MATERNAL NUTRITION, ONE-CARBON METABOLITES, AND PROGRAMMING OF FETAL DEVELOPMENT DURING EARLY GESTATION

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DOCTOR OF PHILOSOPHY

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ABSTRACT

Crossbred Angus heifers (n = 14) were bred via AI, assigned to nutritional treatment (CON = 100% of requirements for 0.45 kg/d gain and RES = 60% of CON) and ovariohysterectomized on d 50 of gestation. Fetal liver, muscle from the hind limb, and cerebrum were analyzed by RNA-sequencing, and a total of 548, 317, and 151 genes, respectively ($P < 0.01$) were differentially expressed. Functional categories affected by nutritional treatment included: 1) Liver: metabolic pathways and nucleosome core, 2) Muscle: skeletal muscle and embryogenesis, and 3) Cerebrum: hippocampus and neurogenesis. Bovine embryonic fibroblast cells were cultured in Eagle's Minimum Essential Medium with 1 g/L glucose (LOW) or 4.5 g/L glucose (HIGH). Control medium contained basal concentrations of one-carbon metabolites (Choline, folate, vitamin B_{12}, and methionine). One-carbon metabolites (OCM: methionine, choline, folate, vitamin B_{12}) were supplemented to the media at 2.5, 5, and 10 times (2.5X, 5X, and 10X, respectively) the control media, except for methionine, which was limited to 2X. One-carbon metabolites increased ($P < 0.01$) basal respiration and Reserve Capacity in HIGH 2.5X and 10X compared with all other treatments. ATP-linked respiration was greater ($P < 0.01$) in HIGH OCM supplemented cells compared to Control and was greater in LOW 2.5X compared with LOW Control, 5X, and 10X cells. Total growth rate was greater ($P < 0.01$) for HIGH 2.5X and 10X compared with LOW Control, 2.5X, and 10X. At 24, 36, 48, and 72 h, cell proliferation in HIGH 10X was always greater ($P \leq 0.03$) than Low 10X. Therefore, these data are interpreted to imply that a moderate maternal nutrient restriction during the first 50 d of gestation in beef heifers alters the transcript abundance of genes impacting tissue accretion, function, and metabolism suggesting potential alterations to fetal physiology which should be further investigated. Lastly, supplementation of OCM may improve fetal growth and program increased

metabolic efficiency in the offspring, and thus should be a focus of future research into the effects of maternal nutrition on postnatal physiology.

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LIST OF ABBREVIATIONS

2 GSH.....2 Reduced Glutathione

2.5X.....Media containing 2.5 times the choline, folate, and vitamin B_{12} of the control media.

5X.....Media containing 5 times the choline, folate, and vitamin B_{12} of the control media.

5-mTHF.....5-methyltetrahydrofolate

5,10-CH2-THF.....5,10 methylene-tetrahydrofolate

5,10-CH = THF.....5,10 methenyl-tetrahydrofolate

10-f-THF.....10-formyl-tetrahydrofolate

10X.....Media containing 10 times the choline, folate, and vitamin B_{12} of the control media.

αKγMB.....α-keto-γ-methybutyrate

α-KB.....α-ketobutyrate

ADG.....Average daily gain

ACTB.....β-actin

ADP.....Adenosine diphosphate

AHCY.....A-adenosylhomocysteine hydrolase

AI.....Artificial Insemination

ARG1.....Arginase 1

ATIC.....5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase

ATP.....Adenosine triphosphate

ATP1A1.....ATPase NA+/K+ transporting subunit alpha 1

BAD.....BCL2 associate agonist of cell death

BAMBI .....BMP and activin membrane bound inhibitor

BCKDC.....Branched-Chain α-Ketoacid Dehydrogenase Complex

BCS.....Body condition score

BHMT.....Betaine homocysteine methyltransferase

CBS.....Cystathione β-synthase

CHDH .....Choline Dehydrogenase

CIDR.....Controlled internal drug release

CL .....Corpus luteum

CON .....Nutritional treatment in which heifers are fed nutrient requirements to gain 0.45 kg/d

CP.....Crude protein

CpG.....Regions of DNA dense with cytosine and guanine bases

CTH.....Cystathione gamma-lyase

CV.....Coefficient of variation

d.....Day

dADP.....Deoxyadenosine Diphosphate

DAPI 4,6-diamidino-2-phenylindole

dATP.....Deoxyadenosine Triphosphate

DEG .....Differentially expressed genes

dGDP.....Deoxyguanosine Diphosphate

dGTP.....Deoxyguanosine Triphosphate

DHF.....Dihydrofolate

DHFR.....Dihydrofolate Reductase

DMG .....Dimethyl glycine

DMI.....Dry matter intake

DNA.....Deoxyribonucleic acid

DNMT.....DNA Methyl Transferase

DNMT1.....DNA methyltransferase 1

DNMT3A.....DNA methyltransferase 3A

DNMT3B.....DNA methyltransferase 3B

dTMP.....Deoxythymidilate Monophosphate

dTTP.....Deoxythymidilate Triphosphate

dUDP.....Deoxyuridine Diphosphate

dUMP.....Deoxyuridine Monophosphate

EBTr.....Embryonic tracheal fibroblast cells

EMEM.....Eagle's Minimum Essential Medium

FA.....Folic Acid

FAD.....Flavin adenine dinucleotide

FCCP.....Carbonyl cyanide-p-trifluoromethoxyphenyhydrazone

FDR.....False discovery rate

GAPDH.....Glyceraldehyde-3-phosphate dehydrogenase

GART.....Phosphoribosylglycinamide formyltransferase

GDP.....Guanosine Disphosphate

GNMT.....Glycine-*N*-Methyl Transferase

GO.....Gene ontology

GPX.....Gluthathione Peroxidase

GSR.....Glutathione-disulfide reductase

GSSG .....Oxidized glutathione

H4.....Histone 4-like

HDAC .....Histone deacetylase

HMT.....Histone Methyl Transferase

HIGH.....4.5 g/L glucose media

IFN-τ .....Interferon-τ

IMP .....Inosine monophosphate

KEGG .....Kyoto encyclopedia of genes and genomes

LOW .....1 g/L glucose media

MAPK.....Mitogen activated protein kinase

MAT.....Methionine adenosyltransferase

MAT2A.....Methionine adenosyltransferase 2A

MAT2B.....Methionine adenosyltransferase 2B

MCEE .....Methylmalonyl-CoA Epimerase

Mo.....Months

mRNA.....Messenger ribonucleic acid

MTA5' .....Methylthiodenosine

MTHFD1.....methylenetetrahydrofolate dehydrogenase 1

MTHFD2.....Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase

MTHFR.....Methyltetrahydrofolate reductase

MTR.....Methionine synthase

MTRR .....Methionine Synthase Reductase

MUT.....Methylmalonyl-CoA Mutase

MYL4.....Myosin light chain 4

MYOD1 .....Myogenic differentiation gene 1

MYOG .....Myogenin

NAD.....Nicatinamide adenine dinucleotide

NADPH.....Nicotinamide adenine dinucleotide phosphate

NDF.....Neutral detergent fiber

NEm .....Net energy of maintenance

NEg .....Net energy of gain

OCM .....One-carbon metabolites

ODC .....Ornithine decarboxylase

$PGF_{2\alpha}$ .....Prostaglandin $F_{2\alpha}$

PRMT.....Protein Methyl Transferase

PCCα/β.....Propionyl-CoA Carboxylase

PVDF .....Polyvinylidene fluoride

RES .....Nutritional treatment in which heifers are fed to maintain weight.

RNA .....Ribonucleic acid

RRBS .....Reduced representation bisulfite sequencing

SAH.....S-adenosylhomocysteine

SAM.....S-adenosylmethionine

SAMDC .....S-Adenosyl Methionine Decarboxylase

SCS .....Succinyl-CoA Synthetase

SDHA.....Succinate Dehydrogenase

SDS .....Sodium dodecyl sulfate

SHMT1 .....Serine Homocysteine Methyltransferase

SMS.....Spermine synthase

SRM .....Spermidine synthase

TBST.....Tris buffered saline with 0.1% Tween 20

TET .....Ten eleven translocation

TF.....Transferrin

THF .....Tetrahydrofolate

TMR.....Total mixed ration

UDP.....Uridine Diphosphate

UMP .....Uridine Monophosphate

Wk.....Weeks

XMP .....Xanthine monophosphate

Yr .....Year

CHAPTER 1. LITERATURE REVIEW

Introduction

In ruminant production systems there is potential for periods of nutritional stress due to inadequate or overnutrition prior to, during, and immediately after pregnancy as a result of factors such as forage quality, overgrazing, or drought (Cline et al., 2009). These nutritional factors may impact the prenatal growth trajectory and affect postnatal growth and development in a process termed Developmental Programming, which can be influenced even from the earliest stages of pregnancy when the nutrient requirements to support the growth of the conceptus are considered to be negligible (Funston et al., 2010; Funston et al., 2012). Therefore, this review addresses current knowledge on the effects of nutritional stress, developmental programming, and epigenetics during early gestation in ruminants on fetal developmental plasticity.

Developmental Programming

Concepts and Initial Observations

Developmental programming, which is also referred to as fetal programming or Developmental Origins of Health and Disease, is the concept that maternal or paternal stressors affect fetal development which can 'program' the increased risk of various postnatal pathologies (Barker et al., 1990; Barker, 1990; Barker, 2004). This concept was elucidated by several epidemiological studies that demonstrated that birthweight and placental size, poor postnatal environment, or other stresses and 'insults' during development, increased the risk of pathologies such as heart disease and metabolic syndrome (Barker et al., 1990; Barker, 1990). Additionally, some of the most well-known programming data comes from the Dutch Hunger Winter famine of 1944-45 which showed that maternal starvation during gestation correlated with an increased

risk for cardiovascular, metabolic, or kidney disease in the offspring which was dependent on the stage of gestation in which the malnutrition occurred (Ravelli et al., 1976). Furthermore, these observations were seen in offspring born of normal birthweight whose mothers were nutrient restricted during early pregnancy. These data demonstrated a programmed effect of early gestational malnutrition which manifested itself later in life, even though all initial signs at birth indicated "healthy" offspring. These studies have provided the launching point from which many epidemiological studies in humans, as well as studies using animal models have elucidated multiple mechanisms (reviewed in detail by Reynolds et al., (2019)) by which developmental programming alters susceptibility to non-communicable diseases, growth abnormalities, altered organ development and function, as well as cognitive dysfunction (Fowden et al., 2006).

Although Dr. Barker and the "Barker Hypothesis" have provided the springboard from which multiple studies have been conducted in developmental programming, it is important to know that the concept that environment can influence development, and the subsequently developed ideology, "Inheritance of Acquired Characteristics" were hypothesized several centuries beforehand. These ideas are most associated with Jean-Baptiste Lamarck in the theory known as "Lamarckian Evolution"; however, these ideas had been accepted by multiple scientists, doctors, and philosophers such as Hippocrates and Aristotle nearly 2,200 years before Lamarck (Zirkle, 1935).

Lamarckian Evolution was developed around two laws: First Law of Use and Disuse which states: "In every animal which has not passed the limit of its development, a more frequent and continuous use of any organ gradually strengthens, develops and enlarges that organ, and gives it a power proportional to the length of time it has been so used; while the permanent disuse of any organ imperceptibly weakens and deteriorates it, and progressively

diminishes its functional capacity, until it finally disappears." The Second Law of Soft Inheritance states: "All the acquisitions or losses wrought by nature on individuals, through the influence of the environment in which their race has long been placed, and hence through the influence of the predominant use or permanent disuse of any organ; all these are preserved by reproduction to the new individuals which arise, provided that the acquired modifications are common to both sexes, or at least to the individuals which produce the young (Lamarck, 1809)". Since the publication of Dr. Lamarck's book, scientists have continued to develop and refine additional hypotheses of inheritance and "programmed" alterations to metabolism and growth.

With the most recent interpretations of the available data, it is understood that regulation of growth, development, and nutrient utilization for adult function are programmed *in utero* (Godfrey and Barker, 2000). These programmed changes may be caused by both paternal and maternal stressors (nutritional, environmental, etc.) which can negatively affect fetal growth and development through modifications of the epigenome. Epigenetic modifications, which will be discussed in further detail in the one-carbon metabolism section, include altering patterns of DNA methylation and covalent modifications to histone tails such as methylation, phosphorylation, and acetylation, all of which impact gene expression, subsequent protein synthesis, and endocrine control which ultimately lead to altered cell physiology, impaired growth, and metabolism of the conceptus (Waterland and Jirtle, 2003; MacLennan et al., 2004; Waterland and Jirtle, 2004; McMillen and Robinson, 2005; Waterland et al., 2006; Meyer et al., 2012; Reynolds et al., 2017; Clare et al., 2019). Interactions between the developing mammalian embryo, fetus, or neonate and its environment involve the process of developmental plasticity (Gluckman et al., 2005). Developmental plasticity is a process evolved to adjust the developmental patterns and trajectories to produce a phenotype that is matched to the anticipated

environment (West-Eberhard, 2005). However, when the prediction of the anticipated environment is inaccurate, or when the environment changes between generations, a mismatched phenotype may result, and then the consequences of plasticity may be maladaptive (Bateson et al., 2004). Thus, maternal environment pre-conception, during the periconceptual period and throughout pregnancy, as well as the immediate post-natal environment of the offspring can have a large impact on development and subsequently impact postnatal growth and production efficiency.

Inadequacies of Knowledge of Developmental Programming in Livestock

The consequences of maladaptive programming in livestock are not well documented. In most production systems, consequences of poor developmental plasticity leading to altered metabolism, cognitive dysfunction, or reproductive failure may not be documented or attributed to maladaptive programming for several reasons: 1) Steers or heifers harvested for slaughter are generally harvested prior to the time they are two years old, and thus may not exhibit symptoms of metabolic or growth dysfunction seen in humans in their later years of life, 2) Animals with poor temperament or cognitive disfunction may be culled from the herd without investigation into developmental stress and poor development as a cause, 3) Animals who fail to conceive may be due to programming; however, inadequate record keeping may not provide significant data for causal relationships to be drawn. This leads to the fact that although maladaptive programming may exist in livestock species, the available records to show correlative or causal relationships do not currently exist to demonstrate these effects. Although this may not yet be of concern to producers, selection based on expected progeny differences therefore may not be fully accurate because the animal may never reach its full genetic potential, which may lead to poor selections for future breeding programs (Reynolds et al., 2010). Inherently, this may cause decreases in

producer profits, whereas understanding programming stresses as well as ways to manage and increase efficiency of production by these animals for reproductive, food, and fiber sources would maximize efficiency and profitability for producers, while also decreasing the carbon footprint of livestock production. This becomes an increased concern to not only food producers, but also to consumers. With the ever-growing concern of how food is raised and the need to significantly increase the output of meats to meet population growth demands, the need for increased production efficiency and decreased loss are essential to meeting these goals. Therefore, we must first begin to understand the nutrient requirements of fetal development; that is, the specific nutrient requirements to establish and maintain pregnancy, as well as the requirements at specific timepoints in gestation to maximize the genetic potential of the animal and program lifelong efficiency.

Early Gestation Nutrient Requirements

Establishment of Pregnancy

Fertilization rates for first service artificial insemination (**AI**) are approximately 90% in beef heifers (Bridges et al., 2013); however, by d 30 of gestation, only 50 to 60% of heifers maintain a viable pregnancy. Moreover, Thatcher et al., (1994) indicated that up to 80% of all embryonic loss occurs before d 40 of gestation in sheep and cattle. Across all livestock species, as well as humans, it is estimated that two-thirds of all embryonic losses occur during the peri-implantation/peri-attachment period of gestation (Bazer and First, 1983). The main cause of this is failed implantation/attachment (humans or ruminants, respectively) of the chorioallantois to the uterine endometrium, which accounts for 50 to 75% of failures to establish pregnancy (Bazer et al., 2011b).

Multiple events must take place during the first 50 days of gestation for a successful pregnancy to be established and maintained, and these events have been previously outlined in reviews and text books (Bazer et al., 2011a; Bazer et al., 2011b; Senger, 2012), and the timeline below has been adapted for cattle. Briefly, fertilization of the ovum by the sperm occurs in the oviduct forming the zygote. Multiple cleavage events take place to form the morula (32 to 64 cell embryo) which migrates into the uterine body by days 4 to 7 of gestation (Senger, 2012). These cells continue to grow and divide forming the spherical blastocyst which consists of the inner cell mass and the trophoblast (Senger, 2012). The inner cell mass is what will give rise to the embryo/fetus, and the trophectoderm will give rise to the placenta (Rowson and Moor, 1966; Bindon, 1971; Wintenberger-Torrés and Fléchon, 1974; Barcroft et al., 1998; Senger, 2012). The blastocyst will then break out of the zona pellucida and transition from its spherical form, and elongate into a large spherical, tubular, and filamentous conceptus consisting of the embryo and the extra embryonic membranes. At d 13 of gestation, the bovine blastocyst is roughly 3 mm in diameter, but by day 17, the blastocyst will become approximately 250 mm in length. This step of elongation of the conceptus is very important for the subsequent attachment steps as well as maternal recognition of pregnancy. As the conceptus elongates, it orients itself within the uterine lumen, and begins to adhere to the uterine endometrium. It is at this time that the mononucleated trophectoderm (formerly described as trophoblast) of the conceptus begins to release interferon-τ (**IFN-τ)** 16 to 17 days post-fertilization; (Senger, 2012), which is the maternal recognition of pregnancy signal in ruminants and is secreted in proportions equivalent to the size of the trophectoderm. Successful release of IFN-τ from the conceptus inhibits upregulation of the uterine oxytocin receptor, silences expression of genes involved in the pulsatile release of prostaglandin $F_{2\Delta}$ (**$PGF_{2\alpha}$**), which would terminate the pregnancy by causing regression of the

corpus luteum (**CL**) and termination of production of progesterone at sufficient levels to maintain pregnancy (Bazer et al., 1997; Bazer, 2013; Bazer et al., 2015). The developing placenta has been forming during this time from the extraembryonic membranes of the pre-attached embryo. This development is an obligatory step in the acquisition of the embryo's ability to attach to the uterus. As the hatched blastocyst begins to grow and expand, layers just beneath the inner cell mass called the mesoderm and endoderm, begins to grow. The endoderm grows downward lining the trophoblast forming the first membrane called the yolk sac. The mesoderm continues to grow and expand forming a sac that surrounds the yolk sac and pushes against the trophectoderm forming a double membrane called the chorion. The chorion then expands surrounding the embryo forming the amniotic sac. The allantois is the final membrane compartment which originates from the primitive gut of the embryo. This membrane collects liquid waste from the embryo. As the embryo continues to grow, the allantois grows in size as well as fusing with the chorion forming the chorioallantois, which will grow and develop into the placenta (Bryden et al., 1972). By days 18-22 the conceptus is successfully adhered to the uterine endometrium, and by day 30, complete attachment of the chorioallantois to the endometrium has taken place. After d 30 of gestation, the chorioallantois begins to differentiate between the cotyledonary tissue of the placenta, and the intercotyledonary tissue. The cotyledons arise due to increased local force of blood pressure from the endometrium on specific points of the placenta. This causes structural changes in the placenta involving a three-step process: 1) cavitation, 2) crowning and extension of anchoring villi, and 3) completion by development of the supplying vascular pattern (Reynolds, 1966). These cotyledons will form the point of attachment between the caruncles on the uterine endometrium and the placenta, allowing for exchange of nutrients through hemotrophic nutrition, and will continue to grow and develop throughout gestation.

Throughout the first 50-day timeframe, the embryo is growing rapidly. By approximately day 45 of gestation, organogenesis (development of the fetal organs) is completed for some organs; however, although differentiated, organ systems like the gastrointestinal system (Noah et al., 2011) or lungs are not completely developed and functional. This indicates, that during the first 50 days of gestation, all the fetal organ systems are growing, developing, and differentiating from one another (Winters et al., 1942), and maturing into functional organ systems throughout gestation and into the neonatal period. Organ differentiation follows the steps of: 1) morphogenesis and cell proliferation, 2) cell differentiation, and 3) functional maturation (Colony, 1983). These developmental events may not require the increased whole energy supply required to support the net energy retained in the fetus as in late gestation; however, to complete the growth and differentiation of the embryo and placenta, there are most likely transitory increases in specific nutrient demands during key times of development to allow for optimal embryonic development.

Heifer and Fetal Nutrient Requirements

Maternal nutrient requirements can be modeled using the most recent publication of the Nutrient Requirements of Beef Cattle (NASEM, 2016). Feed costs are the largest economic burden for beef cattle producers. For beef cattle, more feed resources are dedicated to the parent population (cow herd) than to market bound offspring (Webster, 1989). Establishment of exact nutrient requirements which minimize excessive shedding of nutrients and maximize whole animal nutrient utilization will not only decrease the economic burden of producers, but also decrease the carbon footprint by reducing methane energy loss, as well as nitrogen and phosphorous shedding to the environment. Energy requirements of various production stages for heifers, steers, bulls, and cows have been adapted from the classic publication of Lofgreen and

Garrett (1968) whereby they expressed energy requirements on a basis for maintenance (**NEm**) and gain (**NEg**). Heifers pose unique requirements when compared to cows or steers due to NEg being partitioned to personal growth as well as to gestation and subsequent lactation; however, the current model associates energy cost with NEm or the partial efficiencies of conceptus growth, milk production, and tissue energy which are all dependent upon one another (Freetly, 2019). Freetly (2019) acknowledges that because of the way the calculations in the model are established for maintenance or synthesis of tissues, it is difficult to assign values for energy utilization by support tissues for pregnancy, lactation, and weight fluctuations. Therefore, incorrect estimates in any of the energy efficiencies will result in inaccurate estimates of others and subsequently errors in whole energy requirement calculations. While whole animal studies have been extensively evaluated, studies evaluating individual tissue requirements at specific production stages are needed to more accurately measure maternal nutrient requirements at all stages of gestation and of the production cycle.

Furthermore, maternal nutrient requirements during early gestation are considered to be negligible (Funston et al., 2010; Funston et al., 2012). This is because during the periconceptual period of embryonic development, the nutrient requirements of the gravid uterus do not significantly add to the maintenance requirements of heifers. When evaluating the nutrient and energy contents of utero-placental and fetal tissues throughout gestation, estimated energy requirements of heifers are low during early gestation but increase quickly as gestation progresses (Jakobsen, 1956; 1957; Ferrell et al., 1976). These increases in energy requirements are due to fetal growth throughout gestation as shown in (Figure 1.1A; (NASEM, 2016). Beginning around day 90 of gestation, there begins to be the noticeable increase in fetal weight as a percent of weight at birth (Figure 1.1A). Furthermore, the exponential increase in fetal

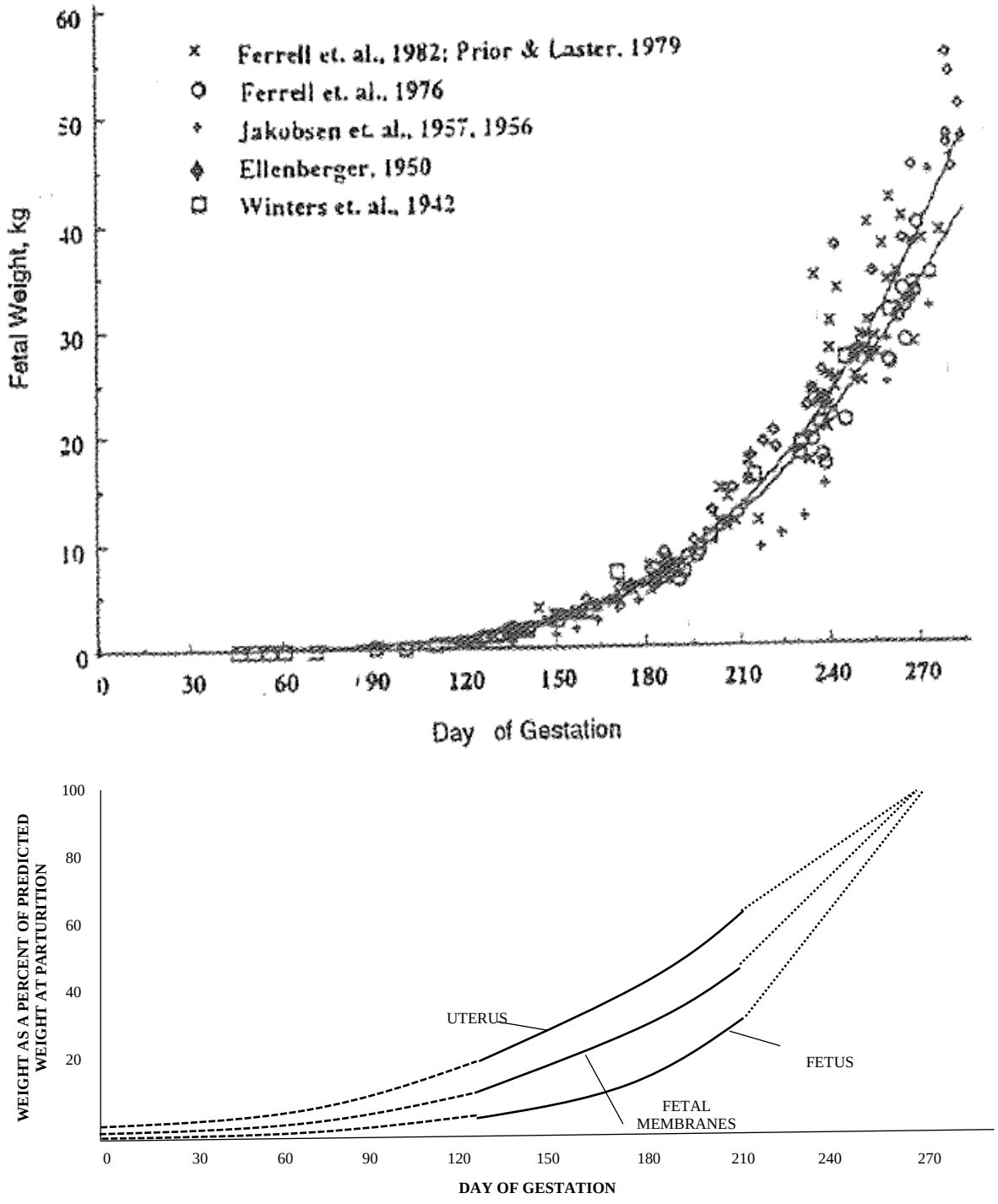

Figure 1.1. A.) Relationship of fetal weight to day of gestation in cattle. (NASEM, 2016). B.) Relationships among weights of the fetus, fetal membranes, and uterus, expressed as percentages of predicted weights at parturition and day of gestation. Solid line represents actual data range, and the dashed line represents extrapolated data (Ferrell et al., 1976).

weight is accompanied by an exponential increase in uterine artery blood flow (Figure 1.1B;(Bollwein et al., 2002). In Simmental heifers, uterine artery blood flow from the uterine horn ipsilateral to the fetus increased from 72 mL/min at 1 month of gestation to over 15,000 mL/min at 9 months of gestation (Figure 1.2B; (Bollwein et al., 2002). Increases in uterine and umbilical blood flow occur after the first trimester to supply the increased energy and nutrient supply of the gravid uterus. Cardiac output directed towards the uterus in ewes is less than 1% in non-pregnant ewes, increases to 8% during the middle trimester, and reaches 16% by the final trimester (Stock and Metcalfe, 1994). These increases in blood flow to the uterus coincide with increased fetal metabolism and oxygen consumption. Maternal oxygen consumption in response to fetal energy requirements increases maternal basal metabolism of which 50% of the increased maternal oxygen consumption is directed toward use by the utero-placental-fetal unit (Metcalfe et al., 1986). The development of the utero-placental-fetal unit occurs majorly first by the uterus, second by the placenta, and lastly by the fetus itself. This is to account for the necessity of the utero-placenta to be able to adequately deliver nutrients to the fetus during the exponential growth period during the final two-thirds of gestation (Figure 1.1B; (Ferrell et al., 1976). While much of the emphasis has been focused on late gestation, data from Ford et al., (1979) measuring uterine blood flow through the first 30 days of gestation in Hereford heifers demonstrated that blood flow to the uterine artery ipsilateral to the conceptus increased two to three-fold from d 14 to 20 of gestation which coincides with maternal recognition of pregnancy (Figure 1.2A). Furthermore, this increase was specific to the ipsilateral uterine horn suggesting a response to estrogen and progesterone from the CL, as well as providing further data to support transient requirements during the time of rapid placental elongation to ensure maintenance of pregnancy.

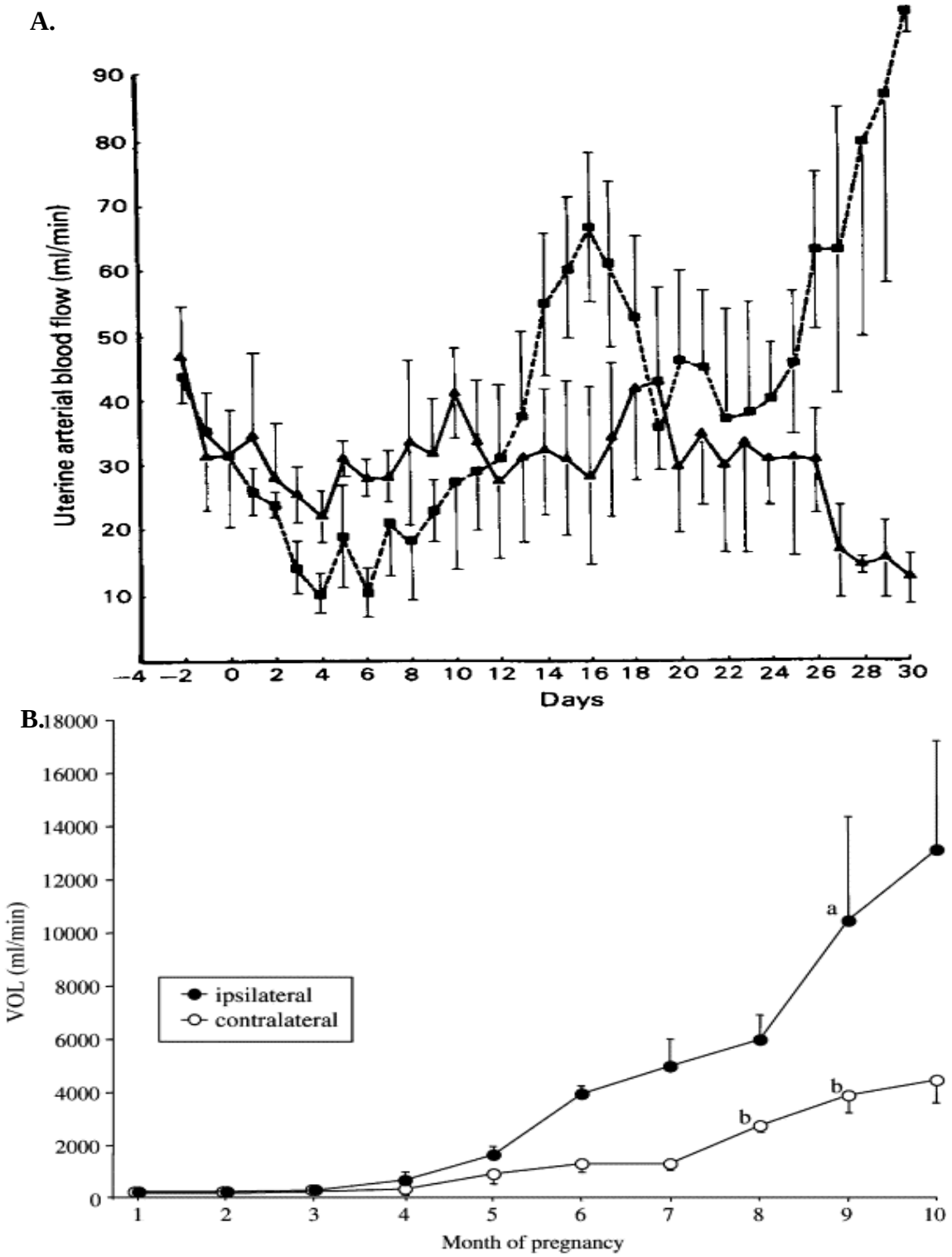

Figure 1.2. A.) Pattern of blood flow to both uterine horns through the first 30 days of pregnancy squares with the dash line represents blood flow to the ipsilateral uterine horn, triangles with the solid line represent blood flow to the contralateral uterine horn (Ford et al., 1979) B.) Volume of blood flow (VOL) in the uterine arteries ipsilateral and contralateral to the conceptus (Bollwein et al., 2002).

Recent data from our laboratories has characterized fetal growth from d 25 to 90 of gestation in beef heifers. Straight crown-rump lengths were measured, and the length increased from 2.2 mm on d 25 to 174.3 mm on d 90 and can be described by the equation $y = 0.0223x^2 + 0.1018x - 16.077$ (R^2 = 0.996, P < 0.01), in which y = fetal crown-rump length and x = day of gestation (Negrin Pereira et al., 2017). The quadratic increase in embryonic growth during the first 90 days of gestation falls under the area in Figure 1.1A that shows almost no growth compared to total; however, these data demonstrate an almost exponential growth rate that agrees with Winters et al., (1942) who showed that relative growth rate of bovine fetuses was greatest in early gestation and declined as pregnancy advanced. This is because at this point in gestation, the cell doubling rate is greater than it will be at any other time during the life of the offspring, and thus should be a focus of future research.

Post Conception Nutrition

Multiple studies in heifers investigating post-insemination nutrition and management have shown that post-insemination nutrient restriction decreases success to timed-AI (Arias et al., 2012; Arias et al., 2013; Lake et al., 2013). Heifers were allocated to one of three nutritional treatments: 1) fed to gain 0.7 kg/d from winter development through the first 21 days of gestation, 2) maintain weight post-AI, or 3) lose weight (80% of NEm requirements). The heifers fed to gain 0.7 kg/d had greater conception rates compared with the maintenance or loss groups. Additionally, the gain group had embryos that were more advanced in their development at d 6 of gestation, greater total cells, and greater live cells compared with their maintenance and loss counterparts (Arias et al., 2012). Furthermore, heifers developed to 65% of mature body weight in a feedlot, bred via timed-AI, maintained in a feedlot for 30 days, and then moved to pasture had greater pregnancy rates than those moved to pasture immediately following breeding (Arias

et al., 2013). These data suggest that heifers have greater pregnancy rates when maintained on the same plane of nutrition post-insemination as they were pre-insemination. Thus, we can interpret these data to imply that moderate negative changes in nutrient supply may reduce pregnancy rates by modifying nutrient partitioning in the heifer away from the utero-placenta during early pregnancy. Subsequently, as previously described, we can hypothesize that although there is not the mass growth of the conceptus during the first trimester of gestation, there are likely transient nutrient requirements to successfully maintain pregnancy in early gestation that if altered, can result in poor programming outcomes or failure to maintain pregnancy during the periconceptual period of gestation in heifers.

Oviductal Fluid

Fertilization takes place in the oviduct which has many secretory epithelial cells which produce oviductal fluid containing nutrients and growth factors necessary for embryonic cleavage events. After fertilization, the embryonic mitochondria are immature with less formed christae (Motta et al., 2000; Dumollard et al., 2007); Therefore, the embryo preferentially utilizes pyruvate, lactate, acyl-coA fatty acids, and amino acids as the main energy sources, with glucose being shunted towards the pentose phosphate pathway for production of the reducing equivalent NADPH as well as synthesis of purines (Sturmey et al., 2009; Absalón-Medina et al., 2014). As the embryo matures forming the morula, energy metabolism shifts towards oxidative metabolism, glycolysis, as well as synthesis of NADPH via the pentose phosphate pathway due to the continued low oxygen environment in the oviduct and uterus during early gestation (Li and Winuthayanon, 2017). Due to the low oxygen environment, enzymatic and non-enzymatic antioxidants are secreted with oviductal fluid (Guérin et al., 2001). Non-enzymatic antioxidants secreted with oviductal fluid include glutathione, taurine, hypotaurine, cysteamine, albumin, and

transferrin which act to reduce reactive oxygen species and hydroxyl radicals (Gardiner and Reed, 1994; Gardiner et al., 1998; Guérin et al., 2001). Enzymatic antioxidants such as catalase, superoxide dismutates, and glutathione peroxidases are also secreted in oviductal fluid to prevent oxidative stress during early phases of cell growth which are susceptible to increased oxidative damage (Guérin et al., 2001; Agbor et al., 2014; Hansen and Harris, 2015).

Histotrophic Secretions

After movement from the oviduct to the uterine lumen, ruminant embryos receive nutrients through histotrophic secretions from the uterine luminal and glandular epithelium. Histotroph includes nutrient transport proteins, ions, mitogens, cytokines, lymphokines, enzymes, hormones, growth factors, proteases and protease inhibitors, amino acids, glucose, fructose, vitamins and other substances (Bazer, 1975; Spencer and Bazer, 2004; Bazer et al., 2011a; Bazer et al., 2011b). In ruminants, histotroph, which is also called uterine milk, is essential to the development of the conceptus throughout pregnancy and is secreted from glands within the uterine endometrium (Bazer, 1975; Gray et al., 2001; Spencer et al., 2008; Bazer et al., 2011b). The uterine endometrium of cattle consists of multiple aglandular caruncular areas, which are dense stromal areas covered by a simple luminal epithelium, as well as intercaruncular areas (Filant and Spencer, 2013). The intercaruncular areas of the endometrium contain many hundreds of glands in a cross-section of the uterine wall (Filant and Spencer, 2013). These glands begin to develop as invaginations of luminal epithelium that progressively invade the stroma, resulting in an extensive network of epithelial glands (Filant and Spencer, 2013). If these glands were to not develop, the conceptus would not receive histotroph for growth and elongation, resulting in insufficient secretion of IFN-τ, which is proportional to conceptus size (Binelli et al., 2001; Rizos et al., 2012). Insufficient IFN- τ results in a failure to inhibit

upregulation of the uterine oxytocin receptor, resulting in lysis of the CL by PGF2α and termination of pregnancy. This was established with the uterine gland knockout model in sheep (Bartol et al., 1988a; Bartol et al., 1988b). Knockdown of uterine gland development in sheep resulted in infertility, and although blastocysts were normal on d 9 after fertilization, conceptuses were severely growth-retarded by d 12 or 14 of gestation (Filant and Spencer, 2013). This lack of elongation emanated from the absence of histotrophic secretions into the uterine lumen from the glandular epithelium (Gray et al., 2001; Gray et al., 2002), and thus suggested the importance of histotroph to the elongation of the conceptus, and subsequent importance of elongation to the maintenance of pregnancy.

Early reports of uterine luminal fluid demonstrated increased reducing sugars and free amino acids throughout the estrous cycle compared with maternal blood (Olds and Vandemark, 1957; Fahning et al., 1967). Although Forde et al., (1979) demonstrated increases in uterine blood flow during the estrus cycle in beef cows, Fahning et al., (1967) showed that the content of uterine fluid was independent to that of the maternal blood. Recent data completed by multiple research groups and described briefly below demonstrates that histotrophic secretions are temporally regulated and subject to hormonal control of the endometrial glands. It should be noted however that although the content of histotroph is independent of maternal blood, that the nutrients secreted in histotroph originated from maternal supply, and thus suggest further metabolism by the uterus or suggests metabolism of the histotroph by enzymes secreted in histotroph.

As previously described, histotroph must be secreted from the uterine glands, and as such, specific nutrient transporters which fall under the solute carrier family (He et al., 2009) are present to secrete histotroph into the uterine lumen. The uterine glands are stimulated by

progesterone and IFN-τ to secrete histotroph. Data in sheep comparing pregnant vs. cyclic ewes determined temporal, progesterone, and IFN-τ effects on hexose and amino acid transporters as well as concentrations of hexoses and amino acids in utero-placental tissues and fluids throughout the peri-implantation period of gestation (Gao et al., 2009b, a; Gao et al., 2009c) and has been completely reviewed by Bazer et al., (2011b).

Protein, amino acid, and hexose characterization of histotroph throughout early pregnancy in beef cows shows similarities to that of the ovine model and supports previous data from Fahning et al., (1967). These data have elucidated temporal changes to uterine secretions throughout the periconceptual period of gestation in cattle. Proteomic analysis of cyclic dairy heifers on day 7 and 13 of the estrous cycle showed increases in copper amine oxidase, catalase, and superoxide dismutase in uterine fluids suggesting a continued importance of redox potential throughout early gestation until gas exchange can be established with a functional placenta (Mullen et al., 2012). Forde et al., (2014) compared amino acid profiles of histotroph in pregnant vs. cyclic cross-bred cows from d 7 to 19 of the estrous cycle (non-pregnant) or post-insemination which demonstrated temporal changes to amino acid abundance in the uterine secretions of both pregnant and cyclic cows as well as the progesterone induced changes in expression of amino acid transporters. Interestingly, the concentrations of most amino acids were numerically decreased in the pregnant compared with the cyclic heifers from day 7 to 16 with arginine, methionine, and glutamine being significantly lower in pregnant compared with cyclic heifers. However, by d 19 of gestation, all amino acids numerically increased in concentration (Forde et al., 2014) which further exemplifies the increased transitory nutrient requirements for the rapid elongation of the placenta and quadratic growth rate (Negrin Pereira et al., 2017).

Data from our lab group built upon these previously published data and investigated the temporal effects of gestation in Angus-cross beef heifers on the expression of nutrient transporters and retroviruses involved in the formation of the ruminant placenta on d 16, 22, 28, 34, 40, or 50 of pregnancy (Crouse et al., 2016b; McLean et al., 2017). From these data, we selected d 16, 34, and 50 of gestation for further investigation due to these days having the greatest expression of multiple target genes in utero-placental tissues. In fact, when evaluating these days of gestation further, they coincide with key developmental time frames. Day 16 in the bovine is the time of maternal recognition of pregnancy (Thatcher et al., 1994), which as described earlier involves rapid placental growth and elongation. By d 34 of gestation, embryonic organogenesis is still taking place with differentiation of the dorsal portion of the conceptus, further organization of the conceptus, and development of the limbs (Winters et al., 1942). Furthermore, by d 45 to 50 of gestation, the conceptus has developed all of its organ systems and is deemed a fetus. As described previously, although not fully functional, all organs are now present and will continue to mature into functional organ systems as gestation progresses. Following previously described work (Arias et al., 2012; Arias et al., 2013; Lake et al., 2013), we imposed nutritional treatments on heifers immediately post-insemination to gain 0.45 kg/d (ADG = 0.51 kg/d; **CON**) or maintain weight (ADG = -0.08 kg/d; **RES**). Data from these studies were interpreted to imply that although a moderate maternal nutritional treatment was imposed upon beef heifers from breeding to d 50 of gestation, the expression of specific hexose and amino acid transporters are regulated temporally with limited effect of maternal nutrition on their expression (Crouse et al., 2017; Greseth et al., 2017b). Data from the same study further demonstrated temporal changes to both amino acids and hexoses in histotroph, allantoic, and amniotic fluids. There were minimal detectable changes in histotroph from d 16 to

50 of gestation; however, concentrations of fructose, alanine, and glutamate increased as gestation progressed (Crouse et al., 2019b). In allantoic fluid, concentrations of glucose, fructose, and almost all amino acids except tyrosine were numerically less in heifers who were nutrient restricted to 60% of CON. In amniotic fluid however, only glucose and fructose were decreased in the restricted compared with control heifers. All amino acids were numerically greater in heifers receiving the restricted diet compared with the controls (Crouse et al., 2019b). Of greatest interest was by day 50, glucose, fructose, and methionine were decreased in fetal fluids and homocysteine was increased in heifer serum (Crouse et al., 2019b).

Simintiras et al., (2019) investigated the metabolome of uterine luminal flushings on d 12, 13, and 14 post insemination with a normal progesterone group or high progesterone group (progesterone-releasing intravaginal device inserted day 3 post-estrus and remained in until collection) to test the effects of temporal and hormonal action on the contents of the uterine luminal fluid metabolome. Of greatest note was the large increase in concentrations of fructose and mannitol/sorbitol of the normal vs. high progesterone and on d 14 compared with d 12 of the estrous cycle. Furthermore, the most enriched pathways affected by a progesterone × day interaction were phenylalanine, glutathione, creatine, polyamine, methionine, cysteine, S-adenosyl methionine, taurine, urea cycle and arginine, proline metabolism, amino sugar, lysine, tyrosine, pentose, glycolysis, gluconeogenesis and pyruvate, as well as aspartate (Simintiras et al., 2019).

Taken together, these data highlight metabolites and cellular processes that may be critical to the establishment and maintenance of pregnancy being: 1) Hexoses such as fructose and glucose supply energy to the developing conceptus, activating nutrient sensing pathways such as the mammalian target of rapamycin (Kim et al., 2012) and play a critical role in

producing NADPH and nucleotides via the pentose phosphate pathway which donate electrons to reduce oxygen radicals, as well as synthesize nucleotides via the production of ribose-5 phosphate, 2) Metabolism of arginine via nitric oxide synthase to produce nitric oxide (an oxygen radical) as well as reduction of free radicals through glutathione peroxidase and NADPH play a key balancing act of oxygen radicals to stimulate cell proliferation such as nitric oxide and prevent cell death due to oxidative stress, 3) In addition to nitric oxide, arginine can be metabolized to polyamines with S-adenosyl methionine donating carbon skeletons (Clare et al., 2019) which further increase cell proliferation and are needed for the successful elongation of the trophectoderm to maintain pregnancy, and 4) Methyl donors such as methionine (metabolized to S-adenosyl methionine) are critical for early embryonic development not only for maintenance of free radicals, and polyamine synthesis, but also for regulating transcription and post translational modifications of DNA and histones as will be further described in the One-Carbon Metabolism section.

It is important to note that by d 50 of gestation, there is still not a functional umbilical cord, and therefore the embryo must receive its nutrients from histotrophic secretions and further transport through the placenta to the allantoic fluid and the amnion to the amniotic fluid. Data from our lab (Crouse et al., 2019b) demonstrating the decrease in amino acids in allantoic fluid and increase in amino acids in the amniotic fluid of the restricted heifers, coinciding with decreases in both glucose and fructose may suggest several outcomes for offspring when exploring past maternal recognition of pregnancy: 1) That these offspring may lack energy for growth due to low reducing sugars and thus are utilizing less amino acids for protein synthesis and will shift towards amino acid oxidation for energy, 2) Conversely, an increase in programmed efficiency of the offspring utilizing less amino acids, which is an unlikely

hypothesis, or 3) There are changes in placental and amniotic tissue metabolism and transport of nutrients from histotroph to allantoic and subsequently to the amniotic fluids. There were no differences in fetal crown-rump length of these offspring on d 16, 34 or 50 of gestation due to nutritional treatment (Negrin Pereira et al., 2017). In the same study, hexose and amino acid transporter expression were affected by day of gestation, and not maternal nutritional treatment (Crouse et al., 2017; Greseth et al., 2017a). Current work is being completed to determine if nutrient restriction and day of gestation alter the abundance of hexose and amino acid transporters in bovine-uteroplacental tissues during early pregnancy. These data would more clearly point to a mode of action upon which future research could be conducted to target nutrient flux to and utilization by the feto-placenta for growth during early gestation. These data would also more clearly delineate the specific transitory nutrient requirements for growth during early gestation.

Nutritional Restriction and Programming of Fetal Organ Development

Timing of insult during pregnancy becomes a critical component to understand the repercussions of the insult on future growth and development. The timing and type of insult as well as the programmed effect of energy programming have been previously reviewed (Reynolds and Vonnahme, 2017; Caton et al., 2019; Reynolds et al., 2019). This section aims to identify the programmed effects of nutrient restriction during early gestation on muscle development, liver function, and begin to highlight the role that maternal nutrition may play in programming temperament in livestock.

Nutritional Programming of Muscle Development

Reduced growth rate and feed efficiency pose a significant economic impact to the beef industry. Undoubtedly, maternal nutritional status is one of the extrinsic factors programming

nutrient partitioning and ultimately growth and development of fetal skeletal muscle (Wallace, 1948; Wallace et al., 1999; Godfrey and Barker, 2000; Rehfeldt et al., 2004; Stickland et al., 2004). Growth-restricted neonates are not only at risk of immediate postnatal complications, but also may be 'programmed' to exhibit poor growth and productivity, as well as diseases later in life (Barker et al., 1993; Godfrey and Barker, 2000). This growth restriction seems to be especially important when fetal muscle development (myogenesis) is adversely affected (Handel and Stickland, 1987a, b; Dwyer et al., 1993). Fetal skeletal muscle has a lower priority in nutrient partitioning compared with the brain and heart in response to the challenges during fetal development, rendering fetal muscle particularly vulnerable to nutrient deficiency (Bauman et al., 1982; Close and Pettigrew, 1990). The fetal period is crucial for lifetime skeletal muscle development because no net increase in the number of muscle fibers occurs after birth (Glore and Layman, 1983; Greenwood et al., 2000; Nissen et al., 2003).

Several studies in a range of mammalian species have shown that maternal under-nutrition during gestation can significantly reduce the number of both muscle fibers and myocyte nuclei in the offspring (Bedi et al., 1982; Wilson et al., 1988; Ward and Stickland, 1991). For example, a lower ratio of secondary to primary myofibers and reduced sizes of muscle fasciculi were observed in muscle of fetuses gestated in nutrient-restricted ewes (Zhu et al., 2004). Therefore, muscle fiber type development may be influenced by nutritional state dependent upon the energy needs of the muscle and species observed.

Nutrient restriction of heifers during the first two-thirds of gestation reduced fetal growth and calf birth weight (Micke et al., 2010). Nutrient restriction to 85% of metabolizable energy (ME) compared with 140% of ME in multiparous Angus-Simmental cows resulted in increased expression of myogenic genes *Myogenin* (***MYOG***) and *Myogenic differentiation gene 1*

(***MYOD1***) in offspring of restricted vs. control cows at d 247 of pregnancy (Paradis et al., 2017). Early prenatal nutritional restriction of ewes resulted in a reduction in the number of myofibers but an increased diameter of muscle fibers in offspring at 8 mo of age (Zhu et al., 2006). The finding of enlarged muscle fibers has been confirmed in other muscles in both bovine and ovine fetuses and at 8-mo-of age in lambs when nutrient intake of dams was restricted during early gestation (Du et al., 2005; Du et al., 2010). Additionally, lambs born from ewes that were feed restricted during early- and mid-gestation had increased subcutaneous fat depots, reduced muscle size, and dysregulated glucose uptake compared with lambs from control ewes (Ford et al., 2007). Ewes nutrient restricted to d 31 of gestation had lambs with decreased muscle fiber density in *triceps brachii* compared with lambs of control fed dams (McCoard et al., 1997).

In the bovine, primary muscle fibers develop during the first 2 mo post conception (Russell and Oteruelo, 1981). Secondary muscle fibers, which make up the majority of muscle fibers, form between 2 and 7 to 8 mo of gestation (Russell and Oteruelo, 1981). The formation of secondary myofibers partially overlaps with the formation of intramuscular adipocytes and fibroblasts (Du et al., 2010). The three cell types: myocytes, adipocytes, and fibroblasts, produce the basic structure of skeletal muscle and form at different time points in gestation as seen in Figure 1.3. These data clearly show that maternal nutrient restriction during pregnancy can impact muscle development in offspring and that timing of restriction may have differential effects on muscle fiber development and growth as seen in postnatal phenotypic responses of offspring.

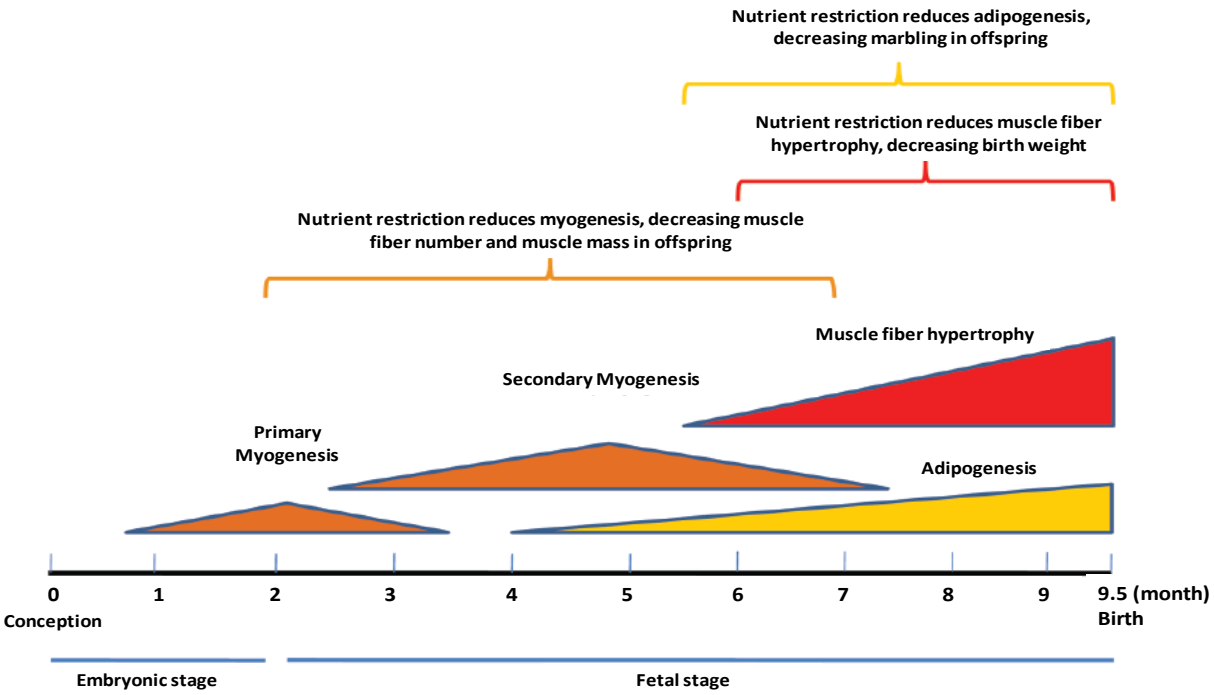

Figure 1.3. Adapted from Du et al., 2010. Effects of maternal nutrition on bovine fetal skeletal muscle development.

Data from our lab (Crouse et al., 2019a) investigating a moderate maternal nutrient restriction (CON = gain 0.45 kg/d, actual ADG = 0.51 kg/d; RES = 60% of CON, actual ADG = -0.08 kg/d) throughout the first 50 d of gestation demonstrated altered expression of genes involved in muscle tissue accretion, function, and metabolism by d 50 of gestation (Table 1.1). Myogenic genes *MYOG* and *MYOD1* were upregulated in offspring of RES treated dams compared with CON which is similar to the previously reported data from Paradis et al., (2017). Furthermore, there was altered expression of genes involved in skeletal muscle function and signaling cascades which suggests that the development of functional contractile proteins may be altered and could potentially lead to altered tenderness; however further experiments would need to be conducted to confirm these hypotheses.

Table 1.1. Adapted from Crouse et al., (2019a). Functional categories and predicted roles for differentially expressed genes that impact tissue metabolism, accretion, and function ($P <$ 0.01) in fetal muscle from hind limb, presented as upregulation or downregulation in fetuses from restricted (RES) heifers compared with control (CON).

Category	Functional annotation[1]	Total genes[2]	Upreg.[3]	Downreg.[4]	*P*-value[5]
Skeletal muscle	Contraction	9	9	0	< 0.001
	Intermediate filament	11	7	4	
	Microtubule	10	2	8	
	Actin	4	3	1	
	Myosin	4	4	0	
	Troponin	6	6	0	
	Calcium-binding	25	14	11	
	ATP-binding	5	0	5	
Embryogenesis	Myogenesis	2	2	0	< 0.001
	Homeobox	12	10	2	
Signaling cascades	Wnt	6	4	2	0.003
	MAPK	12	3	9	

[1]Proposed function of differentially expressed genes that fall under a specific category.
[2]Total number of differentially expressed genes associated within a specific function.
[3]Number of differentially expressed genes that are upregulated in fetuses from RES vs. CON heifers.
[4]Number of differentially expressed genes that are downregulated in fetuses from RES vs. CON heifers.
[5]Probability value associated with a specific category. *P*-value as presented is for the entire pathway, not individual genes within a pathway.

Nutritional Programming of Liver Development

The liver is a key metabolic organ which governs body energy metabolism, and acts as a hub to metabolically connect various tissues, including skeletal muscle and adipose tissue (Rui, 2014). Liver energy metabolism is tightly controlled by multiple nutrient, hormonal, and neuronal signals to regulate glucose, lipid, and amino acid metabolism.

Intrauterine growth restriction is most likely to be associated with the induction of persistent changes in liver tissue structure and functionality (Sookoian et al., 2013). Conversely, a maternal obesogenic environment is most probably associated with metabolic reprogramming

of glucose and lipid metabolism, as well as future risk of metabolic syndrome, fatty liver, and insulin resistance (Sookoian et al., 2013). Exposure to a nutritional insult in early life modulates the functionality of metabolically active target tissues such as the liver, involving a complex network of gene regulation. This effect will result in the programming of a reduced functional capacity of the liver during adult life (Burgueño et al., 2013).

Fetal exposure to utero-placental insufficiency alters the expression of genes encoding enzymes involved with hepatic energy metabolism (Lane et al., 1996), decreasing hepatic oxidative phosphorylation (Ogata et al., 1990) and affecting liver glucose transport. Specifically, protein deficient diets during pregnancy in rat dams induces structural and functional changes in the liver of the offspring affecting glucose production and insulin sensitivity (Burns et al., 1997; Desai et al., 1997a; Desai et al., 1997b).

Maternal realimentation after periods of nutritional insult and the length of the realimentation has the potential to modify tissue mass and energy use which has been observed in cows and fetuses by mid- or late-gestation (Prezotto et al., 2016). Nutrient restriction results in decreased visceral organ mass in sheep (Scheaffer et al., 2004), and impacts maintenance energy expenditure in cattle (Ferrell and Oltjen, 2008). Because different cells in visceral organs develop and differentiate at different stages of gestation (Zaret, 2002; de Santa Barbara et al., 2003), timing of nutrient restriction during gestation may differentially impact specific fetal tissue development and function (Prezotto et al., 2016). Calves from cows nutrient restricted from d 30 to 85 of gestation and realimented through the remainder of gestation had heavier livers than calves from control fed dams on d 254 of gestation (Prezotto et al., 2016). Hepatic oxygen consumption increased in calves from cows restricted from d 85 to 140 of gestation compared with calves from control cows and calves from cows restricted from d 30 to 85

(Prezotto et al., 2016). Additionally, it is hypothesized that these changes during early- and mid-gestation could potentially contribute to adaptations of the fetus to become more efficient in the utilization of nutrients later in life (Camacho et al., 2014).

Liver metabolic programming may also program liver mitochondrial DNA copy number (Burgueño et al., 2013). There is evidence from human studies that epigenetic mechanisms influence mitochondrial function and critically modulate insulin resistance and metabolic syndrome related phenotypes by altering not only the nuclear but also the mitochondrial genome (Sookoian et al., 2010; Pirola et al., 2013).

Data from our lab (Crouse et al., 2019a) investigating a moderate maternal nutrient restriction (CON = gain 0.45 kg/d, actual ADG = 0.51 kg/d; RES = 60% of CON, actual ADG = -0.08 kg/d) throughout the first 50 d of gestation demonstrated altered expression of genes involved in liver tissue function and metabolism by d 50 of gestation Table 1.2. The pathway with the greatest number of differentially expressed genes was the metabolic pathways ontology. These data showed altered expression in metabolism of proteins, fats, and carbohydrates, as well as well differential expression in mitochondrial genes and reducing equivalents. Furthermore, alterations to histone and histone modifying genes which regulate gene expression were altered. Together these data follow previously published data from epidemiological studies in humans demonstrating that early nutritional restriction of dams alters the postnatal metabolism of the offspring (Ravelli et al., 1976) and that some of these changes were due to sustained epigenetic modifications (Heijmans et al., 2008; Tobi et al., 2009).

Table 1.2. Adapted from Crouse et al., (2019a). Functional categories and predicted roles for differentially expressed genes that impact tissue metabolism, accretion, and function ($P < 0.01$) in fetal liver presented as upregulation or downregulation in fetuses from restricted (RES) heifers compared with control (CON).

Category	Functional annotation[1]	Total genes[2]	Upreg.[3]	Downreg.[4]	*P*-value[5]
Metabolic pathways	Amino acid	10	5	5	0.017
	Purine and pyrimidine	7	7	0	
	Carbohydrate	10	5	5	
	Reducing equivalent (NAD/FAD)	5	5	0	
	Steroid and lipid biosynthesis	9	8	1	
	Cytochrome and heme	2	2	0	
Protein kinase	Serine/Threonine protein kinase	22	21	1	0.020
	ATP-binding	19	15	4	
	Nucleotide-binding	6	4	2	
Nucleosome core	Histones	9	9	0	0.005
	Histone modifiers	13	12	1	
mRNA splicing	Spliceosome	7	6	1	0.041
Complement/ Coagulation	Complement factors	3	3	0	0.041
	Coagulation factors	3	3	0	

[1]Proposed function of differentially expressed genes that fall under a specific category.
[2]Total number of differentially expressed genes associated within a specific function.
[3]Number of differentially expressed genes that are upregulated in fetuses from RES vs. CON heifers.
[4]Number of differentially expressed genes that are downregulated in fetuses from RES vs. CON heifers.
[5]Probability value associated with a specific category. *P*-value as presented is for the entire pathway, not individual genes within a pathway.

Nutritional Programming of Cerebrum Development and Potential Impacts on Temperament

The brain may play a greater role in increasing production efficiency than is currently being acknowledged; however, due to limited market value, is not generally considered a

production tissue and little research focus has been placed on understanding brain development and function in livestock. The brain is one of the most conserved and developmentally plastic tissues in the body; however, it is highly susceptible to malnutrition (Georgieff, 2007). Multiple studies have elucidated the detrimental effects of maternal malnutrition (global malnutrition, protein, lipid, carbohydrate, micronutrient, etc.) during gestation on the growth and development of the brain as well as programmed temperament of the offspring due to alterations to functional control centers in the brain (Golub et al., 1994; McEchron and Paronish, 2005).

In addition to the maintenance of neuroendocrine signaling, the hippocampus of the cerebrum plays a critical role in emotion and memory (Engin and Treit, 2007). Hippocampal proteins which regulate synapse development and function include metal-binding proteins as well as actin filaments, which if altered, can lead to cognitive dysfunction and abnormal startle response (Lamprecht, 2014; Cristóvão et al., 2016). Recent advancements in animal science have been made to improve cattle temperament through genetic selection (Haskell et al., 2014) as well as improved working facilities (Grandin, 1997). Further understanding of nutritional programming in livestock may elucidate mechanisms by which cattle temperament may be altered due to maternal nutrition.

Beyond changes to temperament, maternal malnutrition in sheep affected offspring feed intake at 6 years of age (George et al., 2012), increasing feed intake of lambs born to mothers who were nutrient restricted from d 28 to 78 of gestation. The hippocampus has receptors for many metabolic and neuroendocrine hormones including: cholecystokinin, leptin, ghrelin, glucagon-like peptide 1, motilin, and amilin along with receptors for central nervous system neuropeptides including melanocortin-4 and orexin. Although there is a plethora of knowledge about neuroendocrine receptors in the hippocampus, knowledge of receptor mediated

hippocampal modulation of food intake via response to hormonal and neuroendocrine signaling remains a largely unexplored area (Kanoski and Grill, 2017). These gaps in research remain although connections between maternal malnutrition (undernutrition, famine, protein restriction, and high-fat diets) modulating hippocampal function, cognition, and animal intake have been demonstrated (Lucassen et al., 2013).

Data from our lab (Crouse et al., 2019a) investigating a moderate maternal nutrient restriction (CON = gain 0.45 kg/d; RES = 60% of CON, actual ADG = -0.08 kg/d) throughout the first 50 d of gestation altered the expression of genes involved in cerebrum development and function by d 50 of gestation (Table 1.3). These data suggest that hippocampal as well as metal-binding and cytoskeletal genes necessary for the formation and function of synapses may be affected in cattle by early maternal nutrient restriction. Therefore, maternal nutritional restriction during early gestation may "program" offspring towards a temperament that is more sensitive to stimuli.

Of greatest interest of the data reported by Crouse et al., (2019a) was the fact that 78% of all differentially expressed genes in fetal muscle, liver, and cerebrum were upregulated in offspring of restricted compared with control dams. Compensatory gain in offspring born to nutrient restricted cows that were subsequently realimented throughout gestation has been well demonstrated (Camacho et al., 2014). The thrifty phenotype hypothesis further supports the concept of compensatory gain following realimentation in that the phenotype of nutrient restricted offspring has been programmed to rely on fewer nutrients; but these adaptations to programming have produced a mismatched phenotype which once realimented proves to be detrimental (Hales and Barker, 1992). While this may be the case for the upregulated genes establishing for compensatory gain, there may be rationale for investigating

Table 1.3. Functional categories and predicted roles for differentially expressed genes that impact tissue metabolism, accretion, and function ($P < 0.01$) in fetal cerebrum presented as upregulation/downregulation in fetuses from restricted (RES) heifers compared with control (CON)

Category	Functional annotation[1]	Total genes[2]	Upreg.[3]	Downreg.[4]	*P*-value[5]
Hippocampus and neurogenesis	Hippo signaling pathway	5	5	0	< 0.001
	Collagen	9	9	0	
	Netrin	5	5	0	
	SMAD	4	4	0	
	Developmental protein	9	8	1	
Metal-binding	Iron-binding	4	4	0	0.006
	Zinc-binding	10	10	0	
	Copper-binding	2	2	0	
	Nickel-binding	1	1	0	
	Calcium-binding	6	5	1	
Cytoskeleton	Actin remodeling	5	5	0	0.003

[1]Proposed function of differentially expressed genes that fall under a specific category.
[2]Total number of differentially expressed genes associated within a specific function.
[3]Number of differentially expressed genes that are upregulated in fetuses from RES vs. CON heifers.
[4]Number of differentially expressed genes that are downregulated in fetuses from RES vs. CON heifers.
[5]Probability value associated with a specific category. *P*-value as presented is for the entire pathway, not individual genes within a pathway.

whether the upregulation of these genes is in fact because this is the most appropriate nutrient requirement for growth and that the offspring of control treated heifers are down-regulated compared with the restricted. The data produced from our lab cannot confirm these hypotheses; however, due to the fact that the majority of data has not shown a negative consequence of early maternal nutrient restriction in beef cattle on muscle development or metabolism, may support the fact that the CON offspring are truly downregulated. The detrimental effects of maternal nutrient restriction in early gestation may not be evident during early life, and thus future studies should investigate cows and bulls later in their lives and the production cycle to determine if the

reasons for culling may be due to factors that have been programmed *in utero* and are manifesting themselves in later postnatal life.

One-Carbon Metabolism

One-carbon metabolites (**OCM**) include B-vitamins (choline, vitamin B_{12}, vitamin B_6, riboflavin, and folate), minerals (cobalt [component of vitamin B_{12}], sulfur [component of methionine] and amino acids (methionine, serine, and glycine (Clare et al., 2019). One-carbon metabolites are methyl donors and cofactors that play key roles in one-carbon metabolism, which is the network of biochemical pathways in which methyl groups are transferred from one compound to another for methylation processes (Mason, 2003). Perturbation in the one-carbon metabolism cycle, either by nutrient deficiency, or by nutritional, hormonal, and environmental interactions can have a profound impact on cellular function, metabolism, growth, and proliferation, which has the greatest impact on the growing embryo and fetus (Kalhan and Marczewski, 2012). The metabolism of OCM are interrelated and disturbances in one of these metabolic pathways are associated with compensatory changes in the others (Zeisel, 2011).

Linking One-Carbon Metabolism, Growth, and Energetic Pathways

The following section is diagrammed in Figure 1.4 which has been reviewed in detail by Clare et al., (2019), and will be described briefly in this section to demonstrate the interconnected role of OCM, growth, redox reactions, and energetics. Methionine is the precursor for S-adenosylmethionine (**SAM**), which is the physiological methyl group donor for protein, RNA, and DNA methylation (Jeltsch, 2002). The DNA and histone methyltransferases exclusively use SAM as the methyl donor. S-adenosylmethionine availability is directly influenced by diet as it is formed from methyl-groups derived from choline, methionine, or methyl-tetrahydrofolate (Zeisel, 2011). Upon donating the methyl group, SAM is converted to S-

adenosylhomocysteine (**SAH**) and further converted to homocysteine. Homocysteine has multiple fates: 1) Remethylated to methionine by betaine homocysteine methyltransferase (**BHMT**) which methylates homocysteine to methionine using betaine as a methyl donor (Kim et al., 1994; Finkelstein, 2000), 2) Remethylate to methionine by methionine synthase (**MTR**) which is a vitamin B_{12}-dependent enzyme that uses 5-methyltetrahydrofolate as a methyl donor (Kim et al., 1994; Finkelstein, 2000), 3) Transsulfuration of homocysteine to cystathione by cystathione β-synthase (**CBS**) which is a condensation reaction with homocysteine and serine with vitamin B_6 as a cofactor (Clare et al., 2019). These three potential routes for homocysteine are heavily regulated through the abundance of methionine, folate, and SAM (Selhub, 1999). When methionine is limiting, but folate, choline/betaine and vitamin B_{12} are not, homocysteine will be metabolized via BHMT or MTR to synthesize methionine. When folate, methionine, and SAM are in sufficient supply, SAM inhibits the synthesis of 5-methyltetrahydrofolate by 5-methyltetrahydrofolate reductase (**MTHFR**) and stimulates CBS to metabolize homocysteine. for cardiovascular and cerebrovascular diseases (Škovierová et al., 2016), and therefore metabolism via one of the three pathways (BHMT, MTR, or CBS) must occur to prevent subsequent pathologies.

The directionality of homocysteine metabolism is based upon the presence of OCM. When sufficient methionine and SAM are present for methylation processes, SAM can be metabolized for the production of spermidine and spermine. S-adenosylmethionine is a limiting cofactor in the synthesis of polyamines as it provides the aminopropyl group for the synthesis of spermidine and spermine (Pegg, 2016). Furthermore, due to the negative inhibition of MTHFR by increased SAM, 5, 10 methyltetrahydrofolate can be used for the synthesis of purines with

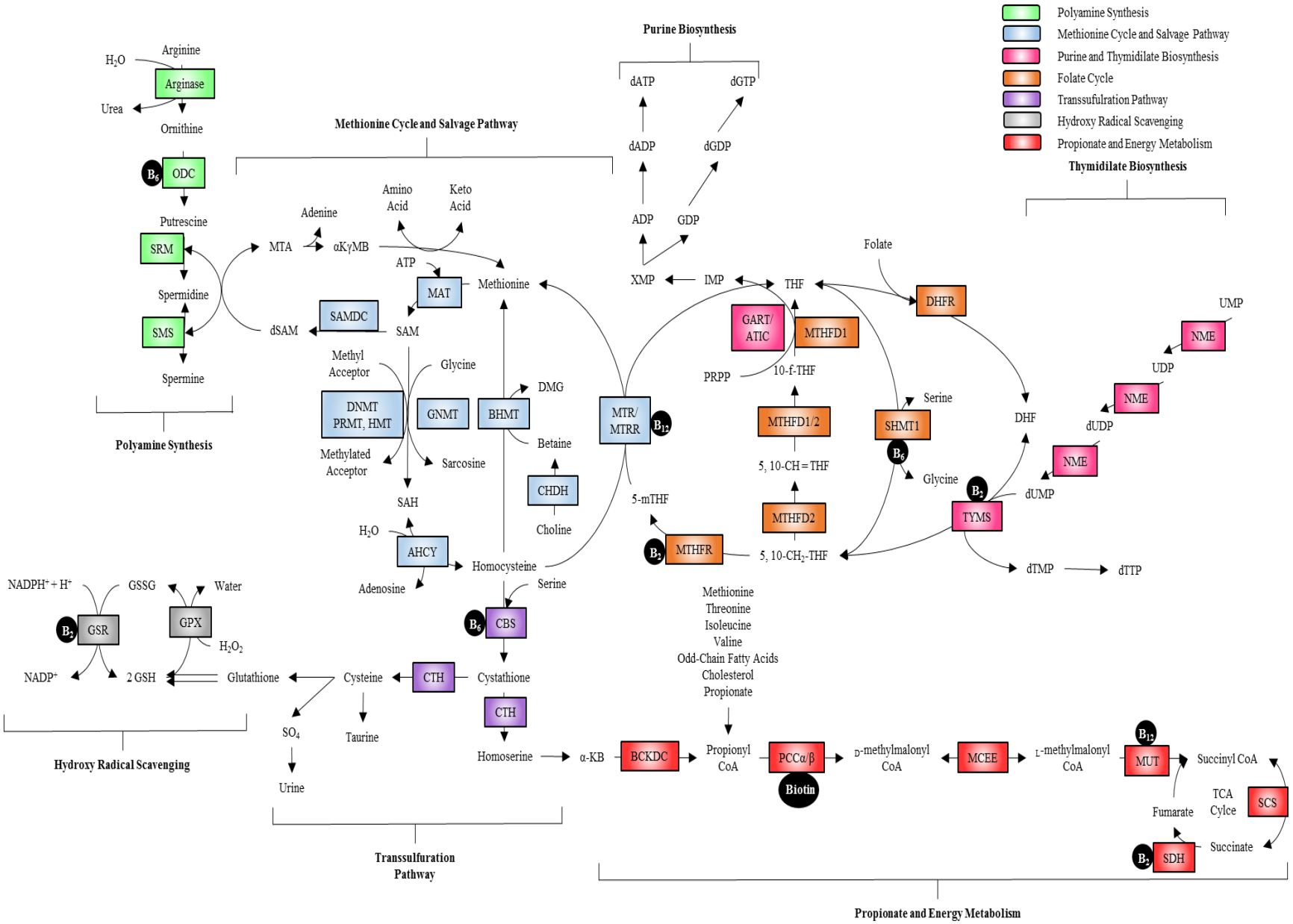
Polyamine Synthesis
Methionine Cycle and Salvage Pathway
Purine and Thymidilate Biosynthesis
Folate Cycle
Transsufulration Pathway
Hydroxy Radical Scavenging
Propionate and Energy Metabolism
Purine Biosynthesis
Thymidilate Biosynthesis
Arginine
Arginase
Urea
Ornithine
ODC
Putrescine
SRM
Spermidine
SMS
Spermine
Polyamine Synthesis
MTA
Adenine
αKγMB
Amino Acid
Keto Acid
ATP
MAT
Methionine
SAMDC
dSAM
SAM
Methyl Acceptor
Glycine
DNMT PRMT, HMT
GNMT
BHMT
DMG
Betaine
CHDH
Choline
Methylated Acceptor
Sarcosine
SAH
AHCY
Adenosine
Homocysteine
Serine
CBS
Cystathione
CTH
Cysteine
Glutathione
GSR
GPX
NADPH + H+
GSSG
Water
H2O2
NADP+
2 GSH
SO4
Taurine
Urine
Hydroxy Radical Scavenging
CTH
Homoserine
Transsulfuration Pathway
dATP
dGTP
dADP
dGDP
ADP
GDP
XMP
IMP
THF
PRPP
GART/ ATIC
MTHFD1
10-f-THF
MTR/ MTRR
5-mTHF
MTHFR
MTHFD1/2
5, 10-CH = THF
MTHFD2
5, 10-CH2-THF
Folate
DHFR
DHF
SHMT1
Serine
Glycine
TYMS
dTMP
dTTP
UMP
NME
UDP
dUDP
dUMP
Methionine
Threonine
Isoleucine
Valine
Odd-Chain Fatty Acids
Cholesterol
Propionate
α-KB
BCKDC
Propionyl CoA
PCCα/β
Biotin
D-methylmalonyl CoA
MCEE
L-methylmalonyl CoA
MUT
Succinyl CoA
TCA Cylce
SCS
Fumarate
Succinate
SDH
Propionate and Energy Metabolism

Figure 1.4. Integration of one-carbon metabolism, energy metabolism, growth, and hydroxy radical scavenging. Enzymes are in boxes. B-vitamin cofactors are shown in black circles.

- Polyamine Enzymes (Green Boxes): ODC- Ornithine Decarboxylase; SRM- Spermidine Synthase; SMS- Spermine Synthase.
- Methionine Cycle and Salvage Pathway Enzymes (Blue Boxes): MAT- Methionine Adenosyltransferase; SAMDC- S-Adenosyl Methionine De Carboxylase; DNMT- DNA Methyl Transferase; PRMT- Protein Methyl Transferase; HMT- Histone Methyl Transferase; GNMT- Glycine-*N*-Methyl Transferase; AHCY- Adenosyl Homocysteinase; CHDH- Choline Dehydrogenase; BHMT- Betaine Homocysteine Methyl Transferase; MTR- Methionine Synthase; MTRR- Methionine Synthase Reductase.
- Methionine Cycle and Salvage Pathway Substrates: MTA- 5'-methylthiodenosine; SAM- S-adenosylmethionine; SAH- S-adenosylhomocysteine; DMG- Dimethyl glycine.
- Purine and Thymidilate Synthesis Enzymes (Pink Boxes): TYMS- Thymidilate Synthase; NME- Nucleoside Diphosphate Kinase A.
- Purine and Thymidilate Synthesis Substrates: IMP- Inosine monophosphate; XMP- Xanthine monophosphate; ADP- Adenosine diphosphate; GDP- Guanosine Disphosphate; dADP- Deoxyadenosine Diphosphate; dGDP- Deoxyguanosine Diphosphate; dATP- Deoxyadenosine Triphosphate; dGTP- Deoxyguanosine Triphosphate; UMP- Uridine Monophosphate; UDP- Uridine Diphosphate; dUDP- Deoxyuridine Diphosphate; dUMP- Deoxyuridine Monophosphate; dTMP- Deoxythymidilate Monophosphate; dTTP- Deoxythymidilate Triphosphate.
- Folate Cycle Enzymes (Orange Boxes): MTHFR- Methyl Tetrahydrofolate Reductase; MTHFD2- Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase; MTHFD1- methylenetetrahydrofolate dehydrogenase 1; GART- Phosphoribosylglycinamide formyltransferase; ATIC- 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase; DHFR- Dihydrofolate Reductase; SHMT1- Serine Homocysteine Methyltransferase.
- Folate Cycle Substrates: 5-mTHF- 5-methyltetrahydrofolate; 5,10-CH2-THF- 5,10 methylene-tetrahydrofolate; 5,10-CH = THF- 5,10 methenyl-tetrahydrofolate; 10-f-THF- 10-formyl-tetrahydrofolate; THF- Tetrahydrofolate; FA- Folic Acid; DHF- Dihydrofolate.
- Transsulfuration Pathway Enzymes (Purple Boxes): CBS- Cystathione Beta Synthase; CTH- Cystathione gamma-lyase.
- Hydroxy Radical Scavenging Pathway Enzymes (Grey Boxes): GPX- Gluthathione Peroxidase; GSR- Glutathione Reductase.
- Hydroxy Radical Scavenging Pathway Substrates: 2 GSH- 2 Reduced Glutathione; GSSG- oxidized glutathione.
- Propionate and Energy Metabolism Pathway Enzymes (Red Boxes): BCKDC- Branched-Chain α-Ketoacid Dehydrogenase Complex; PCCα/β- Propionyl-CoA Carboxylase; MCEE- Methylmalonyl-CoA Epimerase; MUT- Methylmalonyl-CoA Mutase; SCS- Succinyl-CoA Synthetase; SDHA- Succinate Dehydrogenase.
- Propionate and Energy Metabolism Substrates: αKγMB- α-keto-γ-methiolbutyrate; α-KB- α-ketobutyrate

input of phosphoribosyl pyrophosphate which is synthesized from ribose 5-phosphate of the pentose phosphate pathway. Additionally, folate can be utilized to further synthesize thymidylate via thymidilate synthase enzyme. Impaired one-carbon metabolism resulting from protein malnutrition and deficiencies in folate and B_{12} (Castro et al., 2006) increases plasma levels of homocysteine which is a risk factor

Concentrations of homocysteine can increase due reduced remethylation to methionine, and thus, homocysteine will be metabolized to cystathione via CBS, and from here diverges into three different end points: 1) Insufficient OCM leads to metabolism via sulfates to urine through the transulfuration pathway, 2) Metabolized via the transulfuration pathway to the propionate and energy metabolism pathway to synthesize succinyl-CoA which is further metabolized to Succinate and Fumarate in the TCA cycle, which links one-carbon metabolism to energy production and to the electron transport chain as Succinate dehydrogenase (**SDHA**) which is complex two of the electron transport chain, and 3) Metabolism via the transulfuration pathway to produce glutathione to serve as a hydroxyl radical scavenger, which also utilizes NADPH as a cofactor produced from the pentose phosphate pathway. Therefore, one overarching conclusion and hypothesis emerges: When OCM are insufficient, there will be aberrant methylation and transcriptional control during early development with increased homocysteine due to inability to remethylate homocysteine to methionine which must be converted via the transulfuration pathway to sulfates for expulsion in the urine. Conversely, when sufficient OCM are present to remethylate homocysteine to methionine, the coordinated cyclic nature of the methionine and folate cycles will maintain methylation of DNA, RNA, and protein. Furthermore, when sufficient OCM and SAM are present, there is a shift in the pathways to synthesize polyamines, nucleotides, energy metabolites, and hydroxyl radical scavenging metabolites to allow for

increased growth as well as the maintenance of redox potential to prevent oxidative damage to rapidly proliferating cells such as that of the embryo.

Lastly, from the available data presented within and by others, the increases in fructose, arginine, and redox substrates and enzymes such as glutathione and glutathione peroxidase suggest the coordinated nature of strategic supplementation of methionine, folate, vitamin B_{12}, and choline, which are utilized within the one-carbon metabolism cycle to maintain regulated embryonic growth and thus may be key substrates for future targeted nutritional strategies.

Epigenetic Programming During Embryonic Development

Epigenetic modifications include those such as methylation of DNA and RNA, methylation, acetylation, and phosphorylation of histone tails, as well as non-coding RNA molecules, all of which can affect the processes of transcription and subsequent translation to functional proteins (Armstrong, 2014), and thus coordinated embryonic development. DNA methylation can regulate gene expression by modulating the binding of methyl-sensitive DNA-binding proteins (Bird, 1992; Hendrich and Bird, 1998), thereby condensing regional chromatin conformation and inhibiting transcription. Histone acetylation or methylation can alter the positioning of histone binding to DNA; although these effects can result in either a heterochromatic or euchromatic DNA state depending on the modification, and thus a clear understanding of the type of epigenetic modification becomes instrumental to identifying the subsequent change in transcriptional state (Jaenisch and Bird, 2003a; Armstrong, 2014).

Embryos undergo immense epigenetic remodeling during early development including DNA methylation, as well as epigenetic modifications of histone tails such as methylation, acetylation, and phosphorylation to regulate transcription, growth, and cell fate leaving a “memory” on the embryo that will be carried forward through subsequent developmental stages

(Sugden and Holness, 2002; Jaenisch and Bird, 2003a; Waterland and Jirtle, 2004). Methylation is almost exclusively at CpG motifs (with some non-CpG methylation present in the brain). CpG islands are dense clusters of CpG motifs (Armstrong, 2014). During the epigenetic reprogramming of the embryos, there is a wave of global demethylation, resulting in the demethylation of both the maternal and paternal genome (Messerschmidt et al., 2014); however, the kinetics of demethylation differ between the maternal and paternal DNA. Post-fertilization, the paternal DNA is actively demethylated followed by a slower passive demethylation of the maternal DNA occurring through the 8 cell to morula stage of development (Dean et al., 2001; Hou et al., 2007; Dobbs et al., 2013). Active demethylation occurs through the ten-eleven translocation (**TET**) enzyme mediated hydroxylation of 5mC to 5hmC (Gu et al., 2011). The limited remaining 5mC are further diluted by DNA replication (Inoue et al., 2011; Inoue and Zhang, 2011). This is because there is a lack of nuclear DNA methyltransferase 1 (**DNMT1**) enzyme in preimplantation embryos (Hirasawa et al., 2008) which selectively binds to hemimethylated DNA with limited affinity for oxidized 5mC products (Hashimoto et al., 2012). Binding to hemimethylated DNA results in maintenance methylation, and thus a lack of DNMT1 in embryos results in the further decrease in 5mC during subsequent replication stages. The maternal genome, unlike the paternal genome, is demethylated via passive processes due to a protective effect of the protein, developmental pluripotency associated 3 in oocytes which blocks 5mC from conversion to 5hmC (Bakhtari and Ross, 2014; Ross and Sampaio, 2018). Because nuclear DNMT1 is not present in the preimplantation embryo, maternal DNA is demethylated due to dilution of 5mC during replication (Hirasawa et al., 2008). The exception to the rule are imprinted genes which remain at their respective methylated levels throughout epigenetic reprogramming due to a targeting of DNMT1 from the cytosol to specific imprinted control

regions of the DNA by the interaction of a Kreuppel-associated box domain zinc finger protein ZFP57 which mediates the targeted binding of DNMT1 to hemimethylated DNA of imprinted genes thereby maintaining their methylation (Messerschmidt, 2012; Messerschmidt et al., 2014). Subsequent remethylation of the embryonic genome occurs via *de novo* methyl transferases DNMT3A and DNMT3B (Okano et al., 1999) and after establishment of methylation, is maintained by DNMT1 which binds to hemimethylated DNA (Hermann et al., 2004).

Epigenetic reprogramming reflects genotype, developmental history, as well as environmental influences, which ultimately become reflected in the phenotype of the cell and the organism (Morgan et al., 2005). Epigenetic control via methylation of DNA during early embryonic development is important for the commitment of cells to their differentiated cellular fate (Messerschmidt et al., 2014). These epigenetic modifications such as that of methylation are heritable throughout mitotic divisions and thus facilitate specific cell lineages and transcription profiles (Jaenisch and Bird, 2003b; Messerschmidt et al., 2014). Therefore, it is intuitive that methylation profiles differ between the ICM and trophectoderm which differentiate into the embryo and placenta, respectively. Multiple reports have demonstrated differential methylation between the ICM and trophectoderm, though these studies are not in agreement on whether methylation is greater in the ICM or trophectoderm (Dean et al., 2001; Hou et al., 2007; Dobbs et al., 2013). However, these data all point to the importance of OCM for the ability to provide the methyl donor SAM for methylation processes of DNA, RNA, and proteins to occur. Due to these events during the earliest stages of gestation, the proper milieu of OCM is needed to mitigate the effects of poor environmental influences and program the embryo towards improved physiological efficiencies.

One-Carbon Metabolites in Ruminants

The committee drafting the 2016 version of the Nutrient Requirements of Beef Cattle (NASEM, 2016) indicated that the current understanding of the roles of OCM in beef cattle diets during gestation is “insufficient”, and therefore, “additional research in one-carbon metabolism, folic acid, and other factors affecting the metabolic one-carbon pool would likely provide needed insight into developmental and epigenetic events during the fetal and perinatal periods of growth.” When evaluating the current knowledge of OCM, the majority of research has been focused towards transitioning dairy cows with effort focusing on increased milk production (Girard et al., 2005; Girard and Matte, 2005; Preynat et al., 2009a, b; Sun et al., 2016).

Recent publications have investigated the effects of methionine supplementation to dairy cows during the last 28 d of gestation and the programmed effects of offspring growth and metabolism. Rumen protected methionine supplementation for the last 28 days of gestation increased cow dry matter intake (**DMI**) and calf birth weight; however, it is hard to distinguish whether the increase calf birth weight was due to methionine supplementation or the increase in DMI (Batistel et al., 2017). Additionally, there was an increase in plasma methionine and BHMT in methionine supplemented cows as well as total mTOR and the ratio of active to inactive mTOR in the placentome of the methionine supplemented cows (Batistel et al., 2017). The follow up study on the same calves (Alharthi et al., 2018) reported greater average daily gain in calves born to methionine supplemented dams to 9 wk of age despite no difference in intake between the calves born to supplemented and unsupplemented cows. Furthermore, there were day × treatment interactions such that the plasma concentrations of glutamate at d 2 and 7 were greater in calves born to methionine supplemented damns, arginine was greater at d 7 of age in calves born to supplemented dams, taurine was greater at d 2 and less at d 42 of age in calves

born to supplemented dams, and glucose was less at d 2 and greater at d 42 in calves born to supplemented dams. These data were interpreted to imply that supplemental methionine to cows during the last 28 d of gestation increased calf growth *in utero* and programmed the increased growth efficiency postnatally. Furthermore, these data suggest a programming effect of energy and one-carbon metabolism due to alterations in metabolites which play integral parts of one-carbon metabolism and energy supply including methionine, glutamate, glutamine, arginine, and taurine.

Limited data is available on the effects of OCM supplementation to periconceptual or gestating beef cattle. Supplementation of methionine to late gestation beef heifers grazing dormant rangelands improved heifer amino acid utilization but did not improve heifer body weight or calf birth-weight (Waterman et al., 2012). Methionine supplementation to late gestation fall-calving beef cows did not affect cow performance, milk production, subsequent reproductive performance or offspring performance (Clements et al., 2017). Calves from cows supplemented with rumen protected methionine had altered liver metabolism such that methionine, choline, and homocysteine metabolism shifted partly to synthesize taurine and glutathione, which would be advantageous for controlling metabolic-related stress (Jacometo et al., 2017).

In a study by Sinclair et al., (2007) Ewes fed a diet deficient in sulfur and cobalt (components of methionine and Vitamin B_{12}, respectively) for -60 to +6 d relative to conception not only had decrease methionine and Vitamin B_{12} at d +6 relative to conception, but also had decreased folate and increased homocysteine in ewe plasma as well demonstrating the interconnected network in the one-carbon metabolism cycle and the perturbation to increase homocysteine. Furthermore these effects on the one-carbon metabolism cycle were seen in the

ovarian granulosa cells with increased homocysteine, decreased SAM, and a decreased SAM:SAH in the methyl deficient treated ewes. Of greatest interest were the programmed effects to the methyl deficient offspring *in utero* and postnatally. Embryos collected from the control and methyl deficient ewes were transferred to recipient ewes all managed similarly, thus limiting the *in utero* and postnatal effects to the ewes who were on treatment prior to embryo transfer. Fetal liver collected at d 90 of gestation (~60% of gestation) had differential methylation at 57 loci or 4% of the 1,400 CpG loci examined. When comparing methyl deficient to control, 88% of the differentially methylated loci were hypomethylated with the remaining 12% being hypermethylated. Furthermore, sex-specific changes to DNA methylation were elucidated with 52.6% of differential methylation being specific to males and 12.2% being specific to female offspring. At 22 mo of age, there were sex × maternal diet responses to glucose challenge in the offspring, with methyl deficient males having greater plasma insulin than the methyl deficient females, who were greater than control male and females. Furthermore, male offspring had greater resting blood pressure and greater systolic blood pressure than control males at 23 mo of age as well as 25% greater body fat at 22 mo of age when compared to the offspring born to control treated mothers. These data demonstrate that programmed effects of deficient OCM during early gestation on offspring development with greater programmed effects of aberrant metabolism toward male offspring. The greatest inference from this study was that the *in utero* and postnatal effects were all seen from treatments initiated during the periconceptual period of gestation. These data demonstrate the importance of OCM during oocyte maturation and early embryonic development to the lifelong productivity to animals in the herd.

Conclusion and Future Directions

The concepts of nutritional programming of offspring development have been a focal point of increased study across multiple mammalian species; however, this focus has yet to be placed in beef cattle, especially during early gestation. Although the whole energy nutrient requirements of heifers during the first trimester of pregnancy are not much greater than the maintenance requirements of non-pregnant heifers, this does not mean that there aren't specific transient nutrient requirements during this time frame to increase the likelihood of maintaining the pregnancy and improve programming outcomes for calves. There are multiple events which must take place during early embryonic development to ensure successful establishment of pregnancy, and at this time it has been established that the doubling rate of the embryo is greater than it will be at any other time in the life of the offspring. Therefore, further investigation during early gestation in beef cattle should focus on transient nutrient requirements and targeted supplementation to not only maintain pregnancies, but also program whole animal efficiencies throughout the production cycle. These programmed efficiencies may not be seen in calves which are finished for production of food during the first 2 years of life but are most likely to be elucidated once these cows and bulls become mature. Improvements in cow longevity and maintaining cows in the herd for additional breeding years would be a major contributor to increasing producer profits. Furthermore, understanding transient nutrient requirements during specific time points in gestation may decrease excess shedding of nutrients and feed losses, thus decreasing feed expenses as well as the carbon footprint of raising beef cattle.

Work from our lab and others have demonstrated changes to available metabolites in uterine and fetal fluids based on hormone concentrations and maternal nutritional status. Based upon the available data, these transient requirements during early gestation seem to point to

energy metabolites such as fructose, as well as those for integration into the one-carbon metabolism cycle of growth and DNA methylation such as choline, folate, vitamins B_{12}, and B_6 as well as amino acids methionine, serine, glycine, and arginine for the synthesis of polyamines and vasoregulators such as nitric oxide and its precursors.

Lastly, understanding the role of epigenetic programming of offspring development and function will further point to nutrient requirements to allow for epigenetic programming that will increase the health and viability of offspring postnatally. Studies evaluating periconceptual supplementation of OCM may further demonstrate the increased need of OCM to the early embryo in beef heifers and provide a means for which producers can begin to strategically supplement their herd.

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CHAPTER 2. MODERATE NUTRIENT RESTRICTION OF BEEF HEIFERS ALTERS EXPRESSION OF GENES ASSOCIATED WITH TISSUE METABOLISM, ACCRETION, AND FUNCTION IN FETAL LIVER, MUSCLE, AND CEREBRUM BY D 50 OF GESTATION[1]

Abstract

We hypothesized that a moderate maternal nutrient restriction during the first 50 d of gestation in beef heifers would affect transcript abundance of genes associated with tissue metabolism, accretion, and function in fetal liver, muscle, and cerebrum. Angus-cross heifers were estrus synchronized and assigned at breeding to one of two dietary treatments (CON- 100% of nutrient requirements to gain 0.45 kg/d; RES- 60% of CON). At d 50 of gestation, 14 heifers were ovariohysterectomized, and fetal liver, muscle, and cerebrum were collected. Transcriptome analysis via RNA-seq was conducted on the Illumina HiSeq 2500 platform using 50-bp paired-end reads at a depth of 2 × 10.4M reads/sample. Bioinformatic analysis was performed using the Tuxedo Suite and ontological analysis with DAVID 6.8. For fetal liver, muscle, and cerebrum, a total of 548, 317, and 151 genes, respectively ($P < 0.01$) were differentially expressed, of which 201, 144, and 28 genes, respectively were false discovery rate protected (FDR; $q < 0.10$). Differentially expressed genes were screened for fit into functional categories of pathways or ontologies associated with known impacts on tissue metabolism,

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[1]The material in this chapter was co-authored by M. S. Crouse, J. S. Caton, R. A. Cushman, K. J. McLean, C. R. Dahlen, P. P. Borowicz, L. P. Reynolds, and A. K. Ward. It has been published by Translational Animal Science, doi: 10.1093/tas/txz026. M. S. Crouse and K. J. McLean had primary responsibility for collecting samples. M. S. Crouse was the primary developer of the conclusions that are advanced here. M. S. Crouse also drafted and revised all versions of this chapter. A. K. Ward and J. S. Caton served as proofreader and R. A. Cushman helped with bioinformatics and checked the statistical analysis conducted by M. S. Crouse

accretion, and function. In fetal liver, 5 functional categories of interest (n = 125 genes) were affected by nutritional treatment: metabolic pathways, protein kinase, nucleosome core, mRNA splicing, and complement/coagulation cascades, of which 105 genes were upregulated in RES. In fetal muscle, 3 functional categories of interest (n = 106 genes) were affected by nutritional treatment: skeletal muscle, embryogenesis, and signaling cascades, of which 64 genes were upregulated in RES. In fetal cerebrum, 3 functional categories of interest (n = 60 genes) were affected by nutritional treatment: hippocampus and neurogenesis, metal-binding, and cytoskeleton, of which 58 genes were upregulated in RES. These results demonstrate that a moderate maternal nutrient restriction during the first 50 d of gestation in beef heifers alters transcript abundance of genes potentially impacting tissue metabolism, accretion, and function in fetal liver, muscle, and cerebrum. Furthermore, these results indicate that affected categories are tissue-specific and moderate maternal nutrient restriction generally increases expression of genes in fetuses from RES fed dams. Finally, these data lay the foundation upon which further research that identifies phenotypic responses to changes in these pathways may be elucidated.

Key words: developmental programming, fetus, nutrition, RNA-Seq

Introduction

Research investigating developmental programming, or the phenomenon in which maternal metabolic state, physiological traits, or environmental factors influence fetal growth and development leading to permanent changes in postnatal physiology (Barker and Clark, 1997), has emphasized the importance of the uterine environment for the developing fetus. During the early phase of fetal development, differentiation and vascularization of utero-placental tissues as well as fetal organogenesis occur, all of which are critical events for normal fetal development (Funston et al., 2010). Dams that undergo stress (nutritional, environmental,

etc.) during early, but not late gestation, are likely to produce normal birth weight offspring that may still suffer from poor growth and metabolic issues because of the stress early in pregnancy (Ford et al., 2007; Vonnahme et al., 2007; Reynolds and Caton, 2012). These stress-induced phenotypic changes may arise by altered gene expression in tissues impacting future production potential, such as liver, muscle, and brain, thus "programming" offspring for possible susceptibilities to metabolic issues and reduced performance (Waterland and Jirtle, 2004) as well as a temperament that is more sensitive to stimuli (Lamprecht, 2014; Cristóvão et al., 2016; Su et al., 2016). Metabolically and otherwise compromised animals are major deterrents to efficient, sustainable livestock production systems (Reynolds and Caton, 2012). Liver and muscle are key tissues for energy balance as well as a beef product for harvest and sale, and while not considered a traditional tissue for production efficiency, altered cerebrum function leads to more excitable cattle, which is linked to reduced profitability (Cooke et al., 2011). Therefore, we hypothesized that a moderate maternal nutrient restriction during the first 50 d of gestation in beef heifers would affect transcript abundance of genes associated with tissue metabolism, accretion, and function in fetal liver, muscle, and cerebrum.

Materials and Methods

Animals, Experimental Design, and Treatments

Protocols described herein were approved by the North Dakota State University Institutional Animal Care and Use Committee. This study was part of a larger study in which heifers were exposed to treatments and ovariohysterectomized on d 16, 34, and 50 of gestation (Crouse et al., 2017b). The current data were collected from the 14 heifers that were ovariohysterectomized on d 50 of gestation. Briefly, Angus-cross heifers (n = 14 from which tissues were collected; ~16 mo of age; average initial body weight = 313 ± 24.9 kg) were

obtained from the Central Grasslands Research and Extension Center (Streeter, ND); and housed at the NDSU Animal Nutrition and Physiology Center (Fargo, ND). Heifers were acclimated to individual bunk feeding (American Calan, Northwood, NH) for 2 wk before the beginning of the trial. All heifers were exposed to the 5-d CO-Synch + CIDR estrus synchronization protocol (Bridges et al., 2008) and bred via AI to a common sire at 12 h after observed estrus. Immediately post-breeding, heifers were randomly assigned to 1 of 2 treatment groups. Control heifers (CON, n = 7) received 100% of (NRC, 2000) requirements for 0.45 kg/d gain to reach 80% of mature BW at first calving. Restricted heifers (RES, n = 7) were placed on a 40% global nutrient restriction, which was accomplished by reducing total diet delivery to 60% of the control delivery. The diet was delivered via total mixed ration and consisted of grass hay, corn silage, alfalfa haylage, as well as a grain and mineral mix. Dried distillers grains with solubles (53.4% NDF, 31.3% CP) were supplemented in addition to the TMR and fed to achieve the target nutrient content of the CON and RES diets.

Tissue Collection and Analysis

Ovariohysterectomy procedures were conducted as previously described (McLean et al., 2016) on d 50 of gestation for all heifers. Following ovariohysterectomy, fetal liver, muscle from the hind limb, and cerebrum tissues were collected using a stereoscope for increased visualization and to ensure maximum yield of tissue. While under the stereoscope, the entire liver was collected, and the complete left hind limb was severed, skin removed, and muscle was dissected away from the developing skeletal system. The entire cerebrum of the fetuses was examined macroscopically with a dissection-videoscope and collected based on anatomical localization (Fletcher, 2013).

Once collected, all tissues were snap frozen in liquid nitrogen-cooled isopentane (2-Methylbutane; J.T.Baker, Center Valley, PA) and stored at -80°C. Due to the nature of the hysterectomy procedure, tissues were not devoid of blood flow and oxygen until the final ligation and severing of the uterus. From here, samples were immediately taken to the laboratory within the animal facility, yielding snap frozen tissue within ten minutes of being removed from the heifers. RNA extraction and sequencing were performed by the University of Minnesota Genomics Center (Minneapolis-St. Paul, MN). RNA was extracted with the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) and quantified using a NanoDrop for 260/280 (Min = 1.93, Max = 2.10) and 260/230 (Min = 1.53, Max = 2.23) ratios as well as Quant –iT RiboGreen Assay Kit for RNA concentration (Min = 280.3 ng/µL, Max = 2680 ng/µL; Invitrogen, Carlsbas, CA). RNA integrity was measured with the Agilent TapeStation/BioAnalyzer (Agilent Technologies, Santa Clara, CA) for RNA quality (Min = 5.7, Max = 9.5). RNA-seq library creations were strand specific to preserve information about the strandedness of transcripts. RNA-seq analysis was conducted on the Illumina HiSeq 2500 platform (220,000,000 reads in both forward and reverse directions) and multiplexed with 21 samples per lane (42 samples total: 14 liver, 14 muscle, and 14 cerebrum) using 50-bp paired-end reads at a depth of 2 × 10.4M reads/sample in both forward and reverse directions. Minimum required reads were established based on Liu et al. (2014), who determined that at greater than 10M pair-end reads and 7 replications per treatment the number of reads has a diminishing return on power to detect differentially expressed genes. Transcriptome analysis was performed using the Tuxedo Suite (Trapnell et al., 2012). Reads were mapped to the UMD3.1 *Bos taurus* assembly using TopHat; transcripts were assembled using Cufflinks; the assembled transcripts were then merged using Cuffmerge to assemble a final transcriptome; Cuffdiff was then used to

determine differentially expressed genes (DEG) between the CON and RES treated groups. Individual gene significance was set at $q \leq 0.10$ (equivalent to $P < 0.00035$). All genes within a tissue type that were $P \leq 0.01$ were used for pathway and ontological analysis with DAVID 6.8 (Huang et al., 2009a; Huang et al., 2009b), and entered into DAVID 6.8 as ENSEMBL gene ID's to identify pathways and ontologies for further studies. For fetal liver, muscle, and cerebrum, a total of 548, 317, and 151 genes ($P < 0.01$) were differentially expressed and used for pathways analysis, of which 201, 144, and 28 genes were false discovery rate protected (FDR; $q < 0.10$). Differentially expressed genes were screened to determine whether they fit into functional categories of pathways or ontologies associated with phenotypes that could impact animal performance, such as metabolism, differentiation, and growth. Pathways and ontologies were considered significant when the *P*-value for the pathway was $P \leq 0.05$. Pathways were then further broken down to describe the specific roles of genes within a pathway and grouped by function.

RNA- Seq Validation

Validation of RNA-Seq was conducted with 3 randomly selected genes (one $q < 0.10$, one $q > 0.10$ and $P < 0.01$, and one $q > 0.10$ and $P > 0.01$) from each tissue. Validation was completed at the University of Minnesota Genomics Center (Minneapolis-St. Paul, MN) with primers being designed and validated with Roche UPL primer-probe assay with a minimum efficiency of 0.918 being used. Quantitative rt-PCR was run with each UPL probe for each respective tissue. In fetal liver, *histone 4-like* (*H4*), *glutathione-disulfide reductase* (*GSR*), and *arginase 1* (*ARG1*) were used to validate differential expression of a q – value ($q < 0.10$), *P* – value ($q > 0.10$, $P < 0.01$), and non-significant ($q > 0.10$, $P > 0.01$) gene, respectively. In fetal muscle from the hind limb, *myogenin* (*MYOG*), *BMP and activin membrane bound inhibitor*

(*BAMBI*), and *ATPase Na+/K+ transporting subunit alpha 1* (*ATP1A1*) were used to validate a q – value, *P* – value, and non-significant gene, respectively. In fetal cerebrum, *transferrin* (*TF*), *myosin light chain 4* (*MYL4*), and *BCL2 associated agonist of cell death* (*BAD*) were used to validate a q – value, *P* – value, and non-significant gene, respectively. All validated genes for liver, muscle, and cerebrum are presented in Fig. 2.1 as a $2^{-\Delta\Delta}$Ct fold change with *ACTB* (*β-actin*) as the reference gene (Livak and Schmittgen, 2001).

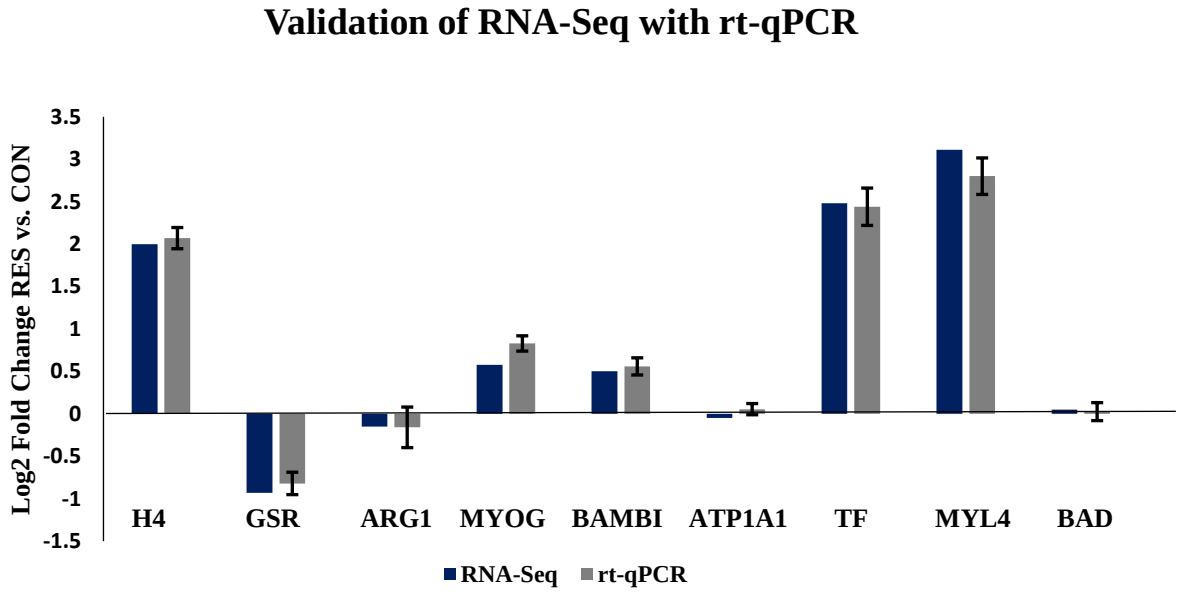

Figure 2.1. Log 2-Fold changes of FDR protected (*H4: Histone 4-like; MYOG: Myogenin;* and *TF: Transferr*in), P-value significant (*GSR: Glutathione Reductase; BAMBI: BMP And Activin Membrane Bound Inhibitor;* and *MYL4: Myosin Light Chain 4*) and non-significant genes (*ARG1: Arginase 1; ATP1A1: ATPase Na+/K+ Transporting Subunit Alpha 1;* and *BAD: BCL2 Associated Agonist Of Cell Death*) genes as measured by RNA-Seq (blue) versus rt-qPCR (grey). Data are presented as a Log2 Fold Change of restricted (RES) vs control (CON). Genes validated in: liver (*H4*, *GSR*, and *ARG1*), muscle (*MYOG*, *BAMBI*, and *ATP1A1*), and cerebrum (*TF*, *MYL4*, and *BAD*).

Results and Discussion

Our model achieved the targeted moderate nutrient restriction. For example, CON heifers were targeted to gain 0.45 kg/d (actual ADG = 0.51 kg/d), and RES heifers were fed to maintain BW throughout the 50-d period (actual ADG = -0.08 kg/d; Crouse et al., 2017b). This restriction changed the physiological fuels available to the conceptus for differentiation and growth. At d

50, glucose (Crouse et al., 2017a) and glutamine concentrations in allantoic fluid tended ($P < 0.10$) to be reduced in RES heifers, and aspartate concentrations were reduced ($P = 0.03$) in RES compared with CON heifers (Crouse et al., 2017a; Greseth et al., 2017). Glutamine concentrations were greater ($P < 0.05$) in the amniotic fluid of RES heifers (Greseth et al., 2017). Because placental circulation is being established during the first 50 d of gestation, there is no shared blood supply between the maternal and fetal systems, and thus, histotroph, allantoic, and amniotic fluids supply the necessary components for growth of the conceptus (Mullen et al., 2012).

Impaired one carbon metabolism affects the availability of methyl donors for DNA and histones, thus modifying gene expression which may result in changes to the phenotype thereby, inhibiting growth and health (Zhang, 2015). Crouse et al., (2019) demonstrated that at d 50 of gestation, methionine in allantoic fluid was less in RES, and homocysteine in maternal serum was greater in RES. The metabolism of choline, folate, vitamin B_{12}, vitamin B_6, and methionine are interrelated, and disturbances to one of these metabolic pathways is associated with compensatory changes in the others (Zeisel, 2011). These data imply that a profile of increased concentrations of serum homocysteine and decreased concentrations of methionine in allantoic fluid observed in the RES heifers could be due to a deficiency in any one of the epigenetic modifiers that affect the methylation of homocysteine to methionine across the maternal-fetal interface (Castro et al., 2006). Elevations in plasma homocysteine concentrations are associated with impaired one-carbon metabolism resulting from compromised nutrition and deficiencies in folate and vitamin B_{12} (Zaret, 2002). These data indicate that our moderate nutrient restriction was successful in altering not only the growth trajectory of the heifers but also the nutrients made available for fetal growth and differentiation. Additionally, differential expressions as reported in

the current manuscript are likely due to changes in fetal energy balance, or availability of one-carbon metabolites.

Liver

The liver is a key metabolic organ that governs energy metabolism and acts as a hub to metabolically connect various tissues, including skeletal muscle and adipose tissue (Rui, 2014). Liver energy metabolism is tightly controlled by multiple nutrient, hormonal, and neuronal signals to regulate glucose, lipid, and amino acid metabolism. Five functional categories of interest were identified based on genes observed to be differentially expressed in fetal liver tissues: metabolic pathways, protein kinase, nucleosome core, mRNA splicing, and complement/coagulation cascades (Table 2.1). The metabolic pathways category (n = 43 genes; $P = 0.017$) comprised 6 proposed functions (Table 2.1): amino acid metabolism (n = 10 genes) consisted of 5 genes upregulated in RES and 5 downregulated in RES; all differentially expressed purine and pyrimidine metabolism genes (n = 7) were upregulated in RES; carbohydrate metabolism (n = 10) comprised 5 genes upregulated in RES and 5 downregulated in RES; all differentially expressed reducing equivalent metabolism genes (n = 5) were upregulated in RES; steroid and lipid biosynthesis (n = 9) were affected by treatment such that 8 genes were upregulated in RES and 1 was downregulated in RES; and cytochrome and heme metabolism (n = 2) was affected by treatment such that both genes were upregulated in RES. The protein kinase category (n = 47 genes; $P = 0.020$) comprised 3 proposed functions (Table 2.11): serine/threonine protein kinase (n = 22) yielded 21 genes that were upregulated in RES, and 1 gene downregulated in RES; ATP-binding function (n = 19) was made up of 15 genes upregulated in RES and 4 downregulated in RES; and nucleotide-binding (n = 6) of which 4

were upregulated in RES and 2 were downregulated in RES. The complement and coagulation cascade category (n = 6 genes; $P = 0.041$) comprised 2 functions (Table 2.1): complement

Table 2.1. Functional categories and predicted roles for differentially expressed genes that impact tissue metabolism, accretion, and function ($P < 0.01$) in fetal liver presented as upregulation or downregulation in fetuses from restricted (RES) heifers compared with control (CON)

Category	Functional annotation[1]	Total genes[2]	Upreg.[3]	Downreg.[4]	*P*-value[5]
Metabolic pathways	Amino acid	10	5	5	0.017
	Purine and pyrimidine	7	7	0	
	Carbohydrate	10	5	5	
	Reducing equivalent (NAD/FAD)	5	5	0	
	Steroid and lipid biosynthesis	9	8	1	
	Cytochrome and heme	2	2	0	
Protein kinase	Serine/Threonine protein kinase	22	21	1	0.020
	ATP-binding	19	15	4	
	Nucleotide-binding	6	4	2	
Nucleosome core	Histones	9	9	0	0.005
	Histone modifiers	13	12	1	
mRNA splicing	Spliceosome	7	6	1	0.041
Complement/ Coagulation	Complement factors	3	3	0	0.041
	Coagulation factors	3	3	0	

[1]Proposed function of differentially expressed genes that fall under a specific category.
[2]Total number of differentially expressed genes associated within a specific function.
[3]Number of differentially expressed genes that are upregulated in fetuses from RES vs. CON heifers.
[4]Number of differentially expressed genes that are downregulated in fetuses from RES vs. CON heifers.
[5]Probability value associated with a specific category. *P*-value as presented is for the entire pathway, not individual genes within a pathway.

factors and coagulation factors (n = 3 and n = 3, respectively) of which all genes were upregulated in RES.

Altered liver function in rats from restricted mothers is reflected by permanent changes in metabolic activities of key hepatic enzymes and kinases in a direction that would potentially bias the liver toward a "starved" setting (Desai and Hales, 1997). Fetal exposure to utero-placental insufficiency alters the expression of genes encoding enzymes involved with hepatic energy metabolism (Lane et al., 1996), thereby decreasing hepatic oxidative phosphorylation (Ogata et al., 1990) and affecting liver glucose transport. These modifications in hepatic metabolism were also demonstrated with sheep in that dietary restriction of ewes from d 28 to 78 of gestation influenced liver function of offspring. Lambs from restricted dams had greater hepatic lipid and glycogen content than controls and altered glucose metabolism and glucose/insulin homeostasis postnatally (George et al., 2012). Due to reduced physiological fuel concentrations in fetal fluids, fetal livers from the present study may be adapting and reflecting the 'starved' state as previously reported. These data may be reflected in our differentially expressed metabolism and protein kinase genes as well as modifications to carbohydrate, amino acid, and especially reducing equivalent metabolism genes, which were all upregulated in RES suggesting a modification to energy metabolism. These metabolic pathways, as presented in Table 2.1, are highly intertwined and may result in the similar metabolic consequences previously observed in sheep and humans.

The liver is highly metabolically active and consumes approximately 20% of maintenance energy in beef cows even though it is a small proportion of maternal BW (Caton et al., 2000). Additionally, the liver is a key metabolic organ that governs body energy metabolism

and acts as a hub to metabolically connect to various tissues, including skeletal muscle and adipose tissue (Rui, 2014). Metabolic pathways in the liver are highly regulated by epigenetic modifications of gene promoters (Gluckman et al., 2009; Pinney and Simmons, 2010). The nucleosome core category (n = 22 genes; $P = 0.005$) comprised 2 proposed functions (Table 2.1): all differentially expressed histones (n = 9) were upregulated in RES; and histone modifiers (n = 13 genes) comprised 12 genes upregulated in RES and 1 gene downregulated in RES. The mRNA splicing category (n = 7 genes; $P = 0.041$) contained 6 genes upregulated in RES, and 1 gene upregulated in CON. Our findings of altered genes related to core histones are also supported by observations of nutrient restriction in mothers resulting in modification of transcriptional regulators such as core histones in rat pups (Tosh et al., 2010). In rats, the exposure to utero-placental insufficiency induces hepatic DNA hypomethylation and histone hyperacetylation of H3K9, H3K14, and H3K18 at birth (MacLennan et al., 2004). These changes persisted through the 21-d postnatal monitoring period indicating a permanent effect on hepatic gene expression. The hyperacetylation of histone H3 in the liver of intrauterine growth restriction rats occurs in association with decreased nuclear protein levels of histone deacetylase 1 (HDAC1) and HDAC activity (Fu et al., 2004). Histone modification is critical as it can impact gene expression, chromosome packaging, and DNA damage/repair (Wood and Shilatifard, 2004) thereby affecting accessibility of transcription factors. Further work on the epigenome using ChIP (chromatin immunoprecipitation) or RRBS (reduced representation bisulfite sequencing) would provide more information as to the functional effects of these changes in the transcriptome.

Muscle

The fetal stage is crucial for skeletal muscle development in mammalian livestock because there is no net increase in the muscle fiber number after birth (Stickland, 1978; Zhu et al., 2004); therefore, any impacts of maternal nutrition on muscle fiber number during gestation have lifelong consequences. Additionally, fetal skeletal muscle has a lower priority in nutrient partitioning compared with the brain and heart in response to challenges during fetal development, rendering fetal muscle particularly vulnerable to nutrient deficiency (Bauman et al., 1982; Close and Pettigrew, 1990).

Three categories of interest were determined for fetal muscle tissue: skeletal muscle, embryogenesis, and signaling cascades (Table 2.2). The skeletal muscle category (n = 74 genes; $P < 0.001$) comprised 8 proposed functions (Table 2.2): contraction genes (n = 9) all of which were upregulated in RES; the intermediate filament genes (n = 11) of which 7 genes were upregulated in RES and 4 downregulated in RES; microtubule associated genes (n = 10) contained 2 genes upregulated in RES and 8 downregulated in RES; actin (n = 4) was made up of 3 genes upregulated in RES and 1 downregulated in RES; all genes associated with myosin and troponin (n = 4 and n = 6 genes, respectively) were upregulated in RES; 25 genes were associated with calcium-binding in skeletal muscle, of which 14 were upregulated in RES and the remaining downregulated in RES; and all differentially expressed ATP-binding genes (n = 5) were downregulated in RES. The embryogenesis category (n = 14 genes; $P < 0.001$) comprised 2 functional ontologies (Table 2.2): myogenesis (n = 2) of which both genes were upregulated in RES; and homeobox related genes (n = 12) of which 10 were upregulated in RES, and 2 were downregulated in RES. The signaling cascades category (n = 18 genes; $P = 0.003$) was made up of 2 functional ontologies (Table 2.2): the Wnt signaling pathway (n = 6) had 4 genes

upregulated in RES, and 2 genes downregulated in RES; and the MAPK pathway (n = 12) comprised 3 genes upregulated in RES and 9 genes downregulated in RES.

Early prenatal nutritional restriction of ewes resulted in a reduced number of myofibers but an increased diameter of muscle fibers in offspring at 8 mo of age (Zhu et al., 2006). Additionally, maternal nutrient restriction from d 32 to 83 of gestation in beef cows resulted in steers with larger muscle fiber area in the complexus muscle compared with steers born to

Table 2.2. Functional categories and predicted roles for differentially expressed genes that impact tissue metabolism, accretion, and function ($P < 0.01$) in fetal muscle from hind limb, presented as upregulation or downregulation in fetuses from restricted (RES) heifers compared with control (CON)

Category	Functional annotation[1]	Total genes[2]	Upreg.[3]	Downreg.[4]	*P*-value[5]
Skeletal muscle	Contraction	9	9	0	< 0.001
	Intermediate filament	11	7	4	
	Microtubule	10	2	8	
	Actin	4	3	1	
	Myosin	4	4	0	
	Troponin	6	6	0	
	Calcium-binding	25	14	11	
	ATP-binding	5	0	5	
Embryogenesis	Myogenesis	2	2	0	< 0.001
	Homeobox	12	10	2	
Signaling cascades	Wnt	6	4	2	0.003
	MAPK	12	3	9	

[1]Proposed function of differentially expressed genes that fall under a specific category.
[2]Total number of differentially expressed genes associated within a specific function.
[3]Number of differentially expressed genes that are upregulated in fetuses from RES vs. CON heifers.
[4]Number of differentially expressed genes that are downregulated in fetuses from RES vs. CON heifers.
[5]Probability value associated with a specific category. *P*-value as presented is for the entire pathway, not individual genes within a pathway.

mothers fed a moderate nutrition diet (Long et al., 2010). Muscle fibers are formed throughout gestation during primary and secondary myogenesis, and at d 50 of gestation, peak primary myogenesis is occurring (Yan et al., 2013), with secondary myogenesis taking place during the second and third trimester (Russell and Oteruelo, 1981). Myogenesis occurs through a tightly-regulated orchestra of gene expression involving the expression of transcription factors and signaling pathways that activate muscle regulatory factors to commit muscle precursor cells to myogenic lineage (Braun and Arnold, 1996; Cossu et al., 1996; Tajbakhsh et al., 1998; Cossu and Borello, 1999; Buckingham et al., 2003; Palacios and Puri, 2006). Additionally, myoblasts proliferate and migrate to the forming muscles where they align and fuse into multinucleated, terminally differentiated myotubes expressing structural and contractile proteins (Relaix et al., 2005; Palacios and Puri, 2006). The myogenesis pathway is tightly regulated by epigenetic modifications, from DNA demethylation at muscle loci to histone tail acetylation and demethylation resulting in chromatin remodeling at muscle loci (Palacios and Puri, 2006). Our data suggest that genes involved in skeletal muscle formation and function, including signaling cascades and myogenic regulatory factors, were altered by maternal nutritional treatment, which may affect total fiber development during gestation. These changes may have been due, in part, to the epigenetic control of the myogenic regulatory factors, MYOG and MYOD1, which were both differentially expressed, as seen in the Myogenesis category of Table 2.2. Although the majority of myogenesis occurs during secondary myogenesis, d50 of gestation falls under primary myogenesis (Du et al., 2010), and changes seen in myogenic differentiation genes, especially stable modifications such as DNA methylation, may result in continued differential expression in the myogenic pathway throughout secondary myogenesis. Additionally, altering

the expression of genes involved in muscle function, more specifically contraction, may affect muscle function postnatally and potentially tenderness after slaughter.

Cerebrum

The brain, while being one of the most developmentally plastic tissues, is also one of the most vulnerable to malnutrition (Georgieff, 2007). Specific nutrients such as amino acids, minerals (zinc, iron, and copper), and vitamins (choline) are responsible for cerebral DNA synthesis, cell proliferation and differentiation, neurotransmitter synthesis, DNA methylation, neuronal energy metabolism, and synaptogenesis (Georgieff, 2007). In addition to autonomic nervous system function, malnutrition during early gestation results in global effects on brain growth and development, influencing long and short-term memory recognition (Golub et al., 1994; McEchron et al., 2005) as well as flight responses (Lamprecht, 2014).

Three categories of interest were determined for fetal cerebrum: hippocampus and neurogenesis, metal-binding, and cytoskeleton (Table 2.3). The hippocampus and neuro-genesis category (n = 32 genes; $P < 0.001$) comprised 5 proposed functional annotations (Table 2.3). Differentially expressed genes in the hippo signaling pathway (n = 5), collagen genes (n = 9), netrin genes (n = 5), and SMAD protein genes (n = 4) were all upregulated in RES. In addition, 8 developmental protein genes were upregulated in RES whereas 1 was downregulated in RES. The metal-binding category (n = 23 genes; $P = 0.006$) comprised 5 metal-binding functional annotation groups (Table 2.3): all differentially expressed iron-binding genes (n = 4) were upregulated in RES; all differentially expressed zinc-binding genes (n = 10) were upregulated in RES; copper and nickel binding genes (n = 2 and n = 1, respectively) were all upregulated in RES; and of the calcium-binding genes (n = 6), 5 were upregulated in RES, and 1 was downregulated in RES. The cytoskeleton category (n = 5) was made up of actin remodeling

Table 2.3. Functional categories and predicted roles for differentially expressed genes that impact tissue metabolism, accretion, and function ($P < 0.01$) in fetal cerebrum presented as upregulation/downregulation in fetuses from restricted (RES) heifers compared with control (CON)

Category	Functional annotation[1]	Total genes[2]	Upreg.[3]	Downreg.[4]	*P*-value[5]
Hippocampus and neurogenesis	Hippo signaling pathway	5	5	0	< 0.001
	Collagen	9	9	0	
	Netrin	5	5	0	
	SMAD	4	4	0	
	Developmental protein	9	8	1	
Metal-binding	Iron-binding	4	4	0	0.006
	Zinc-binding	10	10	0	
	Copper-binding	2	2	0	
	Nickel-binding	1	1	0	
	Calcium-binding	6	5	1	
Cytoskeleton	Actin remodeling	5	5	0	0.003

[1]Proposed function of differentially expressed genes that fall under a specific category.
[2]Total number of differentially expressed genes associated within a specific function.
[3]Number of differentially expressed genes that are upregulated in fetuses from RES vs. CON heifers.
[4]Number of differentially expressed genes that are downregulated in fetuses from RES vs. CON heifers.
[5]Probability value associated with a specific category. *P*-value as presented is for the entire pathway, not individual genes within a pathway.

genes, of which all were upregulated in RES.

The hippocampus in the cerebrum of the brain plays an integral part in emotion and memory and is further linked to anxiety (Engin and Treit, 2007). Key functional proteins in the brain such as collagen, actin filaments, and metal- binding proteins play important roles for maintaining proper synapse and neuronal function, which may lead to brain disorders such as schizophrenia and an abnormal startle response if altered (Lamprecht, 2014; Cristóvão et al., 2016; Su et al., 2016). Although schizophrenia and an abnormal startle response are not measurements that are recorded in livestock operations, common evaluations for temperament markers include chute scores and exit velocity. Calves from dams that were nutrient restricted

during the second trimester of gestation (to lose 1 BCS over the 84-d period), had greater temperament scores (chute score + exit velocity divided by 2) at weaning compared with calves from control dams (Gardner, 2017). In beef steers, more excitable cattle, as determined by greater exit velocities, were linked to producing tougher beef steaks (King et al., 2006; Cooke et al., 2011; Hall et al., 2011). Additionally, more excitable or aggressive cattle reduce profitability in comparison to moderate or calm cattle (Cooke et al., 2011).

The hippocampus has receptors for many metabolic and neuroendocrine hormones. Kanoski and Grill (2017) summarize that the hippocampus contains receptors for cholecystokinin, leptin, ghrelin, glucagon-like peptide 1, motilin, and amilin along with receptors for central nervous system neuropeptides including melanocortin-4 and orexin. Although hippocampal modulation of food intake via response to hormonal and neuroendocrine signaling remains a largely unexplored area (Kanoski and Grill, 2017), maternal malnutrition (undernutrition, famine, protein restriction, and high-fat diets) modulates hippocampal function, cognition, and animal intake (Lucassen et al., 2013). In sheep, nutrient restriction from d 28 to 78 of gestation resulted in increased and more rapid feed intake of lambs once they reached 6 years of age (George et al., 2012), suggesting modulation of feeding behavior due to maternal nutrient restriction. This could further be explained by the thrify phenotype hypothesis which states that changes persist due to a survival advantage in times of nutritional deprivation, even if these changes prove to be detrimental during times of adequate or excess nutrition (Hales and Barker, 1992). Changes to cerebral functions in rats and humans indicate that poor maternal nutrition during early gestation may program cattle for a temperament that is more sensitive to stimuli, alters food intake behaviors, and may thereby decrease tissue metabolism, accretion, and function and thus, producer profits.

Postnatal Phenotypic Ramifications

Due to tissues being collected at d 50 of gestation (resulting in limited yield) all collected tissue was utilized for RNA-sequencing to ensure a high RNA yield. Therefore, acquisition of protein data was not possible to verify whether gene expression changes culminated in actual changes to the proteins. It is unknown if the changes in gene expression observed at d 50 of gestation would lead to postnatal phenotypic changes. From our data, it can be postulated that the number of genes upregulated in offspring from RES dams compared with CON could be due to a recruitment of protein to compensate for nutrient restriction. Previously published data on nutrient restriction during gestation in the bovine model followed by realimentation has shown differential compensatory effects on fetal and placental growth that is dependent on timing of insult and realimentation (Freetly et al., 2000; Long et al., 2009; Gonzalez et al., 2013; Camacho et al., 2018). However, these restrictions were initiated no earlier than d 30 of gestation and are not directly comparable to our model, which initiated restriction at breeding. Additionally, most fetal programming data evaluates effects through finishing, and not over a longer lifespan. Epidemiological studies in humans beginning with the observations on children of the Dutch Hunger Winter have elucidated effects later in life (Roseboom et al., 2001). Therefore, it can be postulated that effects of early maternal malnutrition in pregnancy may not be observed in the steer or heifer calves who are finished early in life or at their first breeding cycle but may manifest and be observed in mature cows. This may be further supported by seven of the ten principles of developmental programming set forth by Nathanielsz (2006): 1) There are developmental windows where there is increased susceptibility to suboptimal conditions, 2) Programming effects are permanent and alter responses in later life, 3) Fetal programming may result in structural changes to organs, 4) Compensation by the offspring may create undesirable

postnatal outcomes, 5) Postnatal compensation to alter prenatal programming may carry additional undesirable consequences, 6) Fetal tissue accretion, metabolism, and function differ from adult processes, and 7) Programming effects may be seen across generations. Therefore, our data suggests that early gestation is a critical developmental window which can be manipulated by maternal nutrient restriction. Furthermore, upregulation of genes in our model to compensate for nutrient restriction may not be beneficial for fetal development, but verification of changes in gene expression resulting in changes to protein must be verified. Finally, it is important to evaluate whether early maternal nutrient restriction results in long-term postnatal ramifications in cattle as seen in humans, and the severity of which those effects result in changes to tissue metabolism, accretion, and function throughout life

Conclusion

Data from the current report clearly indicate that moderate global maternal nutrient restriction during the first 50 d of gestation alters transcript abundance of genes that impact tissue metabolism, accretion, and function in fetal liver, muscle, and cerebrum. Moreover, these data may provide insight into the mechanisms of action by which global maternal undernutrition during the first 50 d of gestation could impact liver metabolism, muscle fiber number and function, as well as the potential for programmed temperament and food intake, which all influence tissue metabolism, accretion, and function, and thus profitability of beef production. Finally, these data indicate that although 75% of fetal growth occurs during the last 2 mo of gestation, cellular processes can be modified during the first 50 d of gestation, emphasizing the need for further research to elucidate the epigenetic mechanisms by which such changes in transcript abundance occur during early gestation, and their respective effects on whole animal lifetime performance.

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CHAPTER 3. ONE-CARBON METABOLITE SUPPLEMENTATION MAY IMPROVE DEVELOPMENTAL OUTCOMES OF OFFSPRING WHO WERE NUTRIENT RESTRICTED DURING EARLY EMBRYONIC DEVELOPMENT

Abstract

Crossbred Angus heifers were bred, assigned to nutritional treatment (CON = 100% of requirements for 0.45 kg/d gain and RES = 60% of CON) and ovariohysterectomized on d 16, 34, or 50 of gestation. Fetal liver, muscle from the hind limb, and cerebrum were collected for RNA-sequencing on d 50 of gestation. Fetuses from RES heifers tended ($P = 0.116$) to have shorter crown-rump lengths compared with CON on d 50. For fetal liver, muscle, and cerebrum, a total of 546, 316, and 144 genes ($P \leq 0.01$) were differentially expressed of which 55, 91, and 13 genes were false discovery rate protected ($q \leq 0.05$). Differentially expressed clusters included nucleosome core, nucleotide metabolism, mitochondira, and embryonic development. Therefore, a second *in vitro* experiment was conducted in which bovine embryonic fibroblast cells were cultured with 1 g/L glucose (LOW) or 4.5 g/L glucose (HIGH). Control medium contained basal concentrations of One-carbon metabolites (OCM: methionine, choline, folate, and vitamin B_{12}). One-carbon metabolites were supplemented to the media at 2.5, 5, and 10 times (2.5X, 5X, and 10X, respectively) the control media, except for methionine (limited to 2X). Total embryonic cell growth rate and reserve mitochondrial capacity were greater ($P < 0.01$) for HIGH 2.5X and 10X compared with LOW CON, 2.5X, and 10X. These data are interpreted to imply that moderate maternal nutrient restriction alters the gene expression profile in fetal liver, muscle, and cerebrum which may affect post-natal development; furthermore, the positive effects supplemental OCM *in vitro* on growth rate and mitochondrial function may also

improve embryonic development trajectory *in vivo* and should be a target of future study in beef heifers.

Introduction

Research in developmental programming has elucidated the importance of embryonic, fetal, and neonatal stresses on postnatal physiology (Godfrey and Barker, 2000). These alterations in postnatal physiology arise due to adaptations *in utero* which have evolved to produce a phenotype to match the predicted environment (Gluckman et al., 2005; West-Eberhard, 2005). These perturbations in the maternal environment bring about epigenetic modifications which change patterns of DNA and histone methylation as well as histone acetylation which alters gene expression, resulting in altered growth and metabolism (Waterland and Jirtle, 2003; MacLennan et al., 2004; Waterland and Jirtle, 2004; McMillen and Robinson, 2005; Waterland et al., 2006; Reynolds et al., 2017). Immediately following fertilization, there is a rapid wave of global demethylation of paternal DNA followed by a slower but complete demethylation of maternal DNA to the morula stage (Reik and Dean, 2001; Reik et al., 2001). At this stage the embryo must properly remethylate its genome for transcriptional regulation. Therefore, although the energy requirements to support the growth of the conceptus during early gestation are not different than that of the maintenance requirements of the mother, there are specific nutrient requirements for embryonic growth a development that aid in the establishment and maintenance of pregnancy. Furthermore, embryos that are born to mothers stressed during early gestation may be of normal birthweight; however, they may still suffer from compromised growth and metabolic syndrome later in life (Ford et al., 2007; Vonnahme et al., 2007; Reynolds and Caton, 2012). Compromised animals pose issues to advancing efficient and sustainable livestock systems (Reynolds and Caton, 2012), and therefore strategies to mitigate compromised

pregnancies through strategic micronutrient supplementation should be investigated. The objectives of these studies are two-fold: 1) determine how a global maternal nutrient restriction during the first 50 d of gestation beef heifers would alter embryonic growth and modify the transcriptome of fetal liver, muscle, and cerebrum, and 2) based on data from experiment 1, determine how supplementation of one-carbon metabolites (OCM: methionine, folate, choline, and vitamin B_{12}) to bovine embryonic fibroblasts cultured in divergent glucose media would impact expression of genes and proteins in the methionine-folate cycle of DNA methylation, mitochondrial respiration, and cell growth and proliferation. We hypothesized that a global nutrient restriction would alter gene expression to program the embryo towards a reduced nutritional environment postnatally, and that supplementation of OCM to bovine embryonic fibroblasts would improve measured paramteres beyond unsupplemented controls.

Materials and Methods

Experiment 1

Animals, Experimental Design, and Treatments

Protocols described herein were approved by the North Dakota State University Institutional Animal Care and Use Committee. Angus-cross heifers (~16 mo of age; average initial body weight = 313 ± 24.9 kg) were obtained from the Central Grasslands Research and Extension Center (Streeter, ND); and housed at the NDSU Animal Nutrition and Physiology Center (Fargo, ND) with a 12 h light/dark cycle. Heifers were acclimated to individual bunk feeding (American Calan, Northwood, NH) for 2 wk before the beginning of the trial. All heifers were exposed to the 5-d CO-Synch + CIDR estrus synchronization protocol (Bridges et al., 2008) and bred via AI to a common sire at 12 h after observed estrus. Immediately post-breeding, heifers were randomly assigned to one of two treatment groups. Control heifers (**CON**,

n = 7 for ovariohysterectomy) received 100% of (NRC, 2000) requirements for 0.45 kg/d gain to reach 80% of mature BW at first calving which is normal practice in the livestock industry. Restricted heifers (**RES**, n = 7 for ovariohysterectomy) were placed on a 40% global nutrient restriction, which was accomplished by reducing total diet delivery to 60% of the CON delivery. The diet consisted of grass hay, corn silage, alfalfa haylage, as well as a grain and mineral mix. Dried distillers grains with solubles (53.4% NDF, 31.3% CP) were supplemented in addition to the total mixed ration and fed to achieve the target protein and energy requirement of the CON and RES diets, respectively. The current data were collected from a larger study in which heifers were exposed to treatments and ovariohysterectomized on d 16, 34, and 50 of gestation (Crouse et al., 2017; Greseth et al., 2017a; McLean et al., 2018; Crouse et al., 2019b).

Fetal Measurements, Tissue Collection, and Analysis

Ovariohysterectomy procedures and tissue collections were conducted as previously described on d 16, 34, and 50 of gestation for all heifers (McLean et al., 2016; Crouse et al., 2019a). Briefly, ovariohysterectomy was performed, and images of the embryo/fetus were captured for size measurements including: vertical and horizontal axis measurements on d 16, as well as straight crown-rump length and straight abdominal girth measurements on d 34 and 50. After images were taken, d 50 fetal liver, muscle from the hind limb, and cerebrum were collected using a dissection-videoscope for RNA-sequencing.

Once collected, all tissues were snap frozen in liquid nitrogen-cooled isopentane (2-Methylbutane; J.T.Baker, Center Valley, PA) and stored at -80°C. RNA extraction and sequencing were performed by the University of Minnesota Genomics Center (Minneapolis-St. Paul, MN). RNA was extracted with the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) and quantified using a NanoDrop for 260/280 (Min = 1.93, Max = 2.10) and 260/230

(Min = 1.53, Max = 2.23) ratios as well as Quant –iT RiboGreen Assay Kit for RNA concentration (Min = 280.3 ng/µL, Max = 2680 ng/µL; Invitrogen, Carlsbas, CA). RNA integrity was measured with the Agilent TapeStation/BioAnalyzer (Agilent Technologies, Santa Clara, CA) for RNA quality (Min = 5.7, Max = 9.5).

RNA-seq library creations were strand specific to preserve information about the strandedness of transcripts. RNA-seq analysis was conducted on the Illumina HiSeq 2500 platform (220 M reads in both forward and reverse directions) and multiplexed with 21 samples per lane (42 samples total: 14 liver, 14 muscle, and 14 cerebrum) using 50-bp paired-end reads at a depth of 2 × 10.4M reads/sample in both forward and reverse directions. Minimum required reads were established based on Liu et al., (2014), who determined that at greater than 10M pair-end reads and 7 replications per treatment the number of reads has a diminishing return on power to detect differentially expressed genes (**DEG**).

Transcriptome analysis was performed using the Tuxedo Suite (Trapnell et al., 2012). Reads were mapped to the UMD3.1 *Bos taurus* assembly using TopHat; transcripts were assembled using Cufflinks; the assembled transcripts were then merged using Cuffmerge to assemble a final transcriptome; Cuffdiff was then used to determine differentially expressed genes (DEG) between the CON and RES treated groups. Individual gene significance was set at $q \leq 0.05$. For fetal liver, muscle, and cerebrum, a total of 546, 316, and 144 genes ($P \leq 0.01$) were differentially expressed of which 55, 91, and 13 genes were false discovery rate protected ($q \leq 0.05$). All genes within a tissue type that were $P \leq 0.01$ were used for pathway and ontological analysis with DAVID 6.8 (Huang et al., 2009a; Huang et al., 2009b). Differentially expressed genes $P \leq 0.01$ were further divided into genes that were upregulated in RES compared with CON or downregulated in RES compared with CON. These groups were then

entered into DAVID and cluster analysis was used to cluster genes with similar functions. For each tissue type, the top three clusters with enrichment scores greater than 1.4 were considered significant. Cluster names were generated based upon gene ontologies and pathways that were found in the cluster.

Experiment 2

Cells, Experimental Design, and Treatments

Based upon data from experiment 1, Bovine Embryonic Tracheal Fibroblasts (**EBTr**; ATCC CCL-44) were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Eagle's Minimum Essential Medium (**EMEM**: Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA), 1% penicillin-streptomycin (Thermo Fisher Scientific) and 0.11g/L Na pyruvate (Sigma). Final glucose concentrations of 1 g/L (**LOW**) or 4.5 g/L (**HIGH**) were achieved by the addition of D-glucose (Sigma). Control medium contained basal concentrations of folate (0.001g/L), choline (0.001g/L), vitamin B_{12} (4µg/L), and methionine (0.015g/L). One-carbon metabolites (folic acid, choline chloride, vitamin B_{12}, and L-methionine [Sigma]) were supplemented to the media to achieve 2.5, 5, or 10 times (2.5X, 5X, or 10X, respectively) the concentrations in the control medium, except for methionine, which was limited at 2X across all supplemented treatments to prevent toxicity. Therefore, the experiment was a completely randomized design with a 2 (glucose [LOW and HIGH]) × 4 (OCM levels [control, 2.5X, 5X, and 10X) factorial arrangement of treatments. Cells were cultured in 10-cm dishes in a 37ºC, 5% CO_2 incubator, and passaged three times in treatment media once cells reached 80% confluence in the dish (avg. 2 d) before being plated for further analyses.

Gene Expression

After the third passage in treatment media in the 10-cm dish, EBTr cells were plated (n = 3/treatment) into 75 cm^2 Nunc EasYFlask Cell Culture flasks (ThermoFisher) and allowed to grow to 90% confluence. Once 90% confluence was reached, cells were trypsinized from the flask and RNA was extracted and purified using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA), and total quantity of RNA was determined using the Take3 module of a Synergy H1 Microplate Reader (BioTek, Winooski, VT). Complementary DNA was synthesized from 1,000 ng RNA for each sample utilizing the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). TaqMan Gene Expression Assays (Applied Biosystems, Grand Island, NY), were purchased from ThermoFisher (Waltham, MA; Table 3.1). Optimal cDNA dilutions (1:10 for all genes of interest, 1:100 for reference genes) were determined by primer validation: m*ethionine adenosyltransferase 2A and 2B* (***MAT2A*** and ***MAT2B****) DNA methyltransferase 1*, *3A,* and *3B* (***DNMT1, DNMT3A,*** and ***DNMT3B***), *S-adenosylhomocysteine hydrolase* (***AHCY***), and *methionine synthase* (***MTR***). Primer efficiency was determined via a five point 10-fold dilution series. For each sample, gene expression was quantified in triplicate using a 7500, Fast, Real-Time PCR System (Applied Biosystems), with Taqman Fast Advanced Master Mix (Applied Biosystems), with 20 µL total reaction volume for all genes (1 µL primer, 2 µL cDNA, 7 µL nuclease free water, and 10 µL master mix). Relative mRNA expression was calculated using the $2^{-\Delta\Delta}C_t$ method (Livak and Schmittgen, 2001) with *succinate dehydrogenase* (***SDHA****)* as the reference gene, as it was the most stable of all reference genes analyzed (Table 3.1). Stability of expression was assessed by geNorm analysis using qBase+ software (Biogazelle, Zwijnarrde, Belgium; Vandesompele et al., 2002). The intra plate CV was 0.12% and the across plate CV was 0.98%.

Protein Expression

After the third passage in treatment media, EBTr cells were plated (n = 3/treatment) into 10-cm Nunc Petri Dishes (ThermoFisher) in treated media and allowed to grow to 90% confluent. Once 90% confluence was reached, whole cells were lysed with 1X SDS sample buffer (10 ml glycerol, 5 ml 2-mercaptoethanol, 2.3 g SDS, 0.756 g Tris, 0.1 g bromophenol blue, sterile water to 100 ml, pH = 6.8), and protein was quantified using a Micro Lowry Total Protein Kit (Sigma) Synergy H1 Microplate Reader (BioTek). Molecular weight ladder (Precision Plus Protein WesternC Blotting Standards; BioRad), all 8 treatments, and the loading control were added to as gel with a 5% acrylamide stacking gel and an 8% acrylamide running gel. Electrophoresis was conducted at 180 V, 400 mA, for 80 minutes at which time the dye front reached the bottom of the gel. Protein was transferred to pre-wet PVDF membranes using the Trans-Blot Turbo Transfer System (BioRad) for 10 min with 25V and 2.5Amp. To normalize across treatments and membranes, total protein load (Fast Green FCF, Sigma) was measured after transfer to the membrane. To block non-specific binding, membranes were treated with 5% non-fat dry milk in TBST for one hour. Optimal protein loading and antibody diluent concentrations were determined for each protein: DNMT3A (ab188470, abcam, Cambridge, UK; anticipated mw: 134 kDa), DNMT3B (SAB2108295, Sigma; anticipated mw: 86 kDa), and MTR (AV48473, Sigma; anticipated mw: 140k kDa). Primary and secondary antibodies were diluted in 1% non-fat dry milk in 1X TBST (Tris 20mM, NaCl 150 mM, Tween 20 0.1% w/v). Each membrane was probed for either DNMT3A, DNMT3B, or MTR overnight and labeled with Goat anti-Rabbit IgG (H + L), Superclonal Recombinant Secondary Antibody, HRP (ThermoFisher) and Precision Protein StepTactin-AP conjugate (BioRad) for 1 h (Table 3.2). Linearity was determined with a 4-point curve with increasing loading volumes. Samples were assayed in

duplicate across two blots (10 well gel; all 8 treatments per gel, molecular weight marker, and loading control). Membranes were exposed for 5 minutes with Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Pittsburgh, PA). Membranes were imaged with Alpha Innotech Fluor Chem FC2 (Alpha Innotech, San Leandro, CA). Image capture times were: DNMT3A-10 mins, DNMT3B-5 mins, MTR-7 mins, and images were analyzed with the AlphaEaseFC software. The across membrane CV's were no greater than 24.8% for each protein.

Mitochondrial Respiration

Oxygen consumption rate was measured using a Seahorse XFe24 Analyzer (Agilent, Santa Clara, CA). EBTr cells were passaged three times in treatment media before being plated (n = 3/treatment) onto Seahorse XFe24 microplates at 5×10^4 cells per well in 150 µL of media to allow cells to attach to the bottom of the well. One-hour after plating, an additional 150 µL of treatment media was added carefully to the well as to not disturb the attached cells. Cells were cultured in Seahorse XFe24 microplates for 18-24 h. Immediately prior to analysis treated media was exchanged for XF Assay Dulbecco Modified Eagle Medium (pH = 7.4) supplemented to contain 4.5 mM glucose (Agilent), 1 mM pyruvate (Agilent), and 2 mM glutamine (Agilent), and plates were incubated at 37ºC in a CO_2-free incubator for 1 h. Baseline rates were measured three times before sequentially adding Oligomycin 12.64 µM (Agilent), FCCP 10 µM (Agilent), and Rotenone/Antimycin A 10 µM (Agilent). Read parameters were: 1 min mixing, 2 min waiting after mixing, followed by 2 min to read, completed three times per measurement.

Table 3.1. Primers for real-time PCR assays.

Symbol	Gene Name	Assay ID	Primer Efficiency	Linearity	M Value
MAT1A	Methionine Adenosyltransferase 1A	Bt03235690_m1	Not Expressed	-	-
MAT2A	Methionine Adenosyltransferase 2A	Bt03271823_m1	89.72	0.987	-
MAT2B	Methionine Adenosyltransferase 2B	Bt03240156_g1	87.61	0.994	-
DNMT1	DNA Methyltransferase 1	Bt03224754_m1	89.82	0.998	-
DNMT3A	DNA Methyltransferase 3A	Bt01027164_m1	96.80	0.951	-
DNMT3B	DNA Methyltransferase 3B	Bt03259818_m1	91.80	0.916	-
AHCY	S-Adenosylhomocysteine Hydrolase	Bt03225928_m1	94.71	0.994	-
MTR	Methionine Synthase	Bt03220598_m1	89.84	0.987	-
BHMT	Betaine-Homocysteine S- Methyltransferase	Bt03210343_m1	Not Expressed	-	-
ACTB	β-Actin	Bt03279174_g1	85.42	0.997	0.279
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Bt03210913_g1	89.05	0.996	0.297
SDHA	Succinate Dehydrogenase	Bt04307498_m1	89.80	0.996	0.254

Table 3.2. Antibodies for Western blot.

Symbol	Protein Name	Assay ID	Antibody Concentration	Protein Load	Linearity
DNMT3A	DNA Methyltransferase 3A	Ab188470	1:2,000 in 1% milk	10 µg	> 0.802
DNMT3B	DNA Methyltransferase 3B	SAB2108295	1:2,000 in 1% milk	25 µg	> 0.929
MTR	Methionine Synthase	AV48473	1:2,000 in 1% milk	10 µg	> 0.633
	Goat anti-Rabbit IgG (H + L), Superclonal Recombinant Secondary Antibody, HRP	A27033	1:10,000 in 1% milk		-

Immediately following analysis, the XF assay medium was removed the wells and the cells were fixed in 10% neutral buffered formalin (Sigma). Cells were treated with Pro-Long Gold with 4,6-diamidino-2-phenylindole (DAPI; Life Technologies, Grand Island, NY) to counterstain all nuclei. Large area (MosaiX, Zeiss) photomicrographs of the wells were taken with a Zeiss Imager M2 epifluorescence microscope using a 5× objective and AxioCam HRm camera. MosaiX images were analyzed using ImagePro Premiere software (Media Cybernetics, Silver Spring, MD) for the total cell number (DAPI stained cells). Mitochondrial respiration parameters were measured as O_2 consumption pmol/min/cell scaled to 10,000 cells. Parameter measure include: basal respiration, O_2-linked ATP synthesis (basal respiration - O_2 consumption after injection with oligomycin), maximal respiration, reserve capacity (maximal respiration – basal respiration), proton leak (O_2 consumption after injection with oligomycin – O_2 consumption after injection with rotenone/antimycin A), and non-mitochondrial respiration. Each reported mean for the measurement is the average of the three reads per parameter across each treatment. The intra plate CV was 6.39% and the across plate CV was 12.53%.

Cell Growth Rate and Proliferation Analyses

EBTr cells were passaged three times in their respective treatment media before being plated onto one of six Seahorse XFe-24 microplates (Agilent) in triplicate at a cell density of 1.8 × 10^3 cells per well. Cells were plated at 0 h and placed in a humidified incubator (37ºC, 5% CO_2) for either 1, 12, 24, 36, 48, or 72 h, after which time the media were aspirated and the cells fixed in 10% neutral buffered formalin (Sigma). Antigen retrieval was performed in 10 mM sodium citrate buffer, pH 6, with 0.05% Tween 20 in a 2100 retriever (Electron Microscopy Sciences, Hatfield, PA). To block nonspecific binding, wells were treated for 1 h with 10% normal goat serum (Vector Laboratories, Burlingame, CA). Each well was stained for cell

proliferation with rabbit anti-Ki67 (Abcam, Cambridge, UK) for 1 h and fluorescently labeled with CF633 goat anti-rabbit secondary antibody (Biotium, Fremont, CA) for 1 h. Cells were treated with Pro-Long Gold with DAPI (Life Technologies, Grand Island, NY) to counterstain all nuclei. Large area (MosaiX, Zeiss) photomicrographs of the wells were taken with a Zeiss Imager M2 epifluorescence microscope using a 5× objective and AxioCam HRm camera. MosaiX images were analyzed using ImagePro Premiere software (Media Cybernetics, Silver Spring, MD) for the total cell number (DAPI stained cells) and proliferating cell number (Ki67 stained cells). Cell growth rate was determined as the slope after natural log transformation of cell number, and cell proliferation with Ki67 was determined by the labeling index (% of cells stained by Ki67).

Statistical Analyses

Experiment 1

Transcriptome analysis was performed using the Tuxedo Suite (Trapnell et al., 2012), and reads were mapped to the UMD3.1 *Bos taurus* genome.

Individual gene significance was set at $q \leq 0.05$ (equivalent to $P < 0.0001$). All genes within a tissue type that were $P \leq 0.01$ were used for pathway and ontological analysis with DAVID 6.8 (Huang et al., 2009a; Huang et al., 2009b). Differentially expressed genes were entered into DAVID 6.8 as a gene list as those upregulated in RES or those downregulated in RES compared with CON for liver, muscle from the hind limb, and cerebrum. Cluster analysis was performed for each gene list to generate functional annotations based on enrichment for Gene Ontology (GO) terms for biological processes and molecular function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Medium stringency was used to define clusters and those clusters with enrichment scores greater than 1.3 were considered. Only the top

three clusters (if enrichment greater than 1.3 and $P \leq 0.05$) are shown for each gene list. Because of the low number of genes (n = 10) downregulated in RES compared with CON in cerebrum, clustering was not used.

Analysis of fetal size measurements on d 16, 34, and 50 were conducted as t-tests within day of gestation. All *P*-values less than or equal to 0.05 were considered significant.

Experiment 2

Gene expression data are presented as a fold change relative to High Control which has been set to 1.00. Gene and protein expression as well as mitochondrial respiration parameters were analyzed with PROC GLM of SAS 9.4 for glucose, OCM, and the interaction. Cell growth rate was analyzed for early growth (1 to 24 h), late growth (24 to 72 h), and total growth (1 to 72 h) rate using PROC REG of SAS 9.4 (SAS, Cary, NY) followed by PROC MIXED with glucose (LOW and HIGH), OCM (Control, 2.5X, 5X, and 10X), and their interaction as fixed effects. Labeling index (cell proliferation) was analyzed at 1, 12, 24, 36, 48, and 72 h. Additional analysis included proliferation post-attachment to the plate (12 to 72 h) and total proliferation (1 to 72 h) using PROC MIXED with glucose, OCM, and their interaction as fixed effects.

Polynomial contrasts were performed within the LOW and HIGH glucose levels to determine whether increasing OCM affected gene expression, protein expression, mitochondrial respiration, cell growth rate or proliferation. To account for unequal spacing of OCM treatments, contrast coefficients were determined using PROC IML and analyzed with the GLM procedure for effects of OCM supplementation within glucose level. All *P*-levels less than or equal to 0.05 were considered significant.

Results

Experiment 1

Fetal Measurements

Embryonic vertical or horizontal axis, and fetal crown-rump length or abdominal girth were not altered by maternal nutritional treatment at either day 16, 34, or 50 of gestation ($P \geq 0.116$; Table 3.3). On d 50 of gestation, offspring from RES heifers tended ($P = 0.116$) to be shorter in crown-rump length compared with offspring from CON heifers (4.149 cm vs. 3.791 cm; Table 3.3).

Table 3.3. Effect of maternal dietary intake on size of the embryo/fetus during early pregnancy in beef heifers.[1]

Maternal Dietary Intake	Day 16		Day 34		Day 50	
	Vertical Axis	Horizontal Axis	C-R Length[2]	Abd. Girth[3]	C-R Length	Abd. Girth
Control	0.623 ± 0.306	1.084 ± 0.497	1.199 ± 0.182	0.751 ± 0.123	4.149 ± 0.082	1.558 ± 0.029
Restricted	0.364 ± 0.183	0.587 ± 0.295	1.174 ± 0.126	0.897 ± 0.111	3.791 ± 0.156	1.625 ± 0.054
P-value[4]	0.236	0.198	0.456	0.200	0.116	0.265

[1]Control = Heifers fed NRC (2000) requirements to gain 0.45 kg/hd/d (Actual ADG = 0.51 kg/hd/d), and Restricted = 60% of Control intake (Actual ADG = -0.08 kg/hd/d) from the day of mating through the day of collection; all measures are in cm.
[2]Crown-Rump length
[3]Abdominal girth.
[4]Control vs. Restricted (t-test).

Fetal Transcriptome

For fetal liver, muscle from the hind limb, and cerebrum, a total of 546, 316, and 144 genes ($P \leq 0.01$; Figure 3.1) were differentially expressed and used for pathways analysis, of which 55, 91, and 13 genes were false discovery rate protected, respectively (FDR; $q \leq 0.05$). Furthermore, of the DEG used for cluster analysis, 508, 144, and 134 genes were upregulated in

offspring from RES compared with CON dams in fetal liver, muscle, and cerebrum, respectively (Figure 3.1). Therefore, 89.1 %, 45.6%, and 93.1% of DEG were upregulated in offspring from RES compared with offspring from CON in fetal liver, muscle, and cerebrum, respectively with 75.9% of all DEG being upregulated in offspring from RES compared with CON across all three tissues (Figure 3.1).

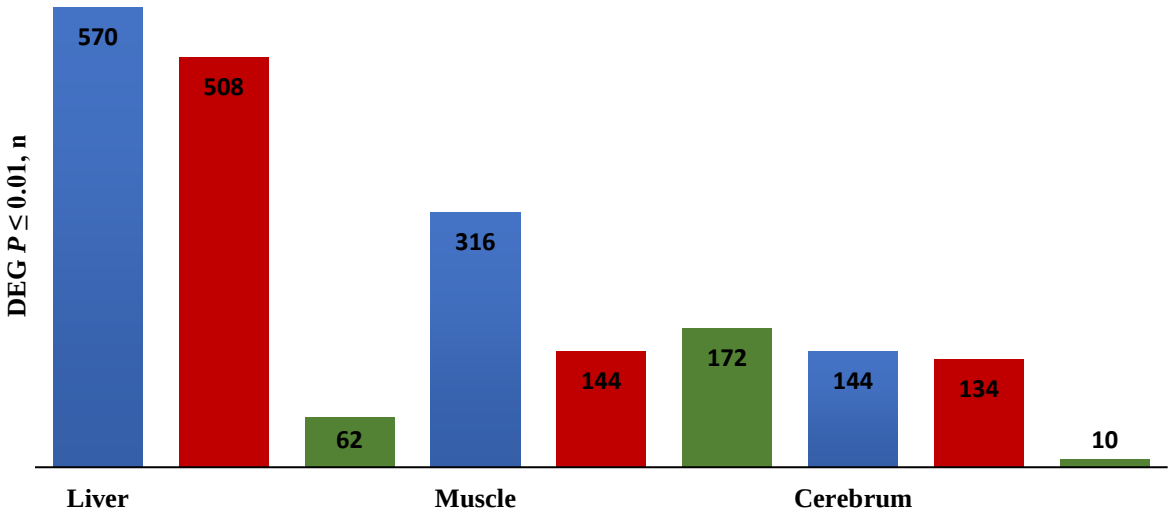

Figure 3.1. Differentially expressed genes (DEG) in fetal liver, muscle from the hind limb, and cerebrum as influenced by maternal nutritional treatment, CON or RES, by day 50 of gestation. Solid blue bars are the total number of differentially expressed genes. Solid red bars are the number of genes upregulated in RES compared with CON offspring. Solid green bars are the number of genes downregulated in RES compared with CON

Table 3.4. Functional Annotation Pathway Cluster.

Tissue	Clusters and Annotation Terms
Liver	***RES Upregulated Clusters[1]***
	Cluster 1: Nucleosome Core, Enrichment = 1.42, Count = 86
	GO[2]: Nucleus, Cytoplasm, DNA binding, Nucleosome, Neucleosome Assembly, Methylation, Acetylation, innate immune response in mucosa.
	KEGG[3]: Systemic Lupus Erythematosus, Alcoholism.
	Cluster 2: Nucleotide Metabolism, Enrichment = 1.3, Count = 34
	GO: Purine Nucleotide Metabolic Process, Nucleoside Triphosphate Biosynthetic Process, Nucleoside Diphosphate Kinase Activity
	KEGG: Pyrimidine Metabolism, Purine Metabolism, Metabolic Pathways.
	RES Downregulated Clusters[4]
	Cluster 1: EF-Hand, Enrichment = 1.53, Count = 4
	GO: Calcium Ion Binding, EF-Hand Domain
	Cluster 2: Mitochondria, Enrichment: 1.5, Count: 11
	GO: Mitochondrion
	KEGG: Biosynthesis of Antibiotics, Metabolic Pathways
Muscle	***RES Upregulated Clusters***
	Cluster 1: Muscle Contraction, Enrichment = 10.25, Count = 9
	GO: Skeletal Muscle Contraction, Troponin Complex
	KEGG: Cardiac Muscle Contraction, Dilated Cardiomyopathy, Hypertrophic Cardiomyopathy, Arrhythmic Right Ventricular Cardiomyopathy, Andrenergic Signaling in Cardiomyocytes.
	Cluster 2: Calcium Binding, Enrichment = 2.26, Count = 14
	GO: Calcium Ion Binding, Myosin Complex,
	KEGG: Focal Adhesion, Regulation of Actin Cytoskeleton, Leukocyte Transendothelial Migration
	Cluster 3: Embryonic Development, Enrichment = 2.74, Count = 14
	GO: Embryonic hindlimb morphogenesis, embryonic forelimb morphogenesis, homeobox, homeobox binding, sequence specific DNA binding, muscle organ development, cellular response to estradiol stimulus, transcriptional activator activity, RNA polymerase II transcription regulatory region sequence-specific binding, positive regulation of transcription from RNA polymerase II promoter, transcription, DNA-templated.
	RES Downregulated Clusters
	Cluster 1: Cytoskeleton, Enrichment = 2.92, Count = 7
	GO: Neuron projection development, regulation of cytoskeleton organization, microtubule depolymerization, tubulin binding.
	Cluster 2: Ion Channel, Enrichment = 2.29, Count = 14
	GO: Membrane depolarization during action potential, voltage-gated sodium channel complex, votage-gated sodium channel activity, neuronal action potential
	Cluster 3: Intermediate Filament, Enrichment = 1.7, Count = 11
	GO: neurofilament, structural molecule activity,
	KEGG: Amyotrophic lateral sclerosis
Cerebrum	***RES Upregulated Clusters***
	Cluster 1: Collagen, Enrichment = 6.86, Count = 14
	GO: Extracellular matrix, collagen trimer, endodermal cell differentiation.
	Cluster 2: Axon Formation, Enrichment = 6.66, Count = 73
	GO: extracellular space, proteinaceous extracellular matrix, collagen binding, heparin binding, axonogenesis
	KEGG: Proteoglycans in cancer.
	Cluster 3: Wnt Signaling, Enrichment: 1.83, Count = 8
	GO: Negative regulation of canonical Wnt signaling pathway, developmental protein, canonical Wnt signaling Pathway.
	KEGG: Wnt signaling Pathway

[1]Clusters from genes which were upregulated in offspring from RES dams compared with CON dams.
[2]GO: Gene Ontology
[3]KEGG: Kyoto Encyclopedia of Genes and Genomes.
[4]Clusters from genes which were downregulated in offspring from RES dams compared with CON dams.

Genes differentially expressed in fetal liver. The genes upregulated in RES compared with CON offspring were grouped into two clusters including: nucleosome core (86 genes) and nucleotide metabolism (34 genes; Table 3.4). The genes downregulated in RES offspring compared with CON were grouped into two clusters including: EF-hand (four genes) and mitochondrion (eleven genes; Table 3.4). The top five DEG with the greatest absolute fold change ($P \leq 0.01$) were all upregulated in RES compared with CON offspring and were: *cytochrome B, carboxypeptidase A1, histone2B, bone gamma-carboxyglutamate, and uncharacterized LOC101907132* (Gene ID unknown in Ensembl database and the gene name is

Table 3.5. Top five genes with the largest absolute fold change that are P-value significant ($P \leq 0.01$) in fetal liver, muscle, and cerebrum.

Symbol[1]	Gene Name[2]	Ensembl ID[3]	*P*-value[4]	*q* Value[5]	CON[6]	RES[7]	Fold Change[8]
Fetal Liver							
CYTB	*Cytochrome B*	00000043580	0.0075	0.1688	1.00	12.59	Up
CPA1	*Carboxypeptidase A1*	00000046169	0.0016	0.0965	1.00	10.67	Up
H2B	*Histone 2B*	00000024188	0.0079	0.1706	1.00	6.23	Up
BGLAP	*Bone gamma-carboxyglutamate*	00000009433	<0.0001	0.0172	1.00	4.99	Up
LOC101907132[9]	*Uncharacterized LOC101907132*	00000025764	0.0039	0.1391	1.00	4.43	Up
Fetal Muscle							
RSPO4	*R-spondin 4*	00000021885	0.0094	0.3827	1.00	8.13	Up
ELAVL3	*ELAV like RNA binding protein 3*	00000014412	0.0033	0.1985	1.00	6.88	Down
TRPVL1	*Transient receptor potential cation channel subfamily V member 1*	00000018880	<0.0001	0.0105	1.00	5.86	Down
TLX3	*T-cell leukemia homeobox 3*	00000010003	0.0057	0.2831	1.00	5.83	Down
ASPHD1	*Aspartate beta-hydroxylase domain containing 1*	00000026319	0.0099	0.3929	1.00	5.24	Down
Fetal Cerebrum							
S10A4	*S100 calcium binding protein A4*	00000019203	0.0022	0.3264	1.00	46.29	Up
PRRX2	*Paired related homeobox 2*	00000002936	0.0011	0.2319	1.00	32.75	Up
MYL2	*Myosin light chain 2*	00000018369	0.0014	0.2603	1.00	29.00	Up
TMEM 119	*Transmembrane protein 119*	00000031849	0.0009	0.2024	1.00	26.86	Up
COL1A1	*Collagen type 1 alpha 1 chain*	00000013103	0.0046	0.5069	1.00	19.28	Up

[1]Approved symbol by HGNC.
[2]Gene name provided when Ensembl gene ID matched a known gene with the Ensembl ID based in DAVID 6.8.
[3]Label for the features such as genes, transcripts, exons, or proteins in the Ensembl database.
[4]Probability value for the effect of treatment.
[5]False discovery rate probability value.
[6]Heifers fed to gain 0.45 kg/hd/d with expression set to 1.0.
[7]Heifers receiving 60% of CON with expression as a fold change relative to CON.
[8]Direction of fold change of RES relative to CON. Up = RES > CON. Down = RES < CON.
[9]Gene has no data in Ensembl. Presented gene is a result of a BLAST of the chromosomal location in GenBank.

the result of BLAST in GenBank; Table 3.5). The top five DEG with the greatest absolute fold change ($q \leq 0.05$) were all upregulated in RES compared with CON offspring and were: *bone gamma-carboxyglutamate, histone H4, proline rich 22, coiled-coil glutamate rich protein 2,* and *TNF receptor superfamily member 4* (Table 3.6).

Table 3.6. Top five genes with the largest absolute fold change that are q value ($q \leq 0.05$) significant in fetal liver, muscle, and cerebrum.

Symbol[1]	Gene Name[2]	Ensembl ID[3]	*P*-value[4]	*q* Value[5]	CON[6]	RES[7]	Fold Change[8]
		Fetal Liver					
BGLAP	*Bone gamma-carboxyglutamate*	00000009433	<0.0001	0.0172	1.00	4.99	Up
H4	*Histone H4*	00000040277	<0.0001	0.0172	1.00	4.03	Up
PRR22	*Proline rich 22*	00000025550	<0.0001	0.0172	1.00	3.83	Up
CCER2	*Coiled-coil glutamate rich protein 2*	00000046295	<0.0001	0.0172	1.00	3.41	Up
TNFRSF4	*TNF receptor superfamily member 4*	00000015635	<0.0001	0.0172	1.00	2.35	Up
		Fetal Muscle					
TRPVL1	*Transient receptor potential cation Channel subfamily V member 1*	00000018880	<0.0001	0.0105	1.00	5.86	Down
CNTN2	*contactin 2*	00000014645	<0.0001	0.0105	1.00	5.12	Down
ELAVL4	*ELAV like RNA binding protein 4*	00000021046	<0.0001	0.0105	1.00	5.01	Down
ATP1A3	*ATPase Na+/K+ transporting subunit alpha 3*	00000018653	<0.0001	0.0105	1.00	4.44	Down
INA	*Internexin neuronal intermediate Filament protein alpha*	00000002717	<0.0001	0.0105	1.00	4.39	Down
		Fetal Cerebrum					
AHSG	*Alpha 2-HS glycoprotein*	00000000522	<0.0001	0.04338	1.00	13.85	Up
ACTC1	*Actin alpha cardiac muscle 1*	00000005714	<0.0001	0.04338	1.00	13.38	Up
P4HA3	*Prolyl 4-hydroxylase subunit alpha 3*	00000006579	<0.0001	0.04338	1.00	11.46	Up
MFAP4	*Microfibrillar associated protein 4*	00000006187	<0.0001	0.04338	1.00	10.77	Up
WNT11	*Wnt family member 11*	00000010820	<0.0001	0.04338	1.00	9.17	Up

[1]Approved symbol by HGNC.
[2]Gene name provided when Ensembl gene ID matched a known gene with the Ensembl ID based in DAVID 6.8.
[3]Label for the features such as genes, transcripts, exons, or proteins in the Ensembl database.
[4]Probability value for the effect of treatment.
[5]False discovery rate probability value.
[6]Heifers fed to gain 0.45 kg/hd/d with expression set to 1.0.
[7]Heifers receiving 60% of CON with expression as a fold change relative to CON.
[8]Direction of fold change of RES relative to CON.

Genes differentially expressed in fetal muscle. The genes upregulated RES compared with CON offspring were grouped into three clusters including: muscle contraction (nine genes), calcium binding (fourteen genes), and embryonic development (fourteen genes; Table 3.4). The genes downregulated in RES compared with CON offspring were grouped into three clusters including: cytoskeleton (seven genes), ion channel (fourteen genes), and intermediate filament (11 genes; Table 3.4). There was only one gene upregulated in RES compared with CON with

the greatest absolute fold change ($P \leq 0.01$) which was *R-spondin 4* up with a fold change of 8.13 up (Table 3.5). The remaining four DEG were all downregulated in RES compared with CON ($P \leq 0.01$) and were: *ELAV like RNA binding protein 3*, t*ransient receptor potential cation channel subfamily V member 1*, *T-cell leukemia homeobox 3*, and *aspartate beta-hydroxylase domain containing 1* (Table 3.5). The top five differentially expressed genes with the greatest absolute fold change ($q \leq 0.05$) were all downregulated in RES compared with CON offspring and were: *transient receptor potential cation channel subfamily V member 1*, *contactin 2*, *ELAV like RNA binding protein 4*, *ATPase NA+/K+ transporting subunit alpha 3*, and *internexin neuronal intermediate filament protein alpha* (Table 3.6).

Genes differentially expressed in fetal cerebrum. The genes upregulated RES compared with CON offspring were grouped into three clusters including: collagen (fourteen genes), axon formation (73 genes), and Wnt signaling (eight genes; Table 3.4). There were only ten genes downregulated in RES compared with CON offspring in fetal cerebrum and therefore no cluster analysis was performed. The top five DEG with the greatest absolute fold change ($P \leq 0.01$) were all upregulated in RES compared with CON offspring and were: *S100 calcium binding protein A4*, p*aired related homeobox 2*, m*yosin light chain 2*, t*ransmembrane protein 119*, and *collagen type 1 alpha 1 chain* with a fold change of 19.28 up (Table 3.5). The top five DEG with the greatest absolute fold change ($q \leq 0.05$) were all upregulated in RES compared with CON offspring and were: a*lpha 2-HS glycoprotein*, a*ctin alpha cardiac muscle 1*, *prolyl 4-hydroxylase subunit alpha 3*, *microfibrillar associated protein 4*, and *Wnt family member 11* (Table 3.6).

Experiment 2

Gene Expression

Data in this section are presented in Figure 3.2 and Table 3.7. *Methionine adenosyltransferase 2A* was greater ($P = 0.02$) in LOW compared with HIGH treated cells. Furthermore, there was a linear decrease in expression of *MAT2A* with increasing OCM supplementation in LOW cells. *Methionine adenosyltransferase 2B* was influenced by a glucose × OCM interaction such that HIGH 2.5X, 5X, and 10X were more greatly expressed ($P < 0.01$) compared with all other treatments. Furthermore, there was a cubic effect ($P = 0.03$) of *MAT2B* expression with increasing OCM supplementation in LOW cells, and a quadratic effect ($P = 0.01$) with increasing OCM in high cells. *DNA methyltransferase 1* was influenced by a glucose × OCM interaction such that LOW Control, 5X, and 10X was more greatly expressed compared with all other treatments. Additionally, there was a cubic effect ($P < 0.01$) of *DNMT1* expression with increasing OCM supplementation in LOW cells. *DNA methyltransferase 3A* tended ($P = 0.09$) to be greater in 10X treated cells compared with all other OCM treatments. Furthermore, *DNMT3A* expression tended ($P = 0.09$) to increase linearly with increasing OCM supplementation in HIGH cells. *DNA methyltransferase 3B* tended ($P = 0.07$) to be greater in 10X compared with all other OCM treatments. Additionally, *DNMT3B* expression was affected cubically ($P = 0.01$) with increasing OCM supplementation in LOW cells. *S-adenosylhomocysteine hydrolase* expression was greater ($P < 0.01$) in HIGH compared with LOW glucose treated cells. Lastly, *methionine synthase* expression was greater ($P = 0.03$) in Control and 10X treated cells compared with 2.5X and 5X OCM supplementation levels. Furthermore, *MTR* expression was affected quadratically ($P = 0.01$) with increasing OCM supplementation in LOW cells.

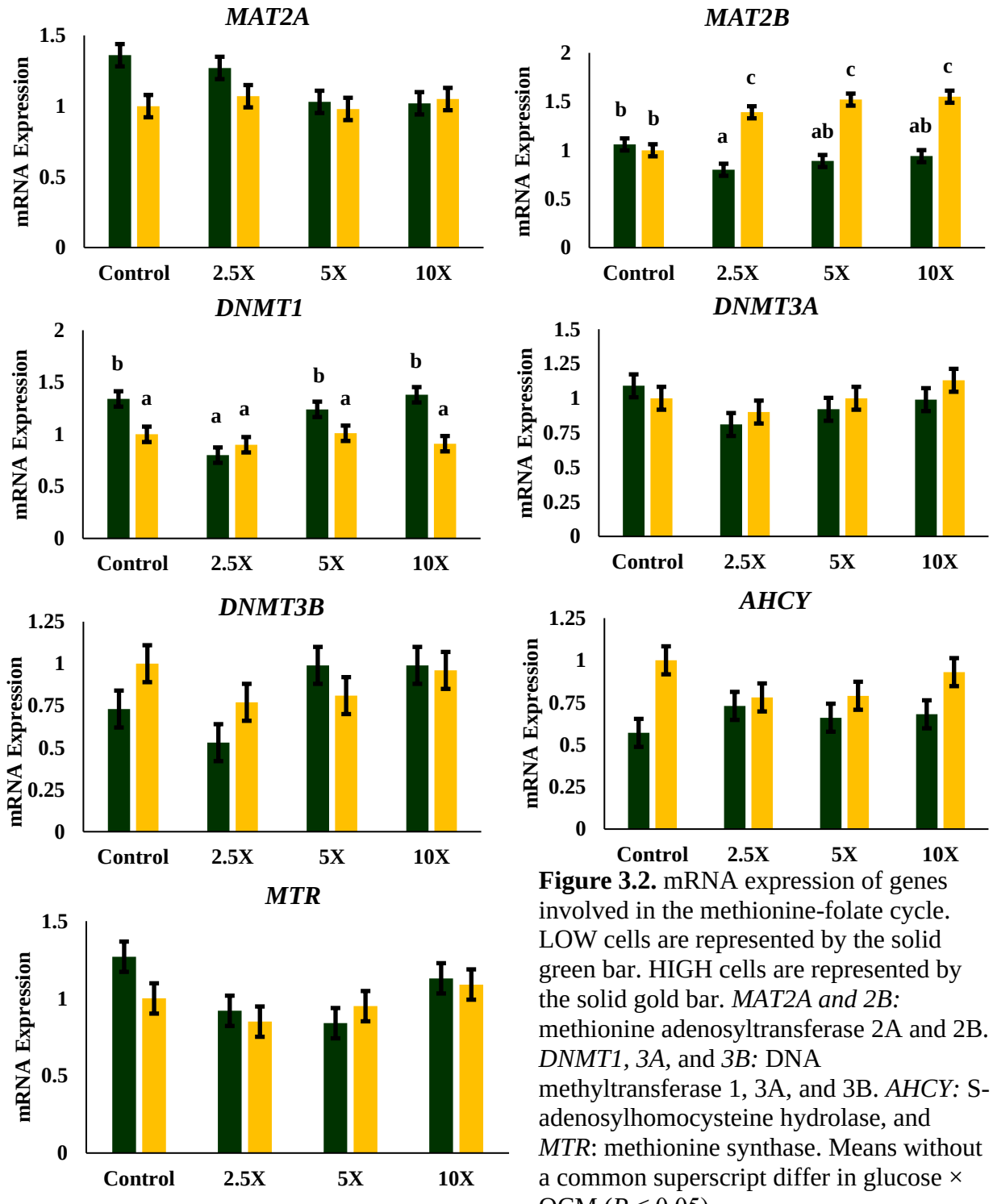

Figure 3.2. mRNA expression of genes involved in the methionine-folate cycle. LOW cells are represented by the solid green bar. HIGH cells are represented by the solid gold bar. *MAT2A and 2B:* methionine adenosyltransferase 2A and 2B. *DNMT1*, *3A*, and *3B:* DNA methyltransferase 1, 3A, and 3B. *AHCY:* S-adenosylhomocysteine hydrolase, and *MTR*: methionine synthase. Means without a common superscript differ in glucose × OCM ($P \leq 0.05$).

Table 3.7. Probability values for the expression of genes for enzymes in the methionine-folate cycle of Embryonic Tracheal Fibroblast (EBTr) cells as influenced by glucose and one-carbon metabolite (OCM) level in Eagle's Minimum Essential Medium (EMEM).

			Glucose ×	LOW Glucose Cells			HIGH Glucose Cells		
Gene[1]	Glucose[2]	OCM[3]	OCM	Linear	Quadratic	Cubic	Linear	Quadratic	Cubic
MAT2A	0.02	0.09	0.11	0.02	0.28	0.38	0.76	0.85	0.25
MAT2B	<0.01	0.02	<0.01	0.43	0.01	0.03	0.01	0.02	0.53
DNMT1	<0.01	<0.01	0.01	0.06	0.01	0.01	0.64	0.92	0.29
DNMT3A	0.36	0.09	0.52	0.84	0.14	0.22	0.09	0.24	0.37
DNMT3B	0.41	0.07	0.20	0.01	0.98	0.01	0.94	0.26	0.65
AHCY	<0.01	0.78	0.15	0.35	0.21	0.11	0.90	0.13	0.63
MTR	0.33	0.03	0.31	0.56	0.01	0.70	0.35	0.28	0.45

[1]*MAT2A and 2B:* methionine adenosyltransferase 2A and 2B. *DNMT1*, *3A*, and *3B:* DNA methyltransferase 1, 3A, and 3B. *AHCY:* S-adenosylhomocysteine hydrolase, and *MTR*: methionine synthase.
[2]LOW: 1 g/L glucose. HIGH: 4.5 g/L glucose.
[3]One-carbon metabolites (methionine, folate, choline, and vitamin B_{12}).

Protein Expression

All data presented in this section can be found in Figure 3.4 and Table 3.8. There were three immunoreactive bands detected for DNMT3A (anticipated mw: 134 kDa; average mw: 132, 115, and 82 kDa for band 1, 2, and 3 respectively; Figure 3.3A). There was no glucose × OCM interaction for any DNMT3A band ($P \geq 0.27$) or main effect of glucose ($P \geq 0.25$) or OCM ($P \geq 0.63$). Band 1 was affected cubically in HIGH ($P = 0.04$) increasing to 2.5X decreasing at below Control at 5X and increasing to Control expression at 10X. Band 2 tended ($P = 0.10$) to increase cubically to 10X in HIGH, and Band 3 was not affected by polynomial contrasts in either the LOW or HIGH cells.

Table 3.8. Probability values for the effect of Glucose, OCM, and Glucose × OCM, as well as linear, quadratic, and cubic polynomial contrasts within glucose level on the mRNA expression of enzymes within the methionine-folate cycle.

				Glucose ×	Low Glucose Cells			High Glucose Cells		
Protein[1]	Band	Glucose[2]	OCM[3]	OCM	Linear	Quadratic	Cubic	Linear	Quadratic	Cubic
	1	0.31	0.71	0.40	0.62	0.74	0.85	0.80	0.34	0.04
DNMT3A	2	0.60	0.63	0.27	0.37	0.33	0.50	0.20	0.07	0.10
	3	0.25	0.68	0.58	0.70	0.24	0.89	0.90	0.86	0.26
	1	0.10	<0.01	0.18	0.56	0.14	0.93	0.08	<0.01	0.69
DNMT3B	2	0.32	0.01	0.03	0.03	0.34	0.68	0.05	0.01	0.69
	3	0.16	0.20	0.08	0.07	0.52	0.72	0.80	0.86	0.03
	1	0.79	0.83	0.99	0.91	0.68	0.97	0.76	0.40	0.80
MTR	2	0.71	0.24	0.25	0.80	0.78	0.15	0.05	0.71	0.80
	3	0.18	0.24	0.06	0.91	0.95	0.47	0.04	0.15	0.01

[1]*MAT2A and 2B:* Methionine Adenosyltransferase 2A and 2B. *DNMT1, 3A,* and *3B:* DNA Methyltransferase 1, 3A, and 3B. *AHCY:* S-Adenosylhomocysteine Hydrolase, and *MTR*: Methionine Synthase.
[2]Low: 1 g/L glucose. High: 4.5 g/L glucose.
[3]One-carbon metabolites (methionine, folate, choline, and vitamin B_{12}).

There were three immunoreactive bands detected for DNMT3B (anticipated mw: 86; average mw: 131, 94, and 60 kDa for bands 1, 2, and 3, respectively; Figure 3.3B). Band 1 was not influenced by a glucose × OCM interaction ($P = 0.18$) but tended ($P = 0.10$) to be more greatly expressed in LOW compared with HIGH cells. Furthermore, DNMT3B band 1 was more greatly expressed ($P < 0.01$) in 10X compared with 2.5 and 5X treated cells. Band 2 was influenced by a glucose × interaction ($P = 0.03$) such that HIGH 10X was more greatly expressed than all other treatment. Furthermore, LOW 10X and HIGH Control were intermediate and more greatly expressed than HIGH 5X. Band 3 tended ($P = 0.08$) to be more greatly expressed in HIGH 2.5X compared with all other treatments. Band 1 expression in HIGH was affected cubically ($P = 0.01$) decreasing to 2.5X and increasing to 10X with similar levels of expression as that of Control. Band 2 expression in LOW increased linearly ($P = 0.03$) with increasing OCM supplementation and was affected quadratically ($P = 0.01$) in HIGH, decreasing to 2.5 and 5X, and subsequently increasing in expression to levels greater than Control at 10X. Band 3 for DNMT3B tended ($P = 0.07$) to increase linearly in LOW cells, and was altered

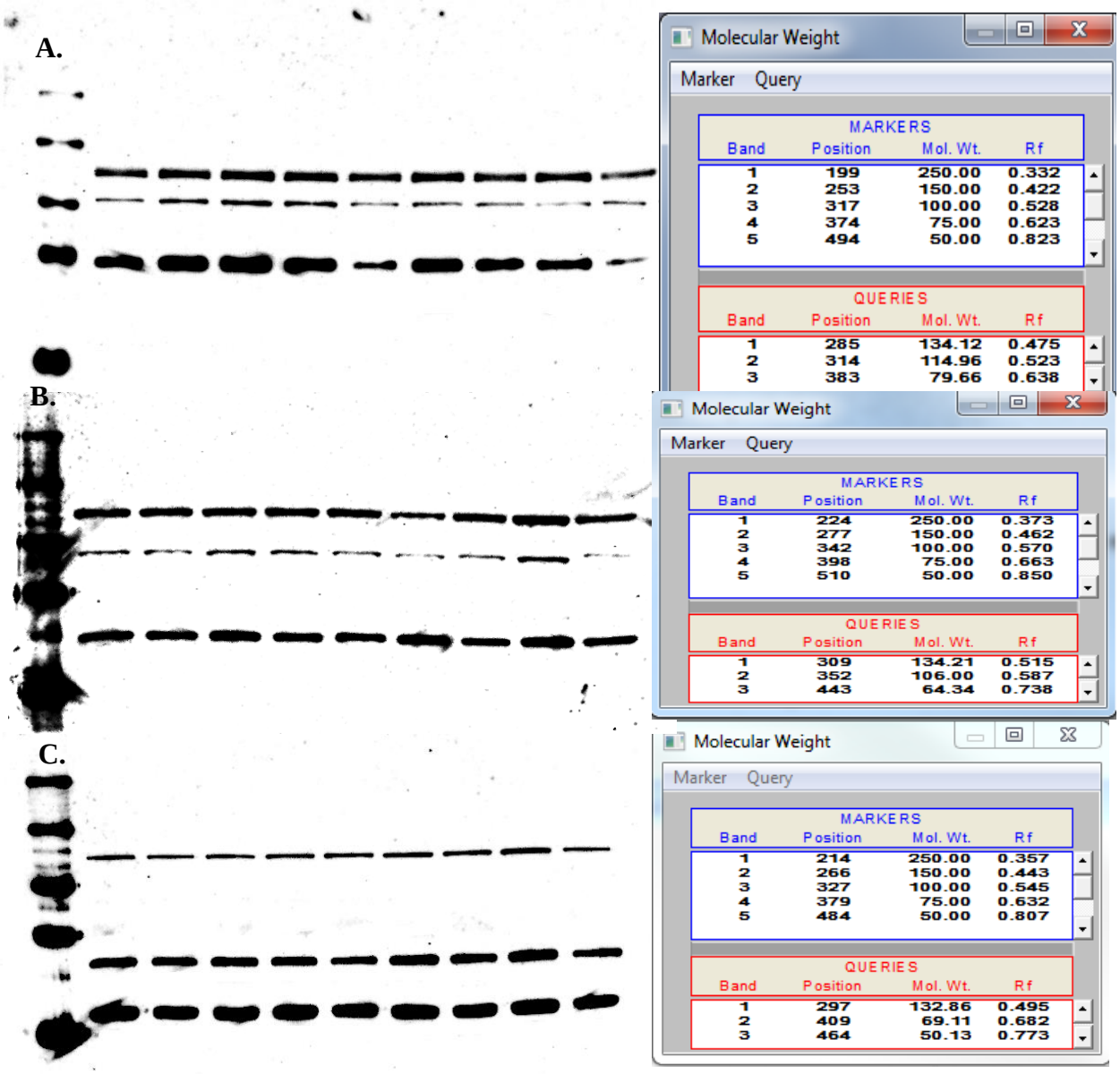

Figure 3.3. Western blot images for DNMT3A (A), DNMT3B (B), and MTR (C) including the molecular weight of each band for the image taken. Lane 1: Molecular Weight Marker, Lane 2: Low Control, Lane 3: Low 2.5X, Lane 4: Low 5X, Lane 5: Low 10X, Lane 6: High Control, Lane 7: High 2.5X, Lane 8: High 5X, Lane 9: High 10X, Lane 10: Pooled normalization control.

cubically (P = 0.03) in High increasing to 2.5X decreasing to levels below Control in 5X and increasing to levels similar to 2.5X at 10X OCM supplementation.

There were also three immunoreactive bands for MTR (anticipated mw: 140 kDa; average mw: 130, 67, and 49 kDa for bands 1, 2, and 3 respectively; Figure 3.3C). There was no

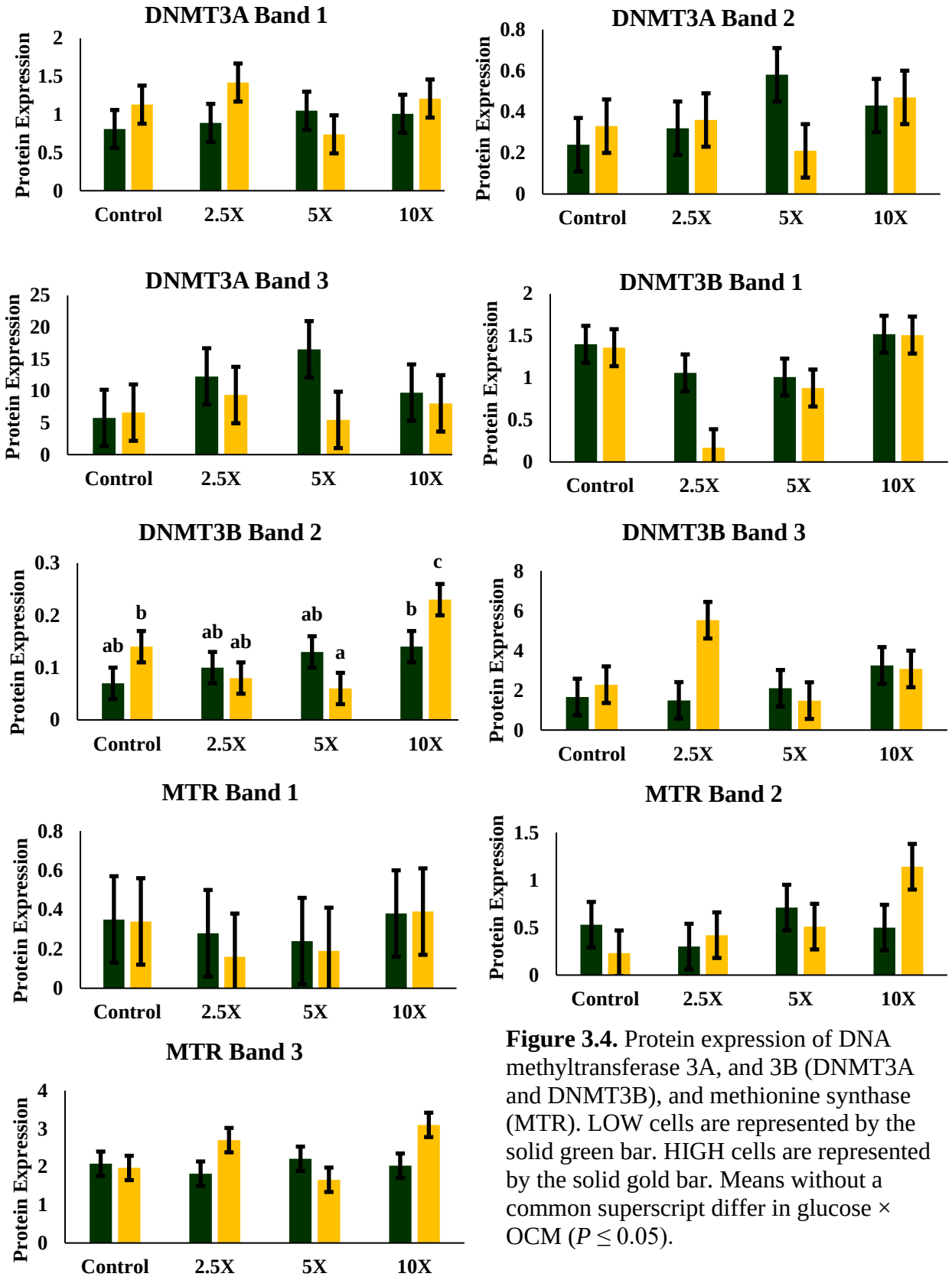

Figure 3.4. Protein expression of DNA methyltransferase 3A, and 3B (DNMT3A and DNMT3B), and methionine synthase (MTR). LOW cells are represented by the solid green bar. HIGH cells are represented by the solid gold bar. Means without a common superscript differ in glucose × OCM ($P \leq 0.05$).

glucose × OCM interaction for any MTR band ($P \geq 0.06$) or main effect of glucose ($P \geq 0.18$) or OCM ($P \geq 0.24$); however, band 3 tended ($P \geq 0.06$) to be greater in HIGH 10X compared with all other treatments. Band 2 expression in HIGH increased linearly ($P = 0.05$) with increasing OCM supplementation. Band 3 expression in HIGH was affected cubically ($P = 0.01$) increasing to 2.5X, decreasing at 5X to levels similar to Control, and increasing to 10X.

Mitochondrial Respiration

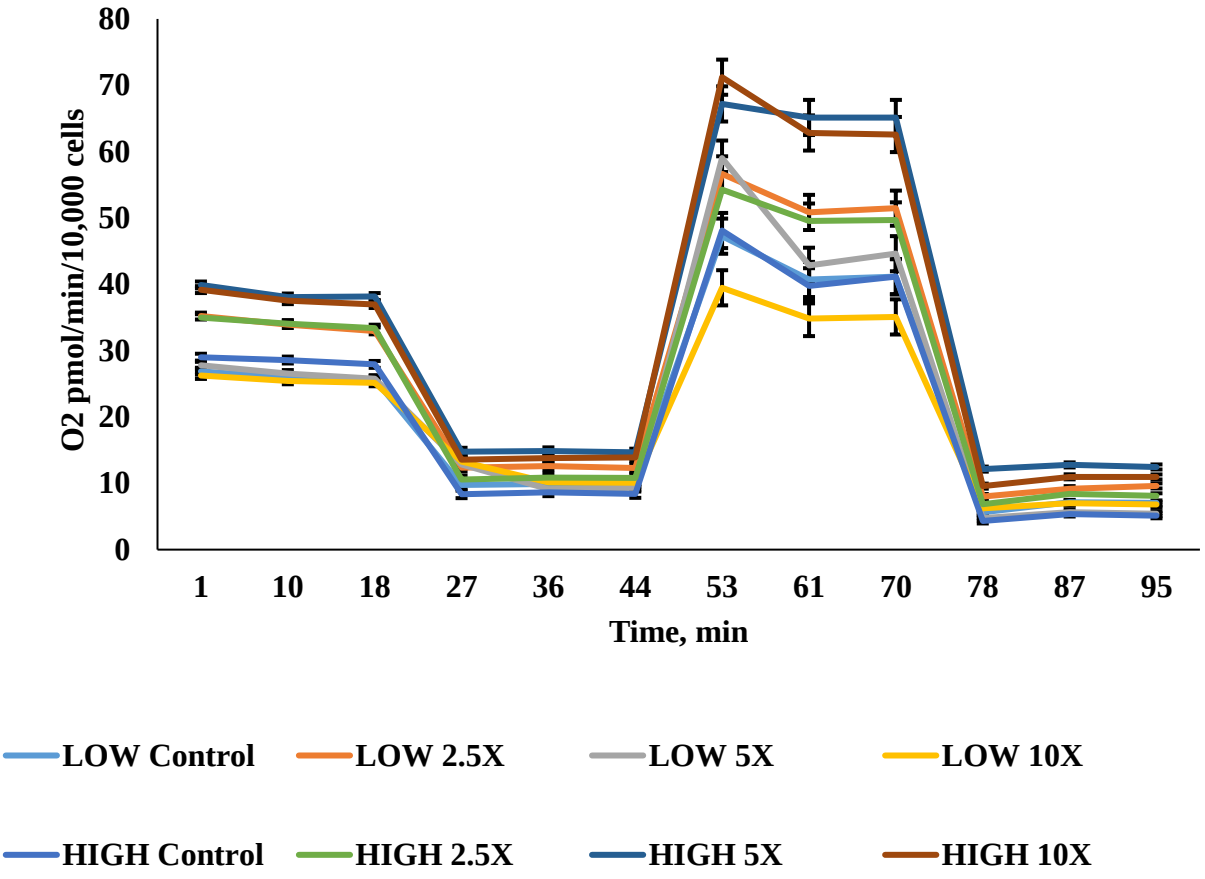

Figure 3.5. Mitochondrial respiration of Embryonic Tracheal Fibroblast (EBTr) cells as influenced by glucose and one-carbon metabolite (OCM) level in Eagle's Minimum Essential Medium (EMEM).

All data presented in this section can be found in Figure 3.5 and Table 3.9. Furthermore, all measurements except for proton leak were influenced by a glucose × OCM interaction ($P \leq 0.05$). Basal respiration was greater ($P < 0.01$) in HIGH 2.5X and 5X compared with all other treatments. Basal oxygen consumption in LOW was altered cubically increasing at 2.5X and returning to Control levels at 5X and 10X OCM supplementation ($P < 0.01$). Basal oxygen consumption in HIGH increased cubically to 10X OCM ($P < 0.01$). O_2-linked ATP synthesis was greater ($P < 0.01$) in HIGH 2.5X, 5X, and 10X compared with all other treatments ($P < 0.01$). O_2-linked ATP synthesis in LOW was affected cubically, increasing to 2.5X and returning to Control levels at 5X and 10X ($P < 0.01$). O_2-linked ATP synthesis in HIGH increased cubically to 10X OCM ($P = 0.04$). Maximal respiration was greater ($P < 0.01$) in High 2.5X an 10X compared with all other treatments ($P < 0.01$). Maximal respiration in LOW increased ($P = 0.01$) quadratically to 2.5X and decreased to 10X. Maximal respiration in HIGH increased ($P < 0.01$) cubically with increasing OCM supplementation. Similarly, reserve capacity was greater ($P < 0.01$) in HIGH 2.5X and 10X compared with all other treatments. In LOW cells, reserve capacity increased ($P = 0.04$) quadratically to 2.5X and decreased to 10X. Reserve capacity in HIGH cells increased ($P < 0.01$) with increasing OCM supplementation. Proton leak was not affected by a glucose × OCM interaction, or a main effect of OCM; however, proton leak tended ($P = 0.06$) to be greater in LOW compared with HIGH glucose cells. Non-mitochondrial respiration was greater ($P < 0.01$) in High 2.5X compared with all other treatments. Non-mitochondrial respiration in LOW cells increased ($P < 0.01$) cubically to 2.5X and returned to levels similar to that of Control at 5X and 10X. In HIGH, Non-mitochondrial respiration increased ($P < 0.01$) cubically to 2.5X and remained intermediate at 5X and 10X OCM.

Table 3.9. Mitochondrial respiration of Embryonic Tracheal Fibroblast (EBTr) cells as influenced by glucose and one-carbon metabolite (OCM) level in Eagle's Minimum Essential Medium (EMEM).[1]

Measurement[4]	Glucose[5]	OCM Concentrations[2]				Glc Avg	SEM	*P* – values[3]					
		Control	2.5X	5X	10X			Glucose	OCM	Glucose × OCM	Lin	Quad	Cub
Basal	LOW	26.15^{a}	34.03^{c}	26.70^{a}	26.61^{a}	28.13	0.52	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
	HIGH	28.50^{b}	38.71^{d}	34.14^{c}	37.89^{d}	34.81					<0.01	<0.01	<0.01
	OCM Avg	27.33	36.37	30.42	31.75								
O_2-linkedATP Synthesis	LOW	16.28^{a}	21.63^{b}	16.28^{a}	14.46^{a}	17.16	0.61	<0.01	<0.01	<0.01	<0.01	0.01	<0.01
	HIGH	20.26^{b}	23.96^{c}	23.40^{c}	24.14^{c}	22.89					<0.01	0.02	0.04
	OCM Avg	18.17	22.79	19.84	19.30								
Maximal Respiration	LOW	43.04^{ab}	52.98^{c}	48.83^{bc}	36.46^{a}	45.33	2.65	<0.01	<0.01	<0.01	0.05	0.01	0.26
	HIGH	43.00^{ab}	64.68^{d}	51.16^{c}	65.53^{d}	56.09					<0.01	0.19	<0.01
	OCM Avg	43.02	58.83	49.99	50.99								
Reserve Capacity	LOW	16.88^{abc}	18.95^{bc}	22.12^{cd}	10.84^{a}	17.20	2.25	0.02	0.06	<0.01	0.11	0.04	0.47
	HIGH	14.5^{ab}	25.97^{d}	17.02^{abc}	27.64^{d}	21.28					<0.01	0.99	<0.01
	OCM Avg	15.69	22.46	19.57	19.24								
Proton Leak	LOW	3.28	3.48	5.14	4.49	4.10	0.75	0.06	0.44	0.48	0.35	0.47	0.46
	HIGH	3.51	2.30	2.96	3.25	3.00					0.85	0.07	0.07
	OCM Avg	3.40	2.89	4.05	3.87								
Non-mitochondrial Respiration	LOW	6.6^{b}	8.93^{d}	5.28^{a}	6.67^{bc}	6.87	0.38	<0.01	<0.01	<0.01	0.13	0.76	<0.01
	HIGH	4.93^{a}	12.45^{f}	7.79^{cd}	10.49^{e}	8.92					<0.01	<0.01	<0.01
	OCM Avg	5.76	10.69	6.54	8.58								

[1]Data presented as O_2 consumption in pmol/min/10,000 cells

[2]Control = Basal concentrations of methionine, folate, and choline in EMEM media with 4 µmol/L vitamin B_{12}. 2.5X, 5X, 10X = 2.5, 5, and 10 times the concentration of folate, choline, and vitamin B_{12} in CON media. Methionine concentrations were limited to 2X methionine in CON media to prevent toxicity.

[3]Probability values for the effect of glucose, one-carbon metabolite, and the interaction. Probability values for linear, quadratic, and cubic polynomial contrasts across OCM supplementation levels within glucose level.

[4]Basal: Initial O_2 consumption measurement. O_2-linked ATP synthesis: basal respiration - O_2 consumption after injection with oligomycin., Maximal respiration: O_2 consumption after injection with FCCP. Reserve capacity: Maximal respiration – basal respiration. Proton leak: O_2 consumption after injection with oligomycin – O_2 consumption after injection with rotenone/antimycin. Non-mitochondrial respiration: O_2 consumption after injection with rotenone/antimycin.

[5] LOW: 1 g/L glucose. HIGH: 4.5 g/L glucose.

[a-f]Means within a measurement without common superscript within row and column differ.

Cell Growth Rate

Data presented in this section are found in Figure 3.6 and Table 3.10. Total growth rate was affected by a glucose × OCM interaction ($P < 0.01$) with HIGH 10X and 2.5X treated cells having greater cell growth compared with HIGH CON and LOW 5X. Furthermore, HIGH CON and 5X as well as LOW 5X treated cells were greater ($P < 0.01$) than LOW CON, 2.5X, and 10X. Early growth rate was not influenced by a glucose × OCM interaction or the main effect of OCM ($P \geq 0.14$); however, HIGH cells had a greater ($P < 0.01$) growth rate compared with LOW treated cells. Late growth rate was affected by a glucose × OCM interaction ($P < 0.01$) with HIGH 2.5X, 5X, and 10X OCM having greater cell growth compared with HIGH CON and

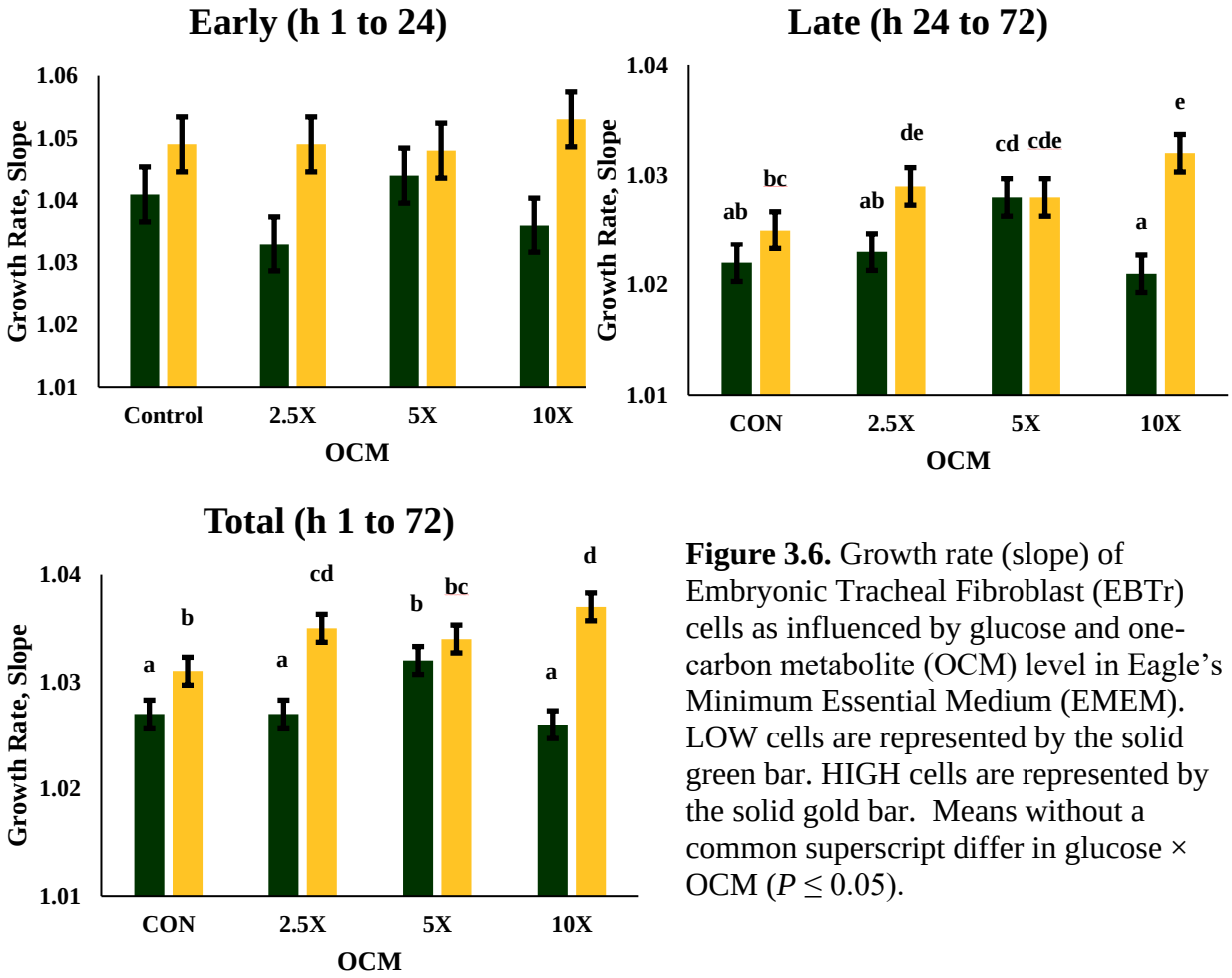

Figure 3.6. Growth rate (slope) of Embryonic Tracheal Fibroblast (EBTr) cells as influenced by glucose and one-carbon metabolite (OCM) level in Eagle's Minimum Essential Medium (EMEM). LOW cells are represented by the solid green bar. HIGH cells are represented by the solid gold bar. Means without a common superscript differ in glucose × OCM ($P \leq 0.05$).

Table 3.10. Growth rate of Embryonic Tracheal Fibroblast (EBTr) cells as influenced by glucose and one-carbon metabolite (OCM) level in Eagle's Minimum Essential Medium (EMEM).[1]

Time, h[4]	Glucose[5]	One-Carbon Metabolites[2]				Average Glc[6]	SEM[7]	*P*-value[3]					
		CON	2.5X	5X	10X			Glc	OCM	Glc × OCM	L	Q	C
1-24 (early)	LOW	1.041	1.033	1.044	1.036	1.039[a]	0.0044	<0.01	0.41	0.14	0.54	0.77	0.04
	HIGH	1.049	1.049	1.048	1.053	1.050[b]					0.28	0.50	0.81
	OCM Avg	1.045	1.041	1.046	1.045								
24-72 (late)	LOW	1.022[cd]	1.023[cd]	1.028[ef]	1.021[c]	1.023	0.0017	<0.01	0.01	0.01	0.54	0.01	0.09
	HIGH	1.025[de]	1.029[fg]	1.028[efg]	1.032[g]	1.029					0.01	0.62	0.17
	OCM Avg	1.023	1.026	1.028	1.026								
1-72 (total)	LOW	1.027[c]	1.027[c]	1.032[d]	1.026[c]	1.028	0.0013	<0.01	0.01	<0.01	0.47	<0.01	0.01
	HIGH	1.031[d]	1.035[ef]	1.034[de]	1.037[f]	1.034					<0.01	0.59	0.04
	OCM Avg	1.029	1.031	1.033	1.031								

[1]Rate = slope of the line of cells after natural log transformation

[2]CON = Basal concentrations of methionine, folate, and choline in EMEM media with 4 µmol/L vitamin B_{12}. 2.5X, 5X, 10X = 2.5, 5, and 10 times the concentration of folate, choline, and vitamin B_{12} in CON media. Methionine concentrations were limited to 2X methionine in CON media to prevent toxicity.

3Probability values for the effects of glucose, OCM, and the interaction. Linear (L), quadratic (Q), and cubic (C) polynomial contrasts for the effect of glucose and increasing OCM supplementation on growth rate of EBTr cells within glucose level.

[4]Hours after plating.

[5]LOW = 1 g/L glucose and HIGH = 4.5 g/L glucose in culture media.

[6]Average growth rate within glucose level.

[7]Standard error of the mean for the interaction of glucose and OCM level.

[8]Average growth rate within OCM supplementation level.

[a-b]Means within a column without a common superscript differ.

[c-g]Means within a time without a common superscript differ.

LOW CON, 2.5X, and 10X. Late and total cell growth in Low treated cells increased quadratically ($P \leq 0.01$) to 5X and decreased at 10X OCM supplementation. In High treated cells, Late and total cell growth increased linearly ($P \leq 0.01$) with increasing OCM supplementation.

Cell Proliferation

Data presented in this section are found in Figure 3.7 and Table 3.11. Labeling index did not differ between glucose concentrations, OCM, or the interaction for total or after plate attachment (1 to 72 and 12 to 72 h, respectively) and was therefore omitted from the tables and results. At 1 h post plating, labeling index was affected by a glucose × OCM interaction (P =0.05) being greater in LOW 10X compared with all other LOW OCM levels and HIGH 10X, with High CON, 2.5X, and 5X being intermediate. There were no differences at 12 h ($P \geq 0.14$). At 24 h, labeling index for HIGH 10X was greater ($P = 0.02$) than all other treatments. Furthermore, labeling index for HIGH 2.5X was greater ($P < 0.01$) compared with HIGH CON and LOW 10X, and was equal to LOW CON, 2.5X, 5X, and HIGH 5X. At 36 h, labeling index was affected by a glucose × OCM interaction ($P = 0.02$) with HIGH 2.5X and 10X being greater than LOW 10X and HIGH 5X. Additionally, HIGH CON and LOW CON, 2.5X, and 5X were greater than LOW 10X. At 48 h, labeling index was affected by a glucose × OCM interaction (P = 0.03) with HIGH 10X being greater compared with all LOW treatments as well as HIGH CON and 5X. HIGH CON, 2.5X, and 5X were greater than LOW 2.5X and 10X. At 72 h, labeling index was affected by a glucose × OCM interaction ($P = 0.01$) with LOW CON being greater than LOW 2.5X and 10X as well as HIGH CON. LOW 5X and HIGH 10X were greater than LOW 10X and HIGH CON. Labeling index linearly decreased ($P \leq 0.04$) in Low cells at 36 and

72 h with increasing OCM supplementation. In contrast, labeling index linearly increased ($P \leq$ 0.05) in High cells at 24, 48, and 72 h with increasing OCM supplementation.

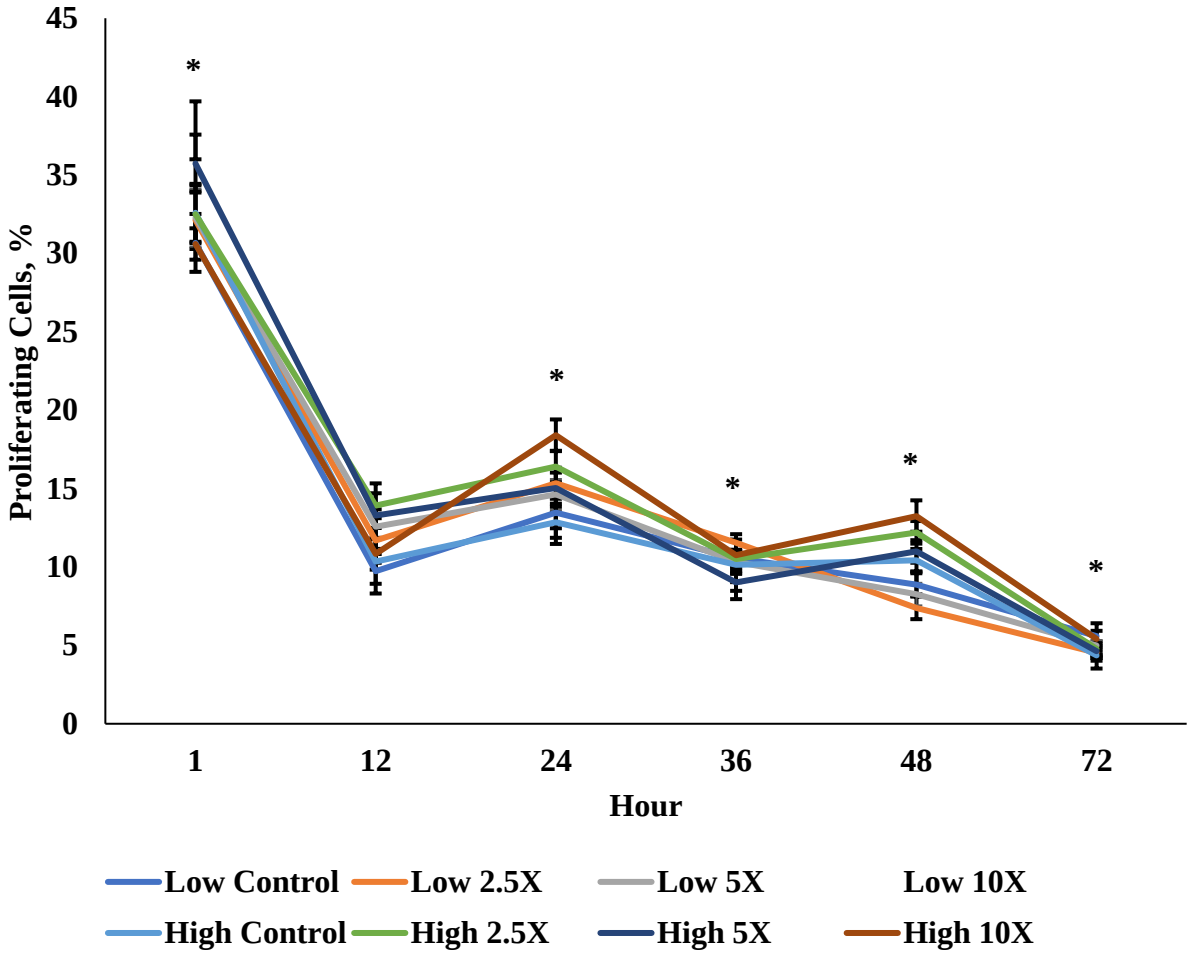

Figure 3.7. Labeling index (cell proliferation) of Embryonic Tracheal Fibroblast (EBTr) cells stained with Ki67 as influenced by glucose and one-carbon metabolite level in Eagle's Minimum Essential Medium (EMEM). *****Means differ glucose × OCM ($P \leq 0.05$).

Discussion

These data are interpreted to imply that a moderate nutrient restriction during the first 50 days of gestation alters gene expression to program the fetus towards a reduced nutritional environment postnatally. Furthermore these data are interpreted to imply that supplementation of

Table 3.11. Labeling index (cell proliferation) of Embryonic Tracheal Fibroblast (EBTr) cells stained with Ki67 as influenced by glucose and one-carbon metabolite level in Eagle's Minimum Essential Medium (EMEM).[1]

		One-Carbon Metabolites[2]						*P*-value[3]					
Time, h[4]	Glucose[5]	CON	2.5X	5X	10X	Average Glc[6]	SEM[7]	Glc	OCM	Glc × OCM	Lin	Quad	Cub
	LOW	30.67^{a}	32.13^{a}	32.28^{a}	37.86^{b}	33.23	1.85	0.44	0.77	0.05	0.50	0.14	0.27
1	HIGH	32.59^{ab}	32.50^{ab}	35.73^{ab}	30.60^{a}	32.85					0.03	0.50	0.67
	OCM Avg	31.63	32.32	34.00	34.23								
	LOW	9.73	11.69	12.57	12.25	11.56	2.01	0.19	0.60	0.65	0.14	0.19	0.91
12	HIGH	10.35	13.91	13.28	10.83	12.09					0.87	0.14	0.55
	OCM Avg	10.04	12.80	12.93	11.54								
	LOW	13.46^{ab}	15.34^{ab}	14.63^{ab}	12.46^{a}	13.97	1.40	0.03	0.07	0.02	0.25	0.09	0.49
24	HIGH	12.85^{a}	16.40^{b}	15.03^{ab}	18.41^{c}	15.67					0.01	0.78	0.10
	OCM Avg	13.15	13.87	14.83	15.44								
	LOW	10.56^{bc}	11.55^{bc}	10.32^{bc}	8.49^{a}	10.23	0.77	0.75	0.06	0.02	0.01	0.10	0.24
36	HIGH	10.14^{bc}	10.50^{c}	9.02^{ab}	10.75^{c}	10.10					0.62	0.15	0.11
	OCM Avg	10.35	11.02	9.67	9.62								
	LOW	8.88^{ab}	7.39^{a}	9.27^{ab}	7.98^{a}	8.38	1.02	<0.01	0.56	0.03	0.71	0.86	0.07
48	HIGH	10.43^{bc}	12.21^{cd}	11.00^{bc}	13.25^{d}	11.72					0.05	0.80	0.11
	OCM Avg	9.65	9.80	10.13	10.62								
	LOW	5.59^{d}	4.53^{abc}	4.99^{bcd}	3.87^{a}	4.74	0.48	0.74	0.83	0.01	0.04	0.94	0.19
72	HIGH	4.36^{ab}	4.78^{abcd}	4.63^{abcd}	5.41^{cd}	4.80					<0.01	0.56	0.19
	OCM Avg	4.97	4.66	4.81	4.64								

[1]Labeling index = % of positively stained cells with Ki67.

[2]CON = Basal concentrations of methionine, folate, and choline in EMEM media with 4 µmol/L vitamin B_{12}. 2.5X, 5X, 10X = 2.5, 5, and 10 times the concentration of folate, choline, and vitamin B_{12} in CON media. Methionine concentrations were limited to 2X methionine in CON media to prevent toxicity.

[3]Probability values for the effects of glucose, OCM, and the interaction. Linear (L), quadratic (Q), and cubic (C) polynomial contrasts for the effect of glucose and increasing OCM on growth rate of EBTr cells within glucose level.

[4]Hours after plating.

[5]LOW = 1 g/L glucose and HIGH = 4.5 g/L glucose in culture media.

[6]Average growth rate within glucose level.

[7]Standard error of the mean for the interaction of glucose and OCM level.

[8]Average growth rate within OCM supplementation level.

[a-d]Means within an hour without a common superscript differ.

OCM to bovine embryonic fibroblasts improves mitochondrial respiration and total growth rate of embryonic fibroblasts beyond unsupplemented controls. Therefore, these data support our hypothesis that: 1) early global maternal nutrient restriction impacts fetal growth and gene expression towards shifting metabolic and growth outcomes, and 2) supplemental OCM increased growth and proliferation of embryonic fibroblasts which may be due in part to greater mitochondrial efficiency *in vitro*.

In ruminant production systems there is potential for periods of nutritional stress due to inadequate or overnutrition prior to, during, and immediately after pregnancy as a result of factors such as forage quality, overgrazing, or drought (Cline et al., 2009). For example, the area impacted by the 2018 drought conditions in the southwest U.S. (Fuchs, 2018) overlapped slightly with areas that were impacted by a long-term drought in 2011, which caused major feed shortages in large portions of Texas, Oklahoma, and Kansas (Miskus, 2011). In addition, a severe drought struck the Upper Great Plains in 2017 (Bathke, 2017) at a time when over 80% of beef cattle breeding seasons in the region were underway, and the drought continued through at least the first trimester of pregnancy (Dahlen, unpublished). Thus, drought or any natural or iatrogenic conditions affecting forage quality and availability during breeding and early gestation are a major concern for producers. Furthermore, post-insemination nutrient restriction to heifers alters pregnancy rates (Arias et al., 2012; Arias et al., 2013; Lake et al., 2013) which suggests that moderate negative changes in nutrient supply may reduce pregnancy rates by modifying nutrient partitioning in the heifer away from the utero-placenta during early pregnancy.

Experiment 1. The moderate nutrient restriction throughout the first 50 days had marginal effects on embryonic/fetal size. During this timeframe there were no differences on d

16 and 34 ($P > 0.19$) in any size measurements taken, but at d 50 of gestation we start to see a tendency ($P = 0.116$) toward crown-rump length being decreased in the offspring from RES compared with CON dams (Table 3.1). It is important to recognize that previously published data from the same study (Crouse et al., 2016a; Crouse et al., 2017; Greseth et al., 2017a) found minor effects of maternal nutritional treatment on the expression of nutrient transporters in the utero-placenta but elucidated differences in glucose and fructose in fetal fluids (Crouse et al., 2019b). The smaller fetal size may be due to a decrease in energetic metabolites available for growth, but the lack of differences in transporters may suggest altered fetal metabolism as well. The current data cannot extrapolate as to whether nutrient concentrations and fetal size differences are due to transport capacity or altered fetal metabolism, but these data may suggest that either of these may be the case and do merit further investigation.

Fetal liver had the greatest number of DEG, followed by fetal muscle, with cerebrum having the least number of DEG (546, 316, and 144, $P < 0.01$, respectively). In cases of fetal nutrient restriction, brain growth is spared compared with other organs (Georgieff, 2007; Lanet and Maurange, 2014), which is why there is asymmetrical growth between the larger head and the smaller body and organs in growth restricted babies (Cox and Marton, 2009). Data presented within support this sparing effect of altered gene expression in contrast to other organs such as the liver and muscle. However, although the brain size and cell number may be spared, there may be structural deviances (Georgieff, 2007). Gene clusters presented within suggest alterations in genes related to collagen and axon formation, were upregulated in offspring from RES dams compared with CON with *COL1A1* (a top-five *P*-value DEG) being 19.28-fold greater in RES offspring compared with CON. These data support previous work suggesting that a moderate nutrient restriction in beef heifers during the first 50 days of gestation may yield altered brain

formation in which embryos may be compensating for by increasing expression of genes related to cerebral structure.

Data published from the same study (Crouse et al., 2019b) showed decreased concentrations of glucose, fructose, and methionine in fetal fluids of RES dams compared with CON, and increased homocysteine in RES heifer serum compared with CON suggesting altered energy availability and one-carbon metabolism across the maternal-fetal interface. Furthermore, RNA-sequencing data reported within had upregulated clusters in nucleosome core, and nucleotide metabolism and downregulated clusters in metabolism. Additionally, upregulated genes *H2B* and *H4* (*P*-value and *q*-value significant greatest fold change, respectively) which are responsible for the formation of histones 2B and 4 were both upregulated in offspring from RES compared with CON offspring. Decreases in methionine and increases in homocysteine coupled with decreases in metabolic fuels may suggest that these upregulations in nucleosome core and nucleotide synthesis may be a compensatory mechanism for decreased availability of energetic substrates and methylation precursors.

In comparison to fetal cerebrum and liver (93.0% and 89.1% upregulated in RES compared with CON, respectively), fetal muscle had a greater percentage of DEG downregulated in RES compared with CON (54.4%; Figure 3.1). The embryonic development cluster was upregulated in offspring from RES dams compared with CON and included genes such as *myogenin* and *myogenic differentiation gene 1* which are involved in myogenic lineage and the formation of primary and secondary myofibers (Palacios and Puri, 2006; Du et al., 2010). Recently, Paradis et al., (2017) showed greater expression of *myogenin* and *myogenic differentiation gene 1* in the *longissimus dorsi* of fetuses from cows nutrient restricted during mid to late gestation as we have reported here in early gestation. Interestingly, there is no discernable

pattern between those genes upregulated and downregulated in RES compared with CON in terms of formation of a contractile muscle fiber. Both clusters show changes in muscle contraction, calcium binding, cytoskeleton, ion channel, and intermediate filament all of which are critical for the formation and function of a contractile muscle fiber. While some data in sheep points to a decline in total muscle fiber number in offspring born to nutrient restricted ewes during early gestation (Zhu et al., 2004), it remains to be seen if this translates to alterations in muscle fiber function or tenderness upon harvest.

Experiment 2. Based upon data presented in experiment 1, elucidating changes in expression of genes related to nucleosome cores, mitochondrial formation and function, as well as the previously published data demonstrating changes to energy metabolites and methionine (Crouse et al., 2019b) the *in vitro* study presented in experiment 2 was conducted to determine whether OCM supplementation to bovine embryonic cells cultured in a moderately divergent glucose media would improve cell growth parameters as measured by cell growth, proliferation, and mitochondrial function.

One-carbon metabolites used in this study such as choline, vitamin B_{12}, folate, and methionine are methyl donors and cofactors that play key roles in one-carbon metabolism which is the network of biochemical pathways in which methyl groups are transferred from one compound to another for methylation processes (Mason, 2003). Choline, via its metabolite betaine, and folate via methyl-tetrahydrofolate, methylate homocysteine to form methionine (Kim et al., 1994; Finkelstein, 2000). Vitamin B_{12} is a cofactor for methionine synthase, the enzyme responsible for folate dependent methylation of homocysteine (Kalhan and Marczewski, 2012). The metabolism of choline, folate, vitamin B_{12}, and methionine are interrelated and disturbances in one of these metabolic pathways are associated with compensatory changes in

the others (Zeisel, 2011). Additionally, elevations in plasma homocysteine concentrations are associated with impaired one-carbon metabolism resulting from protein malnutrition and deficiencies in folate and B_{12} (Castro et al., 2006). In contrast, when methionine and folate levels are present in excess, homocysteine is instead degraded by the transsulfuration pathway (Clare et al., 2019); However, in this case, homocysteine may be further metabolized to cystathione which bridges downstream transulfuration pathways or synthesis of glutathione or succinyl CoA thus linking one-carbon metabolism to redox defense and the TCA cycle (Clare et al., 2019). Methionine is the precursor for S-adenosylmethionine (**SAM**), which is the physiological methyl group donor for protein, RNA, and DNA methylation (Jeltsch, 2002). The DNA and histone methyltransferases primarily use SAM as the methyl donor. S-adenosylmethionine availability is directly influenced by diet as it is formed from methyl-groups derived from choline, methionine, or methyl-tetrahydrofolate (Zeisel, 2011). The metabolism of methionine and folate through the methionine folate cycle influences a large number of processes that directly or indirectly effect cell proliferation and gene expression in the growing organism (Kalhan and Marczewski, 2012). Cell proliferation and gene expression are influenced by the methionine folate cycle due to their role in DNA synthesis and methylation of DNA (and therefore activation or suppression of gene expression through epigenetic modifications), as well as synthesis of polyamines and nucleotides (Clare et al., 2019). Perturbation in the methionine folate cycle, either by nutrient deficiency, or by nutritional, hormonal, and environmental interactions can have a profound impact on cellular function, metabolism, growth, and proliferation, which has the greatest impact on the growing embryo and fetus (Kalhan and Marczewski, 2012).

Multiple genes involved in the methionine-folate cycle of DNA-methylation were altered by glucose, OCM, and the interaction. *Methionine adenosyltransferase 1A* was not expressed in

the EBTr cell line; however, this was to be expected due to being a primarily hepatic enzyme (Nordgren et al., 2011). Furthermore, *BHMT* was not expressed in this cell line despite choline supplementation and choline being an immediate precursor to betaine; however, Ikeda et al., (2010) showed *BHMT* not being expressed from 8 cell embryos onward, but Batistel et al., (2017) showed differential expression of *BHMT* in the placentome of methionine supplemented cows and Alharti et al., (2019) found no differences in liver expression of *BHMT* in calves born to methionine supplemented or control treated dams during late gestation. Therefore, we can interpret these data to imply that the EBTr cells were not expressing *BHMT* due to the stage of development in which these cells were immortalized. Methionine adenosyltransferase 2A is expressed in most tissues (Halim et al., 1999), but MAT2B encodes a regulatory protein that formed a heterotetramer with MAT2A and when bound, increases activity and abundance of MAT2A but decreases the km for methionine and ATP (Nordgren et al., 2011). The interaction seen within increasing expression of *MAT2B* with increasing OCM in High cells may be an effort to increase MAT2A and the functional unit of SAM synthesis. During early epigenetic reprogramming of embryonic development, DNMT1 is excluded from the nucleus and remains a cytosolic protein which allows for complete demethylation of the embryonic genome. Therefore, DNMT3A and 3B provide *de novo* methylation of DNA during this time frame and upon remethylation of the genome (Okano et al., 1999), DNMT1 is the primary methylation enzyme for maintenance methylation (Messerschmidt et al., 2014). While we saw no differences due to treatment in *DNMT3A* or *3B* expression, *DNMT1* expression was lower in HIGH cells compared with LOW in Control, 5X, and 10X supplementation levels.

Western blot analysis of DNMT3A and MTR did not show any differences in bands of the anticipated molecular weight for the either protein. Analysis of DNMT3B was greatest in

HIGH 10X compared with all other treatments and there were linear increases in both LOW and HIGH cells with increasing OCM supplementation. Because of the role in de novo methylation, increased expression to 10X may suggest increases in methylation in 10X treated cells by DNMT3B; however, global methylation analysis would need to be done to confirm these hypotheses.

Methionine supplementation improves mitochondrial respiration in *S. cerevisiae* strains of yeast by increasing protein expression of TCA cycle enzymes, mitochondrial proteins, as well as the influx of pyruvate into the mitochondria (Tripodi et al., 2018). Increases in OCM can increase input of downstream products of the methionine-folate cycle into the TCA cycle as well as provide redox equivalents in the form of glutathione (Clare et al., 2019); therefore, our data demonstrating increases in O_2 consumption in both LOW and HIGH cells in baseline, O_2-linked ATP synthesis, maximal respiration, reserve capacity and non-mitochondrial respiration in at least one OCM treatment compared with Control demonstrates a similar effect of all four OCM in treatments on mitochondrial respiration. In addition, because methionine was limited to 2X across all treatments, and there was a cubic increase in respiration parameters in HIGH cells suggest further action of the remaining OCM in culture on improvements in mitochondrial respiration.

Following a similar pattern as mitochondrial respiration, the cubic responses in total growth rate for both LOW and HIGH treated cells demonstrate that OCM increased growth and proliferation of bovine embryonic cells, which can be explained in part by improvements in mitochondrial efficiency with OCM supplementation. These data further demonstrate that growth rate is not affected by OCM level early in the growth period but diverges as cells are allowed to proliferate and become more confluent. Furthermore, total proliferation is not

different but is time-dependent, with similar trends between 24 to 48 h and differential initial responses at 1 h and final responses at 72 h. Increased levels of SAM, which would be expected with OCM supplementation, downregulate methyl tetrahydrofolate reductase enzyme (Ho et al., 2013). This enzyme catalyzes the conversion of 5,10-methenyl-tetrahydrofolate to 5-methyltetrahydrofolate which serves as the physiological methyl donor for methionine synthesis from homocysteine via the folate cycle. Therefore, high levels of SAM can be used for higher order polyamine synthesis of spermidine and spermine which act as regulators of growth (Landau et al., 2010). Furthermore, inhibition of MTHFR results in increases in 5,10-methylene-tetrahydrofolate and thus folate driven purine synthesis (Clare et al., 2019). Together with increases in mitochondrial respiration in a pattern similar to total growth rate, these data imply that the effect of all four OCM together elicit positive growth effects and mitochondrial respiration which is most likely due to increases in polyamine and nucleotide synthesis as well as downstream synthesis of redox products such as glutathione and increases in proteins in the TCA cycle and mitochondria thus resulting in improved respiration and ATP for growth.

When examining total growth rate and mitochondrial respiration parameters, it is interesting to note the cubic increase in the HIGH cells, as well as the cubic or quadratic increase to 2.5X or 5X and decrease at 10X in the LOW cells. These data indicate that the extreme differences seen between LOW and HIGH 10X suggest that there may be either a supranutritional effect of OCM supplementation when additional energy in the form of glucose is provided, or cellular requirements for OCM are dependent upon energy availability.

Conclusion

We interpret our observation of increased cellular proliferations in cultured embryonic cells to imply that strategic supplementation with OCM may increase embryonic growth rate *in vitro*. Increased growth may be due in part to greater mitochondrial efficiency, which, if sustained, may be an area for increased attention *in vivo* to improve metabolic programming of cattle. Therefore, further studies *in vivo* should be designed to target strategic supplementation during early gestation in beef cows, which may improve pregnancy rates and lead to positive effects on developmental programming.

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CHAPTER 4. GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

General Discussion and Theories

The results of this dissertation, more specifically the timeline of nutrient restriction, and the effects on the fetal transcriptome are novel. Furthermore, previous effects of OCM supplementation either *in vivo* or *in vitro* have been limited to one or two OCM's. Data presented within are novel due to the supplementation of four OCM and the positive effects seen in both LOW and HIGH glucose treatments.

Epidemiological studies in humans investigating global nutrient restriction during early gestation have elucidated multiple effects on postnatal life including heart disease, metabolic disease, and even some behavioral pathologies. In livestock, reduced performance in the form of altered metabolism, body composition, or temperament have not been linked to early gestational events. These are most likely due to a multitude of factors: 1) steers or heifers fed for harvest are slaughtered prior to being two years old. Therefore, the programmed metabolic effects as seen in mature humans may not have had time to culminate, 2) incomplete records which do not include maternal and paternal stressors, are thus not considered by producers or scientists as possibilities for why cows or bulls are performing poorly in the herd, 3) because programmed effects of early nutritional restriction are not seen in animals harvested for human consumption, the majority of focus has been on late gestation. Late gestation is the time when the majority of muscle fibers and adipocytes are developing which can more directly affect muscling and marbling in an animal harvested for human consumption. As a result, focus has not been placed on early gestation, although this may be a time to investigate to determine whether early nutritional factors may program longevity in the herd. If producers were able to keep cows in the herd for more production cycles, producers would be able to generate more revenue due to not having to

replace as many culled cows each year. This focus point needs to be first emphasized on the large number of heifers culled due to failure to re-breed after their first calf, and then extended to cows who are culled after 5 years of age.

Data presented within are significant in that 75% of DEG were upregulated in offspring of RES dams compared with CON. These data suggest two potential thought processes: 1) Multiple studies have shown that nutrient restriction followed by realimentation results in compensatory gain. This is due to the maintenance requirements of the individual decreasing and resulting in more energy being partitioned towards production functions upon realimentation. The data presented within demonstrate a greater number of genes upregulated in RES vs. CON offspring may suggest that these offspring are establishing for compensatory gain upon realimentation. This is the status quo; however, we present an alternative hypothesis that, 2) The difference in DEG may not be that the RES offspring are upregulated compared with CON offspring, but that the CON offspring gene expression is in fact downregulated compared with RES. Unpublished data from the US Meat Animal Research Center suggests that heifers and cows on a stair-step nutritional program have increased longevity in the herd. In the stair step program, these heifers mature more slowly and the available data from these groups suggest that their longevity and thus lifetime performance in the herd are increased. Typical production practice for heifers in the Upper Great Plains is for heifers to grow at roughly 0.45 kg/d to reach 65% of mature body weight by breeding and 80% of mature body weight by parturition of their first calf. Increasing growth rates and early pregnancy adds additional stress to not only the heifer, but also to her offspring. In fact, the greatest number of cows that are culled from the herd is during their two-year-old year because they are unable to maintain a pregnancy due to partitioning energy for their own growth (still only 80% of mature weight), lactation for the calf

that was just born, and further needing to be bred again. Therefore, these data may suggest that those heifers placed on our RES diet and allowed to mature slowly with reduced oxidative stress may give birth to healthier calves who may have increased longevity in the herd, and further, these heifers may stay in the herd longer like the unpublished data previously discussed may suggest.

During early embryonic development, there is a wave of epigenetic reprogramming. First the paternal DNA is actively demethylated, followed by a passive demethylation of the maternal DNA until the morula stage. At this point in time, the embryo must remethylate its genome, and further modify histone tails for transcriptional control. On this point alone, there is justifications for studies in methyl donors during early pregnancy in ruminants; however, data from our whole animal studies have demonstrated that a global nutrient restriction results in decreased methionine in fetal fluids and increased homocysteine in maternal serum suggesting altered one-carbon metabolism across the maternal-fetal interface. Furthermore, all genes except for one relating to histone modification were upregulated in offspring from RES dams compared with CON dams. In response to these data, the cell culture experiment was conducted. Cells supplemented with OCM had increased growth, proliferation, and mitochondrial respiration compared with unsupplemented cells. In both LOW and HIGH glucose cells, 2.5X and 5X had increased growth and mitochondrial respiration; however, only the HIGH glucose cells had increased growth and proliferation at 10X compared with unsupplemented controls.

Pitfalls of the Experiments

While the RNA-sequencing data is very compelling, the interpretation of the data cannot be extrapolated to alterations in cellular function. Because of the size of the fetuses at d 50 of gestation, individual organ mass was not measured and all available tissue utilized for

sequencing. Therefore, validation with protein expression could not be done. Although there were differences in nutrient concentrations in fetal fluids which support some of the changes in gene expression, there were no statistical differences in fetal size although the crown-rump length at d 50 was tended to be less in offspring from RES compared with CON heifers.

The use of cell culture is a powerful technique; however, there are several shortcomings of cell culture and in particular with our study that do limit its applicability to animal production. The cells purchased from ATCC were an immortalized cell line of bovine embryonic tracheal fibroblasts. Immortalized cell lines continuously divide and are of a single cell type, therefore they are not a true representation of tissues and organs *in vivo*. Furthermore, because these are tracheal fibroblasts and some enzymes in the methionine-folate cycle are hepatic enzymes, the true extent, or lack thereof in some cases, of differences may be attributed to these factors. Furthermore, the increased concentration of folate, choline, and vitamin B_{12} in the media may have altered the osmolarity and osmolality of the media, although careful attention was placed to ensure that pH between the treatments remained consistent.

Future Directions

In addition to the data presented within, the EBTr cells were sent off for bisulfite conversion to identify differentially methylated regions. These data are not included because the sequencing and bioinformatics have not been completed, and thus could not be included in this dissertation. Differences seen in CpG methylation may lead us to identify regions of DNA and thus specific genes which can be targets for future studies and may more definitive links between OCM, growth, and mitochondrial respiration in bovine cells.

Epigenetic reprogramming is an important event during early pregnancy. The results presented within regarding alterations to the fetal transcriptome under global nutrient restriction

as well as the *in vitro* data with supplemental OCM, lead to several possible future directions 1) Proof of concept that supplemental OCM to heifers/cows during early pregnancy improves developmental outcomes. 2) Repeat the model with *in vitro* fertilization to more accurately determine how OCM affect early embryo growth in culture and further define an appropriate supplementation level, 3) Determine the concentrations of OCM in histotroph during early gestation to determine what 2.5X or 5X may mean in the context of available nutrients for embryonic growth and development, 4) In order to deliver nutrients to the embryo, we must feed the dam and in turn she provides nutrients for the embryo. If it is determined that the most appropriate OCM level from embryonic development is 2.5X the nutrients currently supplied in histotroph, what would we have to deliver to the mother in order for 2.5X to be delivered via histotroph? Furthermore, does that concentration of nutrients being delivered to the dam negatively affect her performance? 5) The difference in the concentrations of nutrients in the uterine artery and uterine vein (flux to the uterus) do not equal the concentration of nutrients in histotroph. There is a high degree of catabolism and interconversion of nutrients across the uterine endometrium and within enzymes secreted in histotroph. Therefore, using stable isotope labelling of nutrients we can further begin to understand how nutrients are being metabolized across the endometrium and within the histotroph to alter what is available for embryonic metabolism during early gestation. 6) Lastly, although the focus of the latter half of this dissertation has been on OCM, there are multiple other micronutrients which play integral roles in embryonic development. Although epigenetic reprogramming is critical to the establishment and maintenance of pregnancy, it could be that the availability of methyl donors is already sufficient for the embryo and other nutrients may be limiting and thus resulting in the large amount of early embryonic loss that we see in livestock and humans. Therefore, continuing to

investigate micronutrient supply and demand beyond OCM will be critical to tackling the “black box” known as early embryonic development in not only livestock species, but in humans as well.