to construct proteins and has the ability to concentrate substrates up to 1000-fold [107]. It is expressed in a few pharmacologically relevant tissues (such as the lungs and kidneys) and is being evaluated as a possible drug delivery route [108]. In addition, it has been identified as one of the amino acid transporters upregulated in colon cancer [109] and cervical cancer [110] and is possibly upregulated in many other cancer types [111]. These features make it a potential transporter for new cancer chemotherapy drugs [111]. System $B^{0,+}$ has recently been implicated in mercury poisoning as it readily transports environmentally common mercury-conjugates into the kidneys [112]. Its gene, *Slc6a14*, has also been linked to obesity; a comparative genomics study of over 1700 Finnish and Swedish individuals showed significant differences in the allele frequencies in individuals from the obese group when compared to those from the control group [113]. They theorize that varying *Slc6a14* alleles may code for transporter proteins that differ slightly in their rate of tryptophan transport, affecting tryptophan's availability for serotonin synthesis and therefore affecting appetite control. Gestational regulation of amino acid transport across the placenta is extremely important for proper fetal growth and development and $ATB^{0,+}$ has been found on the maternal-facing microvillous surface of the placenta from day 14 onwards in rat gestation [114].

1.4.1 Regulation of System $B^{0,+}$ Activity and Expression

There are no known accessory proteins or other ligands associated with System $B^{0,+}$ regulation and very little is known about its regulation in general. Hypothetically, an increase in extra-cellular sodium levels would stimulate an increase in sodium uptake (and therefore amino

acid uptake) by System $B^{0,+}$ [115]. $ATB^{0,+}$ can be regulated at the transcriptional level. In colorectal cancer cells isolated from patients $ATB^{0,+}$ mRNA levels were found to be 23-fold higher than healthy colon cells from the same patients [109]. In a human intestinal epithelial cell line, CaCo-2, treatment with cholera toxin causes a 2-fold increase in $ATB^{0,+}$ mRNA levels [116]. Cervical carcinoma cells show a 5.6-fold increase in $ATB^{0,+}$ mRNA levels when compared to normal cervical cells [110]. Taylor et. al. found that amino acid supplementation causes adaptive regulation (both up & down regulation) of $ATB^{0,+}$ expressed in a *Xenopus laevis* oocyte system and that cycloheximide (which inhibits translation) and actinomycin D (which inhibits transcription) inhibit this adaptive regulation response [117]. All of the above suggest that System $B^{0,+}$ can be regulated at the transcription level.

1.4.2 System $B^{0,+}$ and Early Development

It is well established that the developing embryo is sensitive to its environment and there is evidence that blastocyst trophectoderm cells will not become motile *in vitro* without adequate amino acids present in the surrounding environment [86, 118, 119]. However, the link between amino acid signaling and trophoblast motility/invasion remains unclear. Martin *et al.* found that trophoblast motility is dependent on the availability of exogenous amino acids, which signal trophectoderm differentiation and the gain of motility about 20 hours before implantation [86, 90]. System $B^{0,+}$ has been linked to this amino acid uptake by Van Winkle *et al.* [120]. He theorized that preimplantation and periimplantation blastocysts are 'poised' to take up leucine; when placed in media which is rich in amino acids they rapidly accumulated leucine, more than even their *in vivo* counterparts [120]. There is evidence to suggest that leucine and isoleucine in particular are rapidly brought into the pre-motile cells and this signals

the cells to start synthesizing the proteins necessary for normal motility and uterine wall penetration [86].

Rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR) nutrient sensing pathway, blocks development of blastocyst motility which is thought to control translation of proteins necessary for motility and invasion [90]. Rapamycin, a macrolide, acts as an immunosuppressant and an antibiotic and suppresses mTOR activity [121, 122]. Leucine is a known stimulator of the mTOR pathway [85, 123]. Disruption of the mTOR gene is an embryonic lethal mutation which is lethal just after implantation [124, 125]. It appears that without mTOR signaling the trophectodermal cells are unable to proliferate further after implantation, and furthermore, the embryonic stem cells (a.k.a. the inner cellular mass) fail to proliferate beyond implantation [124, 125]. This is in consistent with Martin *et al.*'s *in vitro* observations that both rapamycin (a known inhibitor of mTOR) and leucine deprivation prevent development of motility and outgrowth of embryos [86, 90, 118, 119]. Interestingly, in the pig, a mammalian species that does not exhibit penetrative implantation [126], system $B^{0,+}$ is not expressed during implantation [127]. This suggests that species that do not utilize penetrative implantation would not be sensitive to leucine deprivation during preimplantation development in same way that species using penetrative implantation are.

1.5 Rationale

The mechanisms underlying the DOHaD hypothesis remain largely unknown. Epigenetics have been implicated, but how the extracellular environment prompts epigenetic changes remains unclear. The nutrient sensitive mTOR pathway controls translation of approximately 52% of the human proteome and is likely involved in the translation of enzymes which change the epigenetic status of the cell [121]. It has been shown that System $B^{0,+}$ shows a preference for tryptophan and leucine -- upstream signalers of the mTOR pathway [128]. It is possible that mTOR could respond to changes in leucine concentration brought about by changes in System $B^{0,+}$ activity and/or expression. Downstream effects of this response could lead to long term physiological changes in the developing organism. Therefore, investigating System $B^{0,+}$ expression during preimplantation development could help to explain the physiological changes observed in DOHaD research. To that end, this study aims to investigate the effects of a maternal low protein diet on preimplantation rat blastocyst System $B^{0,+}$ expression.

2. Materials and Methods

2.1 Animals

Virgin female Wistar rats (n=14) were maintained in a temperature-controlled room with a 12h light/dark cycle. The colony was kept in the University of Waterloo Biology Animal Care Facility. This room had time-controlled lighting which starts the dark cycke at 3pm. The colony's food was administered twice a week and was never allowed to become depleted. At approximately 6 months of age females were mated overnight with males of the same strain. After a vaginal plug was detected the male was removed and the female was randomly assigned to a diet group; either the 9% casein (low protein diet) or a 18% casein (control diet). The low protein diet was selected based on previous research by Kwong *et. al.* [21]. The diets were provided by Harlan Teklad (Harlan Laboratories; Indianapolis, IN., U.S.A.) and their respective compositions are detailed in table 1.

Table 1. Comparison of the 18% and 9% Casein-based diets.

Diet Details	Control	Low Protein
Casein (g/Kg)	180.0	90.0
DL-Methionine (g/Kg)	5.0	5.0
Sucrose (g/Kg)	198.0	228.0
Corn Starch (g/Kg)	325.0	385.0
Maltodextrin (g/Kg)	95.0	95.0
Corn Oil (g/Kg)	100.0	100.0
Cellulose (g/Kg)	50.0	50.0
Mineral Mix (AIN-76) (g/Kg)	35.0	35.0
Vitamin Mix (AIN-76A) (g/Kg)	10.0	10.0
Choline Bitatrate (g/Kg)	2.0	2.0

After being assigned to a diet group the date was recorded and noted as "Day 0" of gestation, setting the start time of gestation (conception) at approximately 4am that day [129], due to their night starting at 3pm the prior day. At day 4.25 (4d, 6h), 10am on day 4 of gestation, the animals were euthanized for blastocyst collection. Both the dam's weight and remaining food weight was recorded at this time. This gestational age was selected for the following reasons: there is elevated System $B^{0,+}$ activity in mice embryos at this stage [120], evidence suggests that System $B^{0,+}$ mRNA expression peaks in mouse preimplantation embryos at this stage [86], after ~4.25 days rat blastocysts quickly hatch and begin attaching to the uterus; making them extremely difficult to retrieve [130] and previous DOHaD research suggesting exposure to a protein restricted diet for this precisely this length of time is enough to cause an altered phenotype [21, 131].

2.2 Blastocyst Collection

Blastocysts were collected from the euthanized animals using a modified version of an established protocol [132]. First, the animal was opened and the uterus exposed. Then a cotton swab dipped in 0.9% saline was used to clean the vagina. A polyurethane tube (5mm inner

diameter, 10cm in length) was inserted into the vagina. 400 µl of phosphate buffered saline (PBS, 0.01M Na_2HPO_4, 0.0027M KCl and 0.137M NaCl, pH = 7.4) containing 3 mgl/ml bovine serum albumin (BSA, Sigma-Aldrich Canada Ltd., Oakville, Ontario), was then injected into each uterine horn using a 25 gauge, half-inch long needle. The needle was inserted into the lumen of each uterine horn pointed upwards towards the oviduct so that the entire contents of the uterine horn could be flushed down and out into the tubing. The flushing medium was then collected in a customized embryo collection dish (5cm Plexiglas doweling, sliced into a 8mm thick plate, with a convex 4mm divot in the center; created by Mr. Harmen Vander Heide, Science Technical Services) and then examined under a dissection microscope (Nikon SMB-2B). The photograph below shows an animal prepared for blastocyst collection.

Figure 3. Photograph of the blastocyst collection method. The embryo collection dish (bottom-left) is placed under the tubing to catch the flushing medium, which is injected into each uterine horn.

The blastocysts were quickly collected using the dissection microscope with a 10µl micropipette and removed from the flush media. They were then passed through a series of four 25µl droplets of washing solutions; two droplets of phosphate buffered saline (as above) containing 4mg/ml of polyvinyl pyrrolidione (PVP, Sigma-Aldrich Canada Ltd., Oakville, Ontario) and two clean droplets of flushing media. The polyvinyl pyrrolidione was used to reduced blastocyst stickiness, so that they could be separated and analyzed individually [133]. After washing, the individual blastocysts were then immediately passed through a nucleic acid recovery kit.

2.3 DNA and RNA Recovery

DNA and RNA recovery from the blastocysts was then carried out using the Norgen 3-in-1 DNA/RNA/Protein extraction kit. (Norgen Biotek Corp.; Ontario, Canada) This kit uses spin-columns with a proprietary membrane designed to bind nucleic acids. However, the normal kit protocols were modified to extract RNA and DNA from extremely small samples. First, the blastocyst was lysed in 300µl (reduced from 600µl) lysis buffer containing 3µl β-mercaptolethanol. This was done using a vortex mixer set on high for 60 seconds. Then 300µl of 95% ethanol (up from 75%) was added to this mixture, which is passed through the spin-column using a microcentrifuge. Then, two RNA wash buffer steps are performed to clean the column. Finally, the RNA is eluted using RNA elution buffer. The genomic DNA (gDNA), still on the column, is cleaned with a wash buffer and then eluted using an elution buffer. The gDNA was frozen at -20°C and the RNA was placed on ice to be immediately reverse-transcribed to cDNA.

2.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The RNA was reverse transcribed to cDNA using the Qiagen Quantitect Reverse Transcription Kit (Qiagen; Germantown, Md., U.S.A.) following the kit instructions. cDNA synthesized from blastocysts was then amplified with PCR using established rodent $ATB^{0,+}$ and Hprt primers to verify that RNA recovery and cDNA synthesis steps were successful. Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) is a metabolic enzyme with a low basal expression rate in all tissues and was selected as a reference gene to use to evaluate $ATB^{0,+}$ expression. The Qiagen HotStarTaq Plus Kit (Qiagen; Germantown, Md. U.S.A.) was used to prepare a standard 20µl mastermix: 1U Taq, 2µl 10x PCR Buffer, 10.6µl water, 0.4µl dNTPs, 2µl (0.5µM) of each primer pair member and 2µl of blastocyst cDNA. This was loaded into a thermocycler (GeneAmp PCR 9700, Applied Biosystems; Carlsbad, Ca. U.S.A.) and run through the following program: one 5 minute activation step at 95°C, followed by 45 standard cycles (45 seconds at 94°C, 45 seconds at 58°C and 1 minute at 72°C) plus a final extension time of 10 minutes at 72°C. Below are the primer sequences used for both cDNA verification (using RT-PCR) and then again for assessing $ATB^{0,+}$ expression (using quantitative real-time polymerase chain reaction; qRT-PCR).

Table 2. Primers used for both PCR and subsequent qRT-PCR cDNA evaluation.

Target Gene	Upstream Primer	Downstream Primer	Product Size	Ref
$ATB^{0,+}$	5'-ACTTTGCCTTCTTCTAGCTT-3'	5'-TGAGAATCCAGACCCAATGT-3'	551bp	[134]
Hprt	5'-GCTGAAGATTTGGAAAAGGTG-3'	5'-AATCCAGCAGGTCAGCAAAG -3'	157bp	[135]

2.5 Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

After the cDNA had been verified as outlined above it was analyzed with qRT-PCR using Qiagen's QuantiTect SYBR Green PCR Kit (Qiagen; Germantown, Md. U.S.A.) and the University of Guelph's Core-lab facility Roche Lightcycler 3 (Roche Ltd.; Basel, Switzerland) qRT-PCR thermocycler. The same primers used above in cDNA verification were used to evaluate $ATB^{0,+}$ expression using *Hprt* as the reference gene. A mastermix was created using 12µl Qiagen SYBR Green mastermix, 1µl (final working concentration of 1.5µM) of magnesium-chloride (MgCl), 2µl (0.5µM) of each primer pair member, 1µl water and 2µl of blastocyst cDNA. Each sample was amplified in duplicate using both sets of primers. Afterwards, melting curves were acquired by incrementally increasing the temperature from 55°C to 95°C to ensure that a single product was amplified in the reaction. A dilution series of PCR product was created by amplifying pooled embryo cDNA and then diluting the resulting product down to 10^{-10} in ten-fold increments. Then, a co-efficient file containing cycle number and cross-over points for each dilution in the dilution series was created using Roche's RelQuant (Roche Ltd.; Basel, Switzerland) software and applied to all generated data to normalize between runs to control for minute inter-run variances in mastermix volumes and concentrations. To ensure that the qRT-PCR amplification is in the linear range and the correct cross-over points are identified, the LightCycler 3 software (Roche Ltd.; Basel, Switzerland) automatically defines the threshold fluorescence value by assessing the second derivative maximum of each amplification curve to calculate the initial gene copy number in the sample. The resulting calculated values are a ratio of the cross-over points (where fluorescence exceeded the threshold value, which is determined automatically by the software) of $ATB^{0,+}$ to *Hprt*.

2.6 Blastocyst Gender Determination using Nested PCR

Nested PCR with primers for the *Sry*, *Zfy* and *Hprt* genes were used to determine blastocyst gender. It uses two sets of primers in two successive PCR runs with the second primer set, the "inner primers", intended to amplify a target sequence within the sequence produced in the first PCR reaction using the "outer primers". The detailed method was published by Fleming *et al.* in 2002 [136]. The *Sry (*Sex determining Region of the Y-Chromosome) and *Zfy* (male germ cell development) genes are found only in males, but there may be similar sequences on other chromosomes. This is because both *Sry* and *Zfy* are high mobility group (HMG) transcription factors and therefore share a great deal of sequence identity with other transcription factors in the same family [137]. Nested PCR eliminates false positives from these similar sequences since any mis-primed product formed in the first round of amplification should not amplify in the second round of amplification. The third primer set, Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), is used as a positive control for the presence of genomic DNA in a sample. In a female sample only *Hprt* product will amplify, since *Zfy* and *Sry* are not present in female samples. Nested PCR also increases PCR sensitivity one thousand fold and has a proven track record of sexing preimplantation embryos [138].

Genomic DNA recovered from the blastocysts was extremely diluted in 100µl of the elution buffer used during extraction. This was concentrated down to 20µl using a microcentrifuge concentrator set on high for 20 minutes. Then, a 50µl outer PCR reaction mixture was prepared. It was comprised of: 20µl of the concentrated genomic DNA sample, 2.5µl (2.5U) of Taq polymerase, 1µl dNTPs, 3.3µl(0.5µM) of each of the 6 outer primers, 5µl of 10x PCR Buffer and 1.5µl of water. The nested PCR program conditions used were as follows: 30

cycles of 94°C for 1min, an annealing step at 60°C for 2.5min and an extension step at 72°C for 2.5min. A final extension time of 10 minutes at 72°C was performed afterwards. Product from the outer primers was then used as a template for the second, or "inner", PCR reaction. Two separate 20µl inner PCR reaction mixtures were created; one with the *Hprt* and *Zfy* inner primers, and another with the *Sry* inner primers. They were comprised of: 1ul (1U) of Taq polymerase, 2µl 10x PCR Buffer, 0.4µl dNTPs and 2µl of each inner primer pair member (working concentration of 0.5µM), 2µl of outer PCR product DNA (as the template material) and water to top the reaction mixture up to 20µl. The 2 resultant PCR reaction products from the second round of amplification are then run out on a 1.5% agarose gel. If *Zfy* and *Sry* bands were detected, the sample was scored as a male. If only *Hprt* was detected, the sample was scored as a female. Table 3 lists the sequences for all 12 (6 outer and 6 inner) primers used for gender determination.

Table 3. Primers to be used for nested PCR gender determination.

Gene	Upstream Primer	Downstream Primer	Product Size
Sry Outer	5'-CACAAGTTGGCTCAACAGAATC-3'	5'-AGCTCTACTCCAGTCTTGTCCG-3'	300
Zfy Outer	5'-AAGATAAGCTTGCATAATCACATGGA-3'	5'-CCTATGAAACCCTTTGCTGCACATGT-3'	611
Hprt Outer	5'-GTTCTCTTCAATTGCTGGTCCA-3'	5'-TGACAACGATTCACACTGCTGA-3'	618
Sry Inner	5'-AGCATGCAGAATTCAGAGAT-3'	5'-ATAGTGTGTAGGTTGTTGTCC-3'	248
Zfy Inner	5'-GGAAGCATCTTTCTCATGCTGG-3'	5'-TTTTGAGCTCTGATGGGTGACGG-3'	207
Hprt Inner	5'-ATGCTGGTGTTGTCTCTTCAGA-3'	5'-ATCTGTCTGTCTCACAAGGGAA-3'	318

3. Results

3.1 Dam Weight Gain and Food Consumption during Early Pregnancy

Dams were weighed after vaginal plug detection (successful mating) and again at euthanasia just prior to embryo collection. During this time they were given *ad libitum* access to their assigned diet. Upon euthanasia the total food consumption of each dam was recorded. There was not a statistically significant difference in the weight gain or food consumption between the two diet groups.

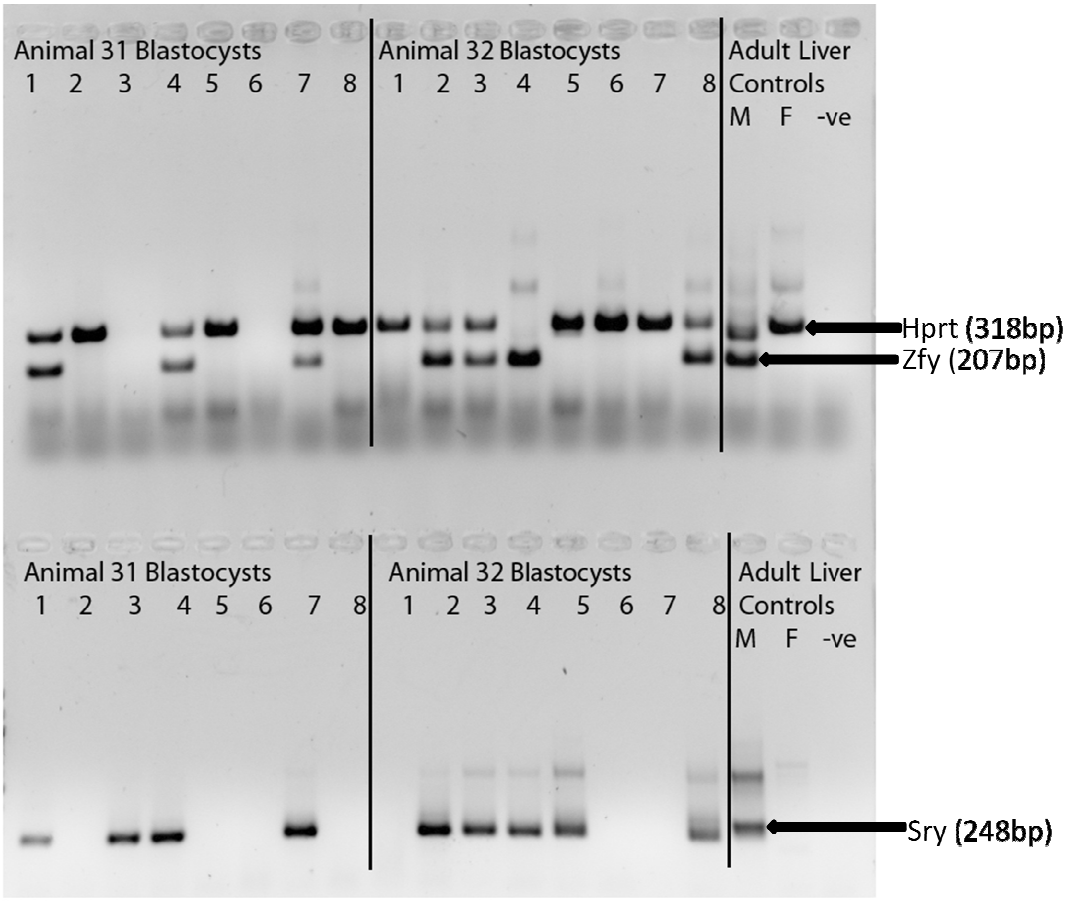
Table 4. Dam Weight Gain and Food Consumption during Early Pregnancy. The two diet groups showed no difference in maternal weight gain or food consumption during early pregnancy. Mean dam weight gain was 16.880 ± 2.376g, mean food consumption was 90.543 ± 5.094g.

	MLP Diet (g) (9% Casein, n = 7)	Control Diet (g) (18% Casein, n= 7)	Difference (g)
Dam Weight Gain During Early Pregnancy (Mean ± SEM)	19.3 ± 2.941	14.4 ± 3.716	4.9 ($p > 0.05$)
Dam Food Consumption During Early Pregnancy (Mean ± SEM)	97.6 ± 6.139	83.5 ± 7.626	14.1 ($p > 0.05$)
Age of Dams at Conception	6.6 ± 0.769 Months	6.3 ± 0.884 Months	-

3.2 Gender Determination of the Recovered Embryos

Using the outlined nested PCR protocol each blastocyst was assessed with male-specific primers targeting X-Chromosome (male) genes. Figure 4 is an image of an electrophoresis gel showing the resultant PCR product. Hprt and Zfy PCR products are different sizes (318bp and 207bp, respectively) and if both bands are present, the sample is male. If only Hprt is present, the sample is female. This is demonstrated in the upper half of figure 4. In addition to the Hprt

and Zfy primers, Sry-specific primers were used in a separate reaction mixture to confirm a sample's gender. The Sry product (expected size of 248bp) is shown in the bottom portion of figure 4. A Zfy product should have corresponding Sry product in the lower part of the gel, confirming a male sample. 110 samples were analyzed this way and 108 were assigned a gender using this technique; 58 males and 50 females. The remaining two samples did not amplify with any primer sets; this probably indicates that no genomic DNA was recovered. In 21 of these samples either *Hprt* amplification or *Zfy* amplification (done in the same reaction mixture) out-competed the other and only one product band was visible on the agarose gel; these samples were flagged to be rerun using two separate PCR reaction mixtures, one for each primer set, to correctly score the sample as male or female.



Upper Half - Hprt (318bp) and Zfy (207bp) double bands indicate male samples.
Lower Half - Sry (248bp) bands indicate male samples.
Ambiguous Samples were flagged to be rerun.

Figure 4. Blastocyst gender determination (Nested PCR) representative results separated on a 1.5% Agarose Gel.

Upper half: Each embryo was assessed for the male-specific *Zfy* gene and the *Hprt* gene. *Hprt* serves as an internal positive control to indicate gDNA was present. A double band indicates a male sample; a single band indicates a female sample.
Lower half: The same embryo was assessed for the male-specific *Sry* gene to verify blastocyst gender.
Notes: Ambiguous samples were flagged to be rerun. Shadow bands above expected product size are likely carry over Nested-Outer PCR product or mis-primed product formed from primers annealing to other transcription factors with similar sequences.

3.3 Relative Expression Rates of ATB$^{0,+}$ mRNA to Hprt mRNA

The blastocysts from the MLP diet group showed no significant difference in expression levels of ATB$^{0,+}$ (relative to Hprt expression) when compared those from the control diet group. This data was then separated into male and female data sets to analyze it for gender specific differences in expression. Both the male and female data sets showed no significant differences in expression. Therefore the MLP diet has no effect on blastocyst expression levels of ATB$^{0,+}$ when compared to the control diet. (See Figure 5)

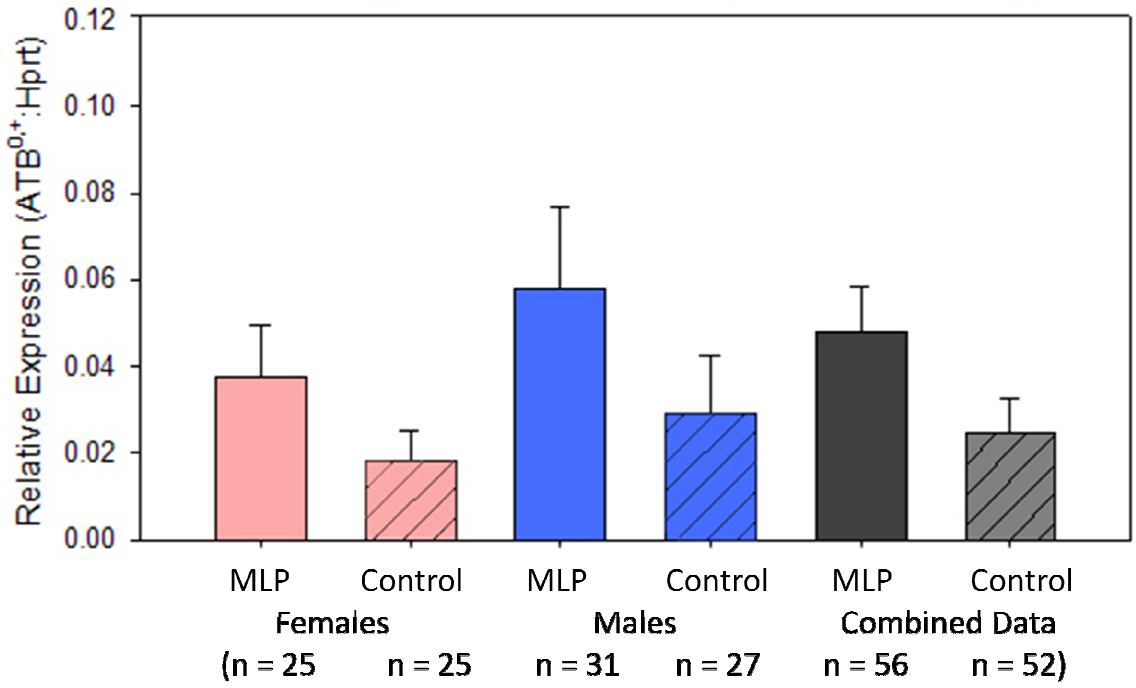


Figure 5. Relative ATB$^{0,+}$:Hprt Expression Rate in rat blastocysts from dams fed either a protein restricted or normal diet. Data has been separated by blastocyst gender to examine it for gender-related effects. Blastocysts from the MLP diet fed dams compared to those from the control diet fed dams did not show any statistical difference in ATB$^{0,+}$ expression rate. Mean relative expression of *ATB$^{0,+}$:Hprt* was statistically analyzed for significance using a two-tailed t-test. ($p > 0.05$ for all groups)

4. Discussion

A protein restricted maternal diet had no significant effect on $ATB^{0,+}$ transcription relative to Hprt in rat preimplantation blastocysts. This finding suggests that changes in the expression of the amino acid transporter System $B^{0,+}$ may not be responsible for the developmental reprogramming observed by many researchers, including Kwong *et al.* [21, 131] and Langley-Evans *et al.* [6].

Although the findings were not statistically significant, there does appear to be a trend in the data. From the graph it appears that there is an upregulation of $ATB^{0,+}$ transcription in the blastocysts exposed to the MLP in all groupings. This trend is inconsistent with a study which found that maternal protein restriction in rats led to reduced transcription rate of System A in the placenta preceding IUGR [98]. It is also inconsistent with an earlier study which found that maternal protein restriction in rats led to reduction in both the activity and the transcription rate of System A during IUGR [100]. However, this trend is consistent with an *in vitro* study wherein a placental cell line was exposed to amino acid restricted conditions. In that study System A responded to the decreased substrate availability by increasing its transcription rate in order to maximize substrate recovery [139]. In addition, System $B^{0,+}$ expression in *Xenopus laevis* oocytes is adaptively regulated at the transcription level in response to amino acid deprivation [117]. If System $B^{0,+}$ is exhibiting this adaptive regulation in rat blatsocysts in response to the MLP diet then it could be having downstream mTOR signaling effects. These effects could lead to the programming of a predictive adaptive response to the protein restricted conditions. Perhaps with a larger sample pool a statistically significant finding could be made.

It is also possible that while expression levels remain unchanged, the activity of System $B^{0,+}$ could be responding to changes in the maternal environment, such as a shift in the uterine sodium content. Furthermore, System $B^{0,+}$ translation rates are regulated separately from transcription rates, meaning changes in translation (and thus overall activity) wouldn't be visible at the transcription level thus reducing the usefulness of qRT-PCR. Such changes in transporter activity without a corresponding change in transcription have been noted before [139]. Insulin-stimulated glucose uptake rates in individual blastocysts has been assessed using tritium labeled 2-deoxyglucose [140]. Non-invasive assessment of pyruvate and glucose uptake using ultramicrofluorescence techniques is also possible [141]. There are techniques that can detect activity changes of amino acid transporter systems in pooled blastocysts [120] and theoretically they could be used to assess System $B^{0,+}$ activity in pooled blastocysts taken from protein restricted dams. However, due to the high number (n = 360) of blastocysts required, the samples used in these studies came from dams artificially stimulated by gonadotropins to produce large litters. The blastocysts used by Van Winkle *et al.* were also allowed to enter diapause first (by ovariectomizing the dams after conception) and then stimulated to develop into "implanting" blastocysts with estrogen and progesterone injections administered to the pregnant dam [142]. Therefore, the blastocysts recovered from these dams are not necessarily analogous to blastocysts recovered from an undisturbed pregnancy, and the activity assays in these studies were done in *in vitro* embryo culture media, which could be very different from *in vivo* conditions. Furthermore, pooling embryos abolishes the ability to detect subtle gender-specific differences, further reducing the usefulness of activity assays using pooled blastocysts. It also eliminates the ability to detect differences between individuals, preventing intra-litter

evaluation. However, if System $B^{0,+}$ activity *is* changing in response to the maternal diet, regardless of its expression levels, it could indeed be leading to reprogramming events. Therefore, System $B^{0,+}$ activity in individual preimplantation embryos, while impossible to determine with current technology, should be investigated in future studies when more sensitive technology becomes available.

Protein abundance levels are a potential variable that could affect System $B^{0,+}$ activity. Immunostaining techniques exist that could be used to assess protein abundance levels in a sample, such as immunohistochemistry and western blotting, which give qualitative information about the protein abundance in that sample. These readings must then be subjectively evaluated for differences. At best, the results can be evaluated using computer software to compare relative brightness between samples (this is called densitometry). Extracts from approximately 100 to 150 blastocysts can be pooled for western blot analysis [143], but doing so would make gender determination impossible and might defeat any attempts at detecting subtle gender specific differences. Unfortunately there is not enough recoverable protein in an individual blastocyst to perform a western blot. Immunohistochemical approaches in conjunction with confocal microscopy (together called 'confocal immunofluorescent microscopy') could theoretically be used to compare System $B^{0,+}$ protein abundance in an individual embryo if a proper antibody was available. Unfortunately at present there are no suitable antibodies available from a commercial source. Confocal microscopy is excellent for detecting dramatic changes in protein abundance in preimplantation embryos. For example, in preimplantation mouse embryos exposed to hyperglycemic conditions the abundance of the GLUT-1 glucose transporter protein was dramatically increased [50]. However this technique

isn't sensitive enough to detect a subtle change in protein abundance levels. Enzyme-linked immunosorbent assay (ELISA) technology is also too insensitive for samples of this size, however two technologies that could assess protein abundance levels in small samples are HPLC and mass spectrometry. In addition, there is a new technology called plasmon resonance which can detect a single molecule of the protein of interest, but for now this technology remains prohibitively expensive. Because of the limited time a masters project affords, and the obvious economical reasons, qRT-PCR was selected for this project. However, System $B^{0,+}$ protein abundance levels should be investigated with some of the aforementioned technologies in case they are changing in response to the maternal diet.

Quantitative RT-PCR, the technique used for this study, has many inherent weaknesses. For example, qRT-PCR yields values that are a ratio of the expression of one gene relative to another. This means that if your reference gene is changing just as much as your target gene then no difference in expression will be detected. In this study, $ATB^{0,+}$ (*Slc6a14*) expression was compared to *Hprt* expression. β-actin (*Actb*; a highly expressed and ubiquitous cytoskeleton component) and *Hprt* are frequently used as reference genes in qRT-PCR studies that use adult tissues [135]. However, *Hprt* is far superior to *Actb* for qRT-PCR analysis using pre-implantation embryos since *Actb* appears to vary significantly in expression rates throughout pre-implantation mouse development, whereas *Hprt* remains constant [144]. Unfortunately, nothing is known about the expression of *Hprt* in rat blastocysts in response to a maternal low protein diet. Therefore, it is conceivable that *Hprt* is responding to the diet, ending its usefulness as a reference gene. $ATB^{0,+}$ expression could be changing in relation to other reference genes and this should be investigated. However, assuming that the reference gene is

not changing in response to experimental conditions, quantitative RT-PCR is a very powerful technique as it provides a relative mRNA copy number present in a given sample and it is sensitive to changes in a large range of starting material concentrations (>5 orders of magnitude) [145], making it extremely useful for expression comparison studies using limited amounts of sample material.

It would be valuable to know what epigenetic changes result from a protein restricted diet in the preimplantation embryo. Although there is a limited amount of genomic DNA available for such analyses, techniques like carrier chromatin immunoprecipitation and micro-arrays ("ChIP-on-chip") can be used to evaluate the epigenetic status of blastocyst DNA [146]. Indeed, there is evidence that certain loci, particularly those near the imprinted *Igf2* locus, have dramatically altered DNA methylation patterns when exposed to an insufficient maternal dietary conditions [147]. There are likely many hundreds of genes being affected at the epigenetic regulation level due to maternal diet perturbations waiting to be discovered using this technology.

System $B^{0,+}$ was investigated in part because its preferred substrate, leucine, is a known upstream signaler of the mTOR pathway [85]. Preimplantation mouse embryos exposed to either amino acid deprivation or rapamycin (an mTOR inhibitor) *in vitro* do not develop the normal cellular outgrowths usually associated with implantation [90]. However, there are many other upstream signalers of mTOR such as insulin, growth factors and oxidative stress [121]. In order to fully understand the mTOR pathway and if it is affecting fetal programming we need to first establish that its activity is indeed changing in preimplantation embryos

exposed to a protein restricted maternal diet. This has not been examined in rat preimplantation embryos exposed to a MLP diet, but it has been established that mTOR activity is altered in the placenta during maternal under nutrition [148], so it seems likely. This could be done using immunohistochemcial approaches to detect increased phosphorylation of mTOR's downstream targets S6K1 and 4EBP1. Previous studies have shown at S6K1 and 4EBP1 phosphorylation changes in a variety of cell types [2, 3, 149]. This work was originally proposed as a part of this study and needs to be done to determine how mTOR in blastocysts is responding to the maternal diet.

The changes in maternal serum amino acid concentrations during MLP conditions have been documented extensively. High Performance Liquid Chromatography (HPLC) is a well developed and broadly used technique that can measure free amino acid and carbohydrate concentrations in a sample. HPLC has been used to assess maternal serum amino acid concentrations from rat dams exposed to the MLP diet during early pregnancy (from conception to day 4 of gestation). In the serum of MLP dams there was a significant increase in glutamine concentrations and a decrease in isoleucine, leucine, methionine, proline and threonine concentrations [21]. Leucine levels in particular dropped by about 10% [21]. In other rat studies, restricted protein during early pregnancy was shown to decrease serum concentrations of threonine [150], increase maternal serum concentrations of glutamine and glycine, and most importantly, decreased maternal serum concentrations of the branched-chain amino acids (leucine, isoleucine and valine) [151]. In pregnant mice, a maternal low protein diet reduces serum concentrations of the amino acids phenylalanine, leucine, isoleucine and valine and these decreases lead to IUGR [152].

These changes in maternal serum amino acid concentrations in response to a protein restricted diet need to be experimentally connected to changes in the amino acid concentrations in the uterine fluids. Some work has already been done with uterine luminal fluid: uterine luminal fluid extracted from mice has been compared to *in vitro* embryo culture media [153] and the normal uterine secretions of rabbits during preimplantation development [154] have been described. Throughout rabbit preimplantation development uterine amino acid concentrations change considerably. For example, leucine drops to about 40% of its initial concentration by the blastocyst stage [154]. If the maternal diet is causing a reduction in uterine leucine concentrations (corresponding with the previously described serum concentrations reductions, above) throughout preimplantation development it has strong implications for System $B^{0,+}$ activity. System $B^{0,+}$ activity is logically dependent on the concentrations of its substrates in the external medium and reducing System $B^{0,+}$ activity would result in lowered intracellular leucine availability. This in turn would alter mTOR activity and could potentially cause a predictive adaptive response.

Predictive adaptive responses could potentially be programmed for by changes in the DNA methylation pattern of a preimplantation blastocyst. The preimplantation embryo is particularly sensitive to methyl donor availability; during preimplantation development the genome is demethylated and the proper methylation pattern of all genes is then established [60]. Methionine's derivative S-adenosyl methionine is the "universal methyl donor" and serves as the primary methyl donor for these reactions. In the MLP fed dams methionine serum levels were markedly reduced [21]. It is then logical to expect a reduction in uterine luminal fluid methionine levels. Interestingly, in adult rat studies, methionine deprivation *or*

supplementation caused a 4 fold and 2.8 fold (respectively) increase in transcription of a liver methionine cycle enzyme, betaine-homocysteine methyltransferase (BHMT) [155, 156]. BHMT catalyzes the transfer of a methyl group from betaine to homocysteine; ultimately producing methionine [157]. It has been detected in human oocytes [158] and is probably present throughout development to maintain the supply of methyl donors for the rapidly developing embryo. BHMT expression and activity is directly linked to methyl donor availability and if it is responding to a protein restricted diet in preimplantation embryos it could provide a valuable clue indicating that the MLP diet is indeed affecting methyl donor availability and potentially the methylation pattern of the developing embryo.

The existence of predictive adaptive responses, as described by the DOHaD hypothesis, is supported by more and more evidence as time passes. However, the mechanisms that cause these adaptive responses remain unexplained. While no significant difference in $ATB^{0,+}$ transcription was noted, the upward trend seen in this research warrants further investigation. System $B^{0,+}$ activity in response to the MLP regime also remains a mystery and further investigation into the nutrient sensing mTOR pathway will likely provide additional insights. There is still much work to be done and uncovering System $B^{0,+}$'s roll in preimplantation development and its possible roll in predictive adaptive responses has only just begun.

Appendix A: Raw qRT-PCR Results

(Expression of $ATB^{0,+}$: Hprt observed in individual embryos)

The relative expression values of $ATB^{0,+}$: Hprt for the individual blastocysts are tabulated below. (4 tables: MLP males, control males, MLP females and control females)

Table 5. Relative expression of ATB0,+ : Hprt in male blastocysts from dams fed the MLP (9% casein) diet.

Dam #:	1	2	3	4	5	6	7
Embryo 1	0.1420	0.0020	0.0609	0.0003	0.1450	0.0327	0.0193
Embryo 2	0.2900	0.0003	0.0468	0.0000	0.0147	0.0281	0.0006
Embryo 3	0.0000	0.0000	0.0146	0.0020	0.0356	0.0011	0.0896
Embryo 4	0.0000	0.0006	0.0353	0.7200	0.0063		
Embryo 5		0.0033	0.0160	0.0002			
Embryo 6		0.0046	0.0853				
Mean	0.1080	0.0018	0.0432	0.1445	0.0504	0.0206	0.0365

Table 6. Relative expression of ATB0,+ : Hprt in male blastocysts from dams fed the control (18% casein) diet.

Dam #:	1	2	3	4	5	6	7
Embryo 1	0.0022	0.0066	0.0114	0.0050	0.0012	0.0119	0.0005
Embryo 2	0.0208	0.0897	0.0060	0.0007	0.0012	0.0101	0.0000
Embryo 3	0.0001		0.0101	0.0013		0.0311	0.0001
Embryo 4	0.0008		0.0200	0.0050		0.0041	0.0009
Embryo 5			0.4310	0.1790			0.0000
Mean	0.0060	0.0482	0.0957	0.0382	0.0012	0.0143	0.0003

Table 7. Relative expression of ATB0,+ : Hprt in female blastocysts from dams fed the MLP (9% casein) diet.

Dam #:	1	2	3	4	5	6	7
Embryo 1	0.0000	0.0024	0.0719	0.0000	0.0620	0.0005	0.0015
Embryo 2	0.0000	0.1550	0.0194	0.0102	0.0121	0.1500	0.0000
Embryo 3	0.0000			0.0291	0.0586	0.0022	0.0461
Embryo 4					0.1790	0.0028	0.0020
Embryo 5						0.0303	0.0025
Mean	0.0000	0.0787	0.0457	0.0131	0.0779	0.0372	0.0104

Table 8. Relative expression of ATB0,+ : Hprt in female blastocysts from dams fed the control (18% casein) diet.

Dam #:	1	2	3	4	5	6	7
Embryo 1	0.0147	0.0056	0.0102	0.0034	0.0010	0.0124	0.0001
Embryo 2	0.0163	0.0116	0.0009	0.0170	0.0016	0.0277	0.0008
Embryo 3	0.0496	0.0385	0.0180	0.0357	0.0011	0.0707	
Embryo 4	0.1110	0.0103			0.0014	0.0359	
Embryo 5		0.0029			0.0012		
Mean	0.0479	0.0138	0.0097	0.0187	0.0013	0.0367	0.0005

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