ONE-CARBON METABOLITES, FETAL GROWTH, MATERNAL OXIDATIVE STRESS, AND FETAL PROGRAMMING DURING EARLY GESTATION

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ABSTRACT

In ruminant production systems, maternal nutrient intake and supplementation practices vary widely throughout the industry. Adequate maternal nutrient supply is critical for fetal development and subsequent offspring performance and health. Therefore, determining strategic supplementation of specific nutrients during critical developmental windows is crucial to fetal growth during early gestation will positively impact beef cattle production systems.

In this dissertation, two experiments using beef heifers were conducted to test the hypothesis that differing maternal rate of gain (achieved through differing intake) during the first 63 d of pregnancy would compromise indices of maternal and fetal development; and that strategic nutrient supplementation would mitigate any negative responses to lower rates of maternal gain. Supplemental nutrients were methionine, choline, folate, and vitamin B_{12} and gain targets were 0.45 kg/heifer daily vs. -0.23 kg/heifer daily. Response variables used included heifer growth and carcass data, maternal oxidative stress, fetal organ weights, and differential expression genes in fetal muscle and liver.

It was found that heifers with lower gains were negatively impacted and critical fetal organs during development were affected by maternal rate of gain and one-carbon metabolite supplementation. Furthermore, measures of maternal oxidative stress were affected by gain and supplementation. Many genes in fetal muscle and liver were differentially expressed due to levels of gain and supplementation. For instance, genes important for cell signaling and death were upregulated when there was one-carbon metabolite supplementation. In conclusion, providing the correct nutrients and amount during early gestation can have an effect on fetal development that may be seen in the offspring's production life. A positive impact during early gestation will result in an increase in the offspring's production capabilities.

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DEDICATION

I dedicate this dissertation to my mother, Dawn. You are an inspiration of strength, perseverance, and altruism. Ember and I would not be where we are today without you. Thank you.

She is strong. Isaiah 40:31

She is valiant. 2 Samuel 13:28

She is fearless. Proverbs 31:25

She is enough. Psalm 46:5

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LIST OF ABBREVIATIONS

+OCM ....One-carbon metabolite supplementation

-OCM ....No one-carbon metabolite supplementation

5,10-CH_2-THF 5,10-methylenetetrahydrofolate

5-mTHF..5-methyltetrahydrolate

AA...Amino acid

AMPK ....Adenosine monophosphate-activated protein kinase

ATG1 ....Autophagy related gene 1

CH_2..Methylene group

CON ....Control

CON+OCM...Control intake with one-carbon metabolite supplementation

CON-OCM..Control intake without one-carbon metabolite supplementation

CpG ....Cytosine-phosphate-guanine

CRE...Copper reduction equivalent; total antioxidant capacity

DHF...dihydrofolate

DNA ....Dinucleotide acid

DNMT ....DNA methyltransferases

eIF4 ....Eukaryotic translation initiation factor 4

GSH...Glutathione

H..Hydrogen

H_2O_2 ....Hydrogen peroxide

HCY ....Homocysteine

IACUC ....Institutional Animal Care and Use Committee

lncRNA ....Long non-coding RNA

miRNA...microRNA

mRNA..Messenger RNA

MS..Methionine synthase

MTHFR..Methylenetetrahydrofolate reductase

mTOR ....Mammalian target of rapamycin

mTORC1-ATG ....Mammalian target of rapamycin complex 1 – autophagy related gene

MUT...Methylmalonyl-CoA mutase

NO..Nitric oxide

O_2- ....Superoxide anion

OCM ....one-carbon metabolites

OH..Hydroxyl radical

ONOO-...Peroxynitrite

P70S6K ....Serine/threonine protein kinase

PC...Phosphatidylcholine

PE...Phosphatidylethanolamine

RES ....Restricted

RES+OCM...Restricted intake with one-carbon metabolite supplementation

RES-OCM..Restricted intake without one-carbon metabolite supplementation

RNA ....Ribonucleotide acid

ROS..Reactive oxygen species

SAH...S-adenosylhomocysteine

SAM...S-adenosylmethionine

SAMTOR..S-adenosylmethionine target of rapamyocin

TAG ....Triacylglycerol

TCA..Tricarboxylic acid

THF ....Tetrahydrofolate

TSC2 ....Tuberous sclerosis complex 2

USDA..United States Department of Agriculture

VLDL..Very low-density lipoprotein

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CHAPTER 1. LITERATURE REVIEW

Introduction

In ruminant production systems, environmental stressors such as drought, temperature extremes, compromised forage quantity and/or quality, other environmental concerns, including toxins (Bova et al., 2014; NASEM, 2016) are significant issues for managers. Consequently, maternal nutrient intake and supplementation management practices vary widely throughout the beef cattle industry. These stressors on the maternal system can influence offspring phenotypes. This review will focus on maternal nutritional stressors and how they can negatively impact the developing conceptus in early gestation and cause postnatal pathologies through developmental programming mechanisms, with an emphasis on beef cattle These “programmed responses” can influence the offspring throughout their production life and are likely driven by epigenetic mechanisms, specifically DNA methylation, histone modification, and micro-RNA. Due to its role in epigenetics, one-carbon metabolism is of considerable research interest as it is driven by several micro-nutrients and physiological stress. This review will cover environmental stressors during early gestation, developmental programming and its mechanisms, and the significance of the one-carbon metabolism nutritional pathway.

Compromised Maternal Nutrition in Ruminant Production Systems

Within ruminant production systems and specifically beef cattle, compromised maternal nutrition is primarily driven by reductions in dietary quality or quantity available to the animal. Nutrient supply issues within ruminant livestock can be related to many factors including environmental and management issues. Extreme weather conditions have historically and recently negatively affected the U.S. cow/calf producers. The majority of the nation struggled with extreme drought during 2011 and 2012 where more than 70% of U.S. crop and livestock

production was affected (Countryman et al., 2016). More recently, in 2020-2021 North Dakota, South Dakota, and Montana experienced drought that significantly decreased cattle numbers. In the USDA cattle inventory reports, all three states showed cattle number declines varying from 2% to 20% in specific categories. This drought also increased feed prices and degraded pasture conditions for grazing. Due to lack of moisture, forage quality can rapidly decline and result in lower crude protein and rate of digestion (Sletmoen-Olsen et al., 2000 a,b). Degraded pasture conditions or low-quality forage can cause compromised maternal nutrition in ruminant production systems and, in beef cattle, most often occurs during gestation. In fact, a common time for this to occur is during a summer drought or during the late fall and winter months, which generally coincide with the first two trimesters of pregnancy in cow-calf production systems (Sletmoen-Olsen et al., 2000 a,b). Specifically, the first trimester is crucial to early fetal development because fetal organogenesis, placental differentiation, and tissue vascularization are occurring during this period (Boss et al., 2018; Kingdom and Kaufmann, 1999).

Oxidative Stress

Oxidative stress can be defined as the imbalance between the production of reactive oxygen species (ROS), also known as free radicals, and antioxidant defenses (Schieber and Chandel, 2014). Free radicals are unavoidable by-products of many biochemical processes and in some cases, they are natural results of life processes. For example, in eukaryotic cells, ROS are produced by biochemical reactions in the mitochondrial respiration processes. Specifically, complex I and III of the mitochondrial electron transport chain are major sources of superoxide anion and hydrogen peroxide (Juan et al., 2021). Another example of this would be activated neutrophils and their production of ROS to contribute to killing microbes and inflammation control (Dahlgren et al., 2019).

Once the antioxidant defenses are surpassed by ROS production, cellular damage will occur in a variety of tissues. Free radicals are described as chemical species that contain unpaired electrons which can increase the chemical reactivity of an atom or molecule (Jakubczyk et al., 2020). Some examples of free radicals are hydroxyl radical (OH), superoxide anion (O_2-), hydrogen peroxide (H_2O_2), transition metals such as iron and copper, nitric oxide (NO), and peroxynitrite (ONOO-). The most potent oxidant is OH because it has the ability to attack most biological molecules, resulting in the creation of free radical chain reactions. Superoxide anion is a weak oxidizing agent and can act as a reducing agent. For example, superoxide is considered both a radical and a -1 charged anion, making it an unstable and strong oxidant that can be reduced to hydrogen peroxide and act as a reductant and convert to oxygen (Andres et al., 2023). and can be produced more when peroxidase activity of iron complexes (cytochrome C) is increased. Cytochrome C has a role in mitochondrial electron transport and has antioxidant and peroxidase activity. However, under oxidative stress the peroxidase activity is increased resulting in more hydrogen peroxide production and superoxide radical formation (Velayutham et al., 2011). Finally, nitric oxide acts as a mediator of vascular tone and is an example of a radical with physiological functions (Betteridge, 2010; Jakubczyk et al., 2020). Under physiological conditions, small quantities of ROS production are necessary and are primarily signaling molecules (Jakubczyk et al., 2020). However, a balance between the generation and removal of free radicals is necessary to avoid damage to proteins, DNA, lipids, and other biomolecules (Jakubczyk et al., 2020).

Free radicals are highly reactive and can interact with more stable nonradicals (macromolecules, lipids, proteins, nucleic acids, and carbohydrates). When a radical reacts with a nonradical it will produce a free radical chain reaction forming new radicals, and the process

continues until consumed or terminated by an antioxidant. This chain reaction can cause severe damage to healthy cells by repeating the steps of forming radicals and causing harm until it is terminated. Some examples of this are lipid peroxidation and protein damage (addition of carbonyl groups). If carbonyl groups are added, the derivatives of amino acid (AA) residues will make the protein vulnerable to proteolysis, DNA base conversion, strand breaks, and protein/DNA crosslinks (Betteridge, 2010).

The free radical chain reaction begins with a reactive free radical that is able to capture a hydrogen atom (H) from, for example, a methylene group (CH_2) of fatty acids. This abstraction will leave behind an unpaired electron on the carbon. The remaining radical will then experience molecular rearrangement that will produce a conjugated diene which can combine with oxygen, forming a peroxyl radical. Once this radical is formed the process can start all over again from the beginning, creating a chain reaction that will continue until all substrate is consumed or terminated by a chain-breaking antioxidant. This process can have damaging effects on cellular function resulting in changes in fluidity, increased permeability, a decrease in membrane potential, and eventually membrane rupture (Betteridge, 2000). Lipid peroxidation also depletes antioxidant enzymes which will lead to oxidative stress (Chatterjee et al., 2016).

Another way oxidative stress will damage tissues is through the mammalian target of rapamycin (mTOR) pathways. The mTOR pathway is involved in varying cell functions that regulate cellular protein anabolism and catabolism (Laplante et al., 2012; Sengupta et al., 2010; Liu and Sabatini, 2020). For example, if the cell has enough AA, mTOR can signal other molecules within the cell to build new proteins. Contrastingly, if the cell is depleted of nutrients, the existing proteins will break down to free AA (building blocks) to be reused within the cell. This process of the cell breaking down its own components is called autophagy. Additionally,

mTOR has effects on the control of apoptosis which has an essential role in physiological and pathophysiological circumstances (Chatterjee et al., 2016). When oxidative stress occurs it will begin transcriptional alterations of cellular proteins that impair the mTOR network signaling. Reactive oxygen species will inhibit mTORC1 by direct phosphorylation of TSC2 and indirect activation of adenosine monophosphate-activated protein kinase (AMPK). Adenosine monophosphate-activated protein kinase will result in ROS generation, phosphorylation, and activating TSC2 leading to more mTORC1 inhibition. Stress can also reactivate mTORC1 and break the mTORC1-ATG protein complex to free ATG1 which will lead to the formation of mature functional lysosomes by autophagic lysosome reformation. Reactive oxygen species can trigger cellular pathways involving processes such as death and survival signals. Each organ has its own critical threshold towards ROS that is regulated by an intricate signaling system (Chatterjee et al., 2016).

Due to the variability seen in ruminant production systems, oxidative stress can be induced through numerous environmental conditions such as feed availability, transport stress, and weather. Oxidative stress can lead to systemic inflammation which will disrupt the growth efficiency of livestock (Reith et al., 2022). Reith et al., 2022 induced heat induced oxidative stress in beef cattle and found that metabolic pathways and gene expression were altered in white adipose tissue. Furthermore, the supplementation of β-adrenergic agonists helped moderate these adverse effects in metabolic and immune function pathways. It was also found in a study transporting beef cattle a long distance that ROS production was increased. Alfaro et al., 2022, provided rumen-protected methionine (RPM) and extracted skeletal muscle tissue to analyze gene expression. From this, they found that RPM supplementation enhanced DNA methylation and reduced oxidative stress levels after transport. Reactive oxygen species are also generated

throughout the pre-implantation period and are crucial for normal embryo development. However, at increased levels it can reduce the viability of the embryo (Deluao et al., 2022). Thus, external environmental stressors during gestation should be minimized. The antioxidant glutathione (GSH) appears to be the main defense against over accumulation of ROS in mammalian embryos (Deluao et al., 2022; Luciano et al., 2006). There is evidence that pre-implantation embryos have a decrease of total GSH content and an increase in H_2O_2, which can result in oxidative stress and alteration of fetal development (Bueno et al., 2020). These data suggest that even though the majority of fetal growth occurs towards the end of gestation, it is crucial to avoid nutritional insults or external stressors during the peri-conceptional period.

Developmental Programming

History and Concept

Developmental programming is the concept that maternal stressors can affect fetal development and can "program" the increased risk of various postnatal pathologies (Barker, 2004). Developmental programming is also termed "Fetal Programming" or "The Barker Hypothesis". This theory was based on human epidemiological studies that showed maternal stresses (maternal malnutrition) during fetal development increased the risk of pathologies such as heart disease and metabolic syndrome during the offspring's adult life (Barker et al., 1990 and 1989). Furthermore, it was suggested that maternal nutrition restriction during the first half of pregnancy can lead to smaller infants at birth, resulting in a greater risk of health problems later in life. For instance, studies indicated that low birth weight infants tend to have greater mortality ratios from coronary heart disease. However, the impact is dependent on the type, timing, and duration of the maternal nutritional insult (Hoffman et al., 2014).

Because of the accumulated data in humans the potential ramifications that a maternal stimulus (including plane of nutrition) may result in developmental programming responses that can impact fetal growth and development and adult responses have gained traction in animal agriculture (Caton and Hess, 2010). In a review by Reynolds et al., (2019); the studies that brought developmental programming into livestock trials are described. From this data in livestock, it is seen that altered growth and development of fetal organs can lead to 1) irreversible alterations in tissue, organ structure, and organ size, and 2) permanent changes in tissue function. For instance, in a study done by Long et al., 2012 cows were fed either 70% or 100% of their nutrient requirements from day 45 to 185 of gestation and then all cows were fed to nutrient requirements until calving. The progeny birth weights were similar, however, cows fed at 70% calved heifers with smaller ovaries and luteal tissue (Long et al., 2012). Since reproductive performance is a major economic contribution to ruminant production systems, this emphasizes the importance of maternal nutrition during gestation. These alterations are associated with increased risk of conditions such as growth abnormalities, and reproductive, immune, behavioral, or cognitive dysfunction in the offspring. Furthermore, the studies described have shown that virtually every organ system and metabolic function can be affected by developmental programming in livestock (Reynolds et al., 2019). Maternal nutrient restriction is often associated with intrauterine growth restriction. For example, Pillai et al. (2017), observed in sheep that a restriction from day 30 of gestation to parturition resulted in offspring with lower birth weights. In addition, when heifers were provided 55% of requirements versus 100% it was seen that lungs and trachea weighed less and the DNA concentrations in muscle tissue and fiber were affected (Long et al., 2010).

Developmental Programming and Maternal Nutrition

Maternal nutrition plane has been implicated as a major factor affecting developmental programming and offspring health and performance (Wu et al., 2004; Caton and Hess, 2010; Reynolds et al., 2012). Specifically, it has been seen that developmental programming can cause alterations in organ structure resulting in chronic pathologic conditions in offspring (Reynolds et al., 2019). Prenatal growth trajectory is dependent on maternal nutrient intake from the early stages of embryonic life, even when nutrient requirements for the conceptus are reported to be negligible (Robinson et al., 1999; NRC, 1996; NASEM 2016; Caton et al., 2020; Reynolds et al., 2022). Within ruminant production systems, it is likely for animals to go through periods of undernutrition during gestation due to seasonal changes in food availability, which can result many faults such as fetal growth restriction (Toschi and Baratta, 2021). If undernutrition occurs, it can result in fetal growth restriction, which is imperative because fetal growth restriction is connected to a greater risk of neonatal mortality and morbidity. Growth restricted fetuses are at risk of postnatal complications and can exhibit poor development with significant consequences later in life (Barker, 2004; Wu, 2006). This can be reduction in growth that begins as placental growth and vascular development. The placenta supplies gases, nutrients, and exchanges waste between the maternal and fetal systems (Redmer et al., 2004). Thus, placental size and proper function is a determining factor for subsequent fetal growth trajectory, which was found through experimental models in sheep (Anthony et al., 2003). Supplying the dam with sufficient nutrients to allow for proper placental function will improve fetal growth trajectory. When investigating fetal organ development, it was demonstrated by Long et al, (2021) that fetuses of heifers that were on a restricted diet had a reduced pancreas weight compared to control fetuses. Additionally, a study done by Marquez et al., (2017) found that supplementing cows that were

strictly grazing improved the offspring's skeletal muscle development. Additionally, Café et al., (2006) showed that poor maternal nutrition can result in a reduced weaning weight of offspring. Furthermore, the decreased growth can result in suboptimal carcasses that cost feedlot producers millions of dollars annually (Gardner et al., 1998).

Recently, gene expression has been analyzed in varying tissues to further investigate developmental programming. For instance, it was found that dams fed a diet to either achieve 60% or 100% of energy requirements had differential tissue (cerebrum, liver, and muscle) regulation in fetuses (Diniz et al., 2021). Diniz et al., (2023) found that maternal vitamin and mineral supplementation in the first 83 days of pregnancy affected fetal hepatic function through altered expression of energy- and lipid-related genes. For instance, vitamin and mineral supplementation resulted in greater fetal liver and intestine weights as well as greater concentrations of selenium, copper, and cobalt in the liver (Menezes et al., 2022). Additionally, Crouse et al., (2019) reported 373 differentially expressed genes from fetal liver, muscle, and cerebrum due to maternal nutrient restriction during the first 50 d of gestation in beef cattle. In the mitochondria, Crouse et al., (2022) demonstrated in bovine embryonic tracheal fibroblast cells that basal respiration, O_2-linked ATP synthesis, and maximal respiration was increased when provided more epigenetic modifiers (folic acid, choline chloride, vitamin B_{12}, and methionine). These supplemental nutrients not only improved mitochondrial respiration, but also cell growth rate.

Maternal undernutrition can cause permanent changes in fetal development that may be seen postnatally and cause challenges to livestock producers. Nutritional management decisions focus on the cow versus the developing fetus and are often based on averages of a group of

animals. This can lead to undernutrition for a subset of animals and cause perturbed growth in fetuses.

Epigenetics Mechanism

A major biological mechanism behind developmental programming is epigenetics (Meyer et al., 2012; Reynolds et al., 2017). Epigenetics was first described in 1942 by Conrad H. Waddington and is now described as the set of marks on the genome that induce changes in gene expression without altering the DNA sequence (Berger et al., 2009). These genetic marks are heritable and include DNA methylation, post-translational histone changes, chromatin remodeling, and non-coding RNAs that can transmit information through mitosis by regulating gene expression (Beaujean et al., 2020). The epigenome is controlled by complex interactions between genetic and environmental factors (Kouzarides, 2007). Depending on the cell's state, information is delivered by activating or inhibiting epigenetic marks which result in alterations in gene expression. These modifiable epigenetic marks are a response to the environment and can have long-term consequences. Furthermore, embryo development and the period surrounding conception is a crucial window that is sensitive to different environmental factors. In early embryogenesis, a reprogramming event of DNA methylation will occur causing global methylation profiles to be remodeled (Breton-Larrivee et al., 2019). After fertilization, the zygotic genome is still separated in two distinct paternal and maternal pronuclei which must undergo global demethylation to erase the germ cell-specific methylation profiles and implement totipotency before the embryo implants (Seisenberger et al., 2013). Thus, environmental variations that occur during early gestation can exert effects on the phenotype of the next generation (Junien et al., 2016).

Along the genome, there are cytosine-phosphate-guanine (CpG) dinucleotides that are grouped together forming "CpG islands" and can be methylated to 5-methylcytosine (Panel B, Figure 1A). Cytosine-phosphate-guanine islands methylation was first found in sea urchin embryos and linked as an epigenetic mark with gene activity during development. Methylation of DNA is necessary to prevent replication of viral mobile elements on the genome in CpG island regions to limit undesirable chromosomal recombination and segregation. In regions surrounding the islands, DNA methylation is dynamic as a function of cell type, developmental stage, the animal's physiology, or the environment (Beaujean et al., 2020). Furthermore, DNA methylation can inhibit gene expression when associated with regulatory elements while also activating by limiting the initiation of illegitimate transcription (Schubeler, 2015). Methylation of DNA is governed by nuclear proteins that carry a methylated DNA binding domain and can recruit transcriptional repressors or histone modifying enzymes. The enzymes that catalyze the transfer of methyl groups to the 5-position of cytosines from the S-adenosylmethionine metabolite (SAM, produced from folic acid) are called DNA methyltransferases (DNMT). Some of these DNMT are associated with *de novo* methylation, which takes place during cell differentiation. De novo methylation is the process by which methyl groups are added to unmethylated DNA at specific CpG sites (Smallwood and Kelsey, 2012). Therefore, the epigenetic DNA methylation is crucial to development of fetuses and can have a significant impact if perturbed. In a study done by Liu et al., (2021), alternative splicing patterns were investigated in skeletal muscle of beef calves when the dams were provided a prenatal diet rich in methionine. Since DNA methylation has a role in regulation of alternative splicing (Shayevitch et al., 2018) it was found that alternative splicing patterns were significantly altered by the maternal diet high in methyl donors.

The genes altered had implications in muscle development, muscle physiology, and DNA methylation (Liu et al., 2021).

Genomic DNA is wrapped around octamers of four types of histones to form the nucleosome in the nucleus. The histones are H2A, H2B, H3, and H4 (Panel B, Figure 1A). These histones are basic proteins that have an N-terminal tail which can be targeted by varying modifications. Acetylation, methylation, phosphorylation, and ubiquitylation are examples of some of the post-translational histone modifications that can be seen. The accessibility of genomic DNA to the transcription machinery is affected by the addition or removal of the modifications. Therefore, histone modifications will influence the activation or repression of gene expression (Kouzarides, 2007). When histone marks interact with DNA methylation, specific chromatin states are transmitted to daughter cells, which will continue through mitosis with cell identity (Beaujean et al., 2020). For instance, chromosomes have two distinguishable forms: euchromatin and heterochromatin. Euchromatin is less condensed which allows for transcription to occur while heterochromatin is highly condensed closing off the ability for transcription (Tamaru, 2010). These chromatin states are depicted in Figure 1A. Similar to DNA methylation, histone modifications are based on the use of dietary metabolites (acetyl-coA and SAM).

Non-coding RNAs are a category of RNAs that do not encode functional proteins and play an essential role in epigenetic control. Recent research has shown that non-coding RNAs have roles in different pathologies and physiological functions (Bayoumi et al., 2016). Non-coding RNAs are split up into sub-classes based on size, function, or genomic location. Some examples of non-coding RNAs are microRNA (miRNA) and long non-coding RNA (lncRNA). For instance, the microRNA subclass is 19-24 nucleotides long and at the genomic level are

organized in clusters (Griffiths-Jones et al., 2008). Micro RNA are single stranded RNAs, and fifty percent are located in chromosomal regions that are prone to structural changes. Mirco RNA can target a hundred or more varying mRNA (messenger RNA) and participate in regulation of gene expression that play a role in all biological functions (Bushati and Cohen, 2007). In a study done by Paradis et al., (2017) where beef cattle were put on a mild feed restriction (85% of total requirement), it was demonstrated that the restricted fetuses had a lower mRNA abundance for insulin-like growth factors in the liver. Therefore, the lower nutrient resources caused a metabolic response in the liver. Altered expression of insulin-like growth factors has also been seen in fetal sheep at the end of a restriction period (Brameld et al., 2000). Additionally, evidence shows that the primary function of miRNA is mediating transcriptional gene silencing. Control of miRNA expression is imperative to maintain the physiological functions of a cell. The deregulation of miRNA expression has been associated with diseases such as cancer (Landgraf et al., 2007). There are some implications that miRNA is what maintains the epigenetic memory when early embryos undergo global demethylation and remethylation (Vaskova et al., 2013) and can also change DNA or chromatin state by inhibiting chromatin remodeling enzyme activity.

When fertilization occurs, the paternal and maternal genomes are reprogrammed which allows for embryo development. The reprogramming is characterized by a series of epigenetic modifications (Ross and Sampaio, 2018). Therefore, parental pre-breeding and early gestation factors are significant for fetal development. Since a newly fertilized embryo is transcriptionally inactive, embryo development will depend on the stock RNA and proteins that are present in the oocyte (Beaujean et al., 2020).

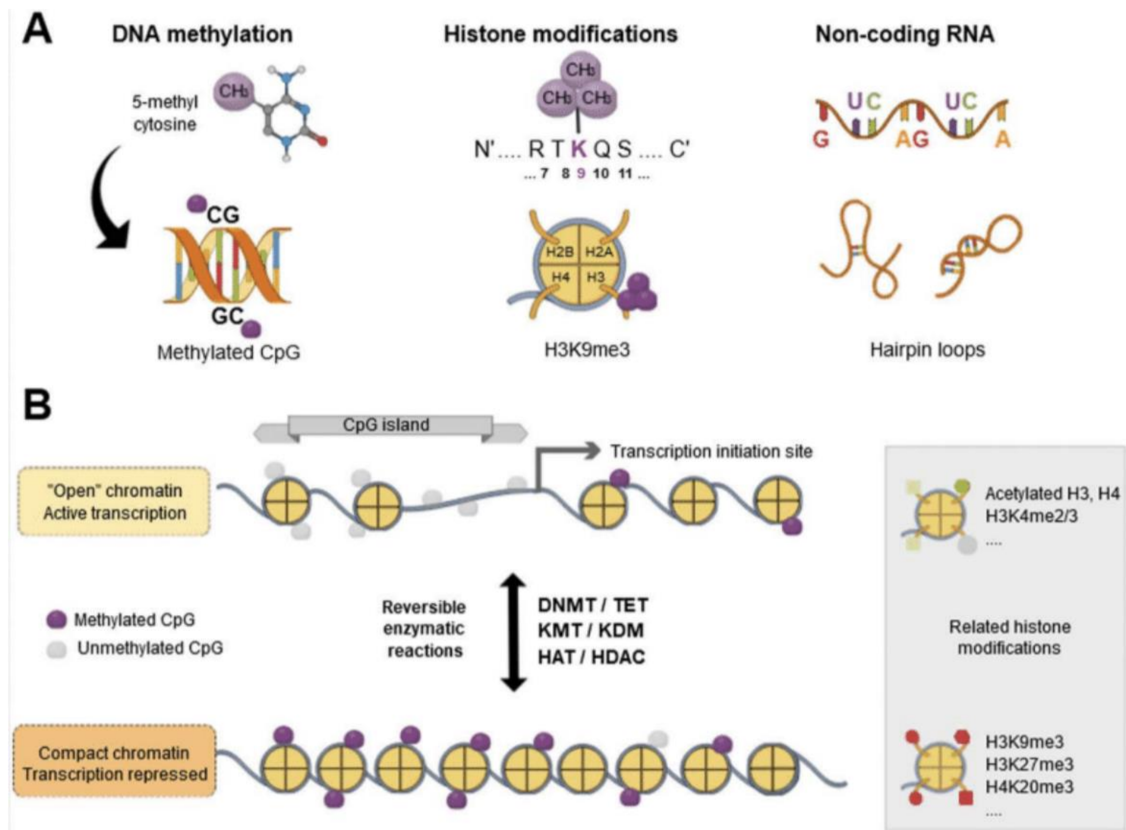

Figure 1.1 Epigenetics Mechanism. (Beaujean et al., 2020).

A. DNA methylation on CpG dinucleotides, Histone modifications in nucleosome cores, and non-coding RNAs
B. Chromatin states depending on environment will allow for transcription or not.

One-Carbon Metabolism

One-carbon metabolism is a metabolic process that activates and transfers one-carbon units for biosynthetic processes. For example, homocysteine remethylation and purine and thymidine synthesis. Furthermore, a function of one-carbon metabolism is to manage the provision of methyl groups for methylation reaction, which includes DNA and histone proteins. Methylation at specific sites on the DNA sequence and histone tails are a crucial epigenetic feature for regulating gene expression. One-carbon metabolism is dependent on several nutrients such as folate, vitamin B12, vitamin B6, betaine, choline, and methionine, which are referred to as one-carbon metabolites (OCM). These nutrients are either co-factors or methyl

donors/acceptors. The dietary supply of these methyl donors is necessary for normal growth, development, and physiological functions (Rush et al., 2014). There is a strong relationship between OCM/nutrients and epigenetic phenomena (Friso et al., 2017). Nutritional intervention influences regulatory pathways and can eventually influence disease prevention and outcomes.

The Folate Cycle

Unlike bacteria and plants, animals require dietary folate intake and a deficiency can result in anemia and offspring developmental complications and birth defects. Because of its involvement in methyl group delivery, folate is an extensively studied nutrient regarding DNA methylation. Furthermore, it plays an essential role in epigenetics, amino acid synthesis (Fournier et al., 2002), and the formation of the S-adenosylmethionine (SAM; Linhart et al., 2008).

Folate molecules are versatile methyl donors that function as carriers for one-carbon units which allow for them to be manipulated and can support metabolic processes (Clare et al., 2019). Animals are unable to synthesize folates endogenously, therefore, they must be derived entirely from dietary sources (Ducker and Rabinowitz, 2017). Folates are hydrophilic and need transport systems such as reduced folate carrier, the proton-coupled folate carrier, and folate receptors (Zhao et al., 2009). Once folate monoglutamate molecules reach the body's target cells, they are transformed to 5-methyltetrahydrolate (5-mTHF) where they are polyglutamated for cellular retention and one-carbon coenzyme function (Clare et al., 2019). The synthetic form of folate, folic acid, is fully oxidized and not an active coenzyme (Shane, 2008). Thus, it is reduced to dihydrofolate (DHF) and then reduced further to the active form tetrahydrofolate (THF) before entering the folate cycle (Ducker and Rabinowitz, 2017). Via the vitamin B6-dependent enzyme serine hydroxy-methyltransferase, THF is converted to 5,10-methylenetetrahydrofolate (5,10-

CH_2-THF). Following this, it is irreversibly reduced to 5-mTHF by the vitamin B2-dependent enzyme methylenetetrahydrofolate reductase (MTHFR). The demethylation of 5-mTHF completes the folate cycle as one-carbon is donated for re-methylation of homocysteine (HCY) to methionine (Mentch and Locasale, 2016). The reaction is catalyzed by the vitamin B12-dependent enzyme, methionine synthase (MS; Škovierová et al., 2016). Quantitatively, choline, serine, and glycine are important dietary sources of folate one-carbon units by being precursors and cofactors of the folate cycle. There have been decades of research that confirms the crucial requirement of dietary folate for the developing animal. Recently, more evidence has been discovered that there is a role in specific folate-utilizing enzymes that support growth and development in fetuses as well. These enzymes are involved in immune proliferation and tissue homeostasis (Ducker and Rabinowitz, 2017).

Folate is only provided via diet or produced via de novo mechanisms by plants and micro-organisms in the rumen and the amount produced is not enough to achieve high performance (Abbasi et al., 2014). Therefore, there is evidence that dietary folate given to dairy cattle improved production parameters. For instance, studies have shown that folic acid injection resulted in a significant increase in milk protein content in multiparous dairy cows (Girard and Matte 1998). More recently, rumen-protected folic acid was supplemented promoted microbial growth, increased microbial enzyme activity, and improved total VFA production in beef cattle (Wang et al., 2017).

The Methionine Cycle

Methionine is an essential amino acid that is not only needed for protein synthesis but also for one-carbon metabolism. In ruminants, methionine is supplied through dietary ruminal escape methionine, ruminal microbial methionine passage to the intestine (NASEM, 2016) or via

supplemental ruminal bypass methionine. Methionine is adenylated by methionine adenosyl transferase to SAM, which is a universal methyl donor involved in many downstream cellular reactions (Mentch and Locasale, 2016). S-adenosyl-methionine is then converted to S-adenosylhomocysteine (SAH) which is hydrolyzed back to HCY and adenosine via S-adenosylhomocysteine hydrolase to complete the cycle. S-adenosyl-methionine is essential for one-carbon metabolism and can be used to methylate DNA (epigenetic processes) and/or phosphatidylcholine (PC) synthesis which supports fatty acid metabolism. This is important because PC can improve very low-density lipoprotein (VLDL) synthesis which will reduce hepatic triacylglycerol (TAG) accumulation (Du et al., 2018). During gestation, hepatic TAG accumulation can result in mitochondrial dysfunction, inflammation, and reduced liver function, which can potentially have negative consequences on fetal development. An adequate supply of methionine; therefore, can reduce inflammation and improve liver function and health status (Du et al., 2018). Another major pathway for SAM metabolism is the production of polyamines (Lu, 2000). When production of polyamines occurs, SAM is converted to decarboxylated SAM by SAM decarboxylase. Next, the aminopropyl group is transferred to putrescine to form polyamines. This process is increased during times of liver regeneration (Lu, 2000). Synthesized SAM can also transform into an S-adenosylmethionine-binding protein (SAMTOR), which is a SAM sensor that regulates mammalian target of rapamycin complex 1 (mTORC1) activity, which aids in the mTOR pathway involvement in cell growth and metabolism. (Tang et al., 2022).

Methylation of DNA nucleotides is an important epigenetic mechanism to regulate gene expression. This is crucial during periods of growth and development and explains why nutritional imbalances are associated with the increased risk for subsequent diseases in offspring

(Jimenez-Chillaron et al., 2012). Following the pathway (Figure 1B) SAM is transformed into S-Adenosyl-Homocysteine leading to synthesis of homocysteine. Homocysteine can either be remethylated into more methionine; thereby, recharging the process. It can also transition into the transsulfuration pathway to synthesize cystathionine which is used to make cysteine. Cysteine can then be used to produce the antioxidants taurine and GSH. Therefore, enhanced SAM production with increased methionine supply can help enhance the transsulfuration pathway resulting in more antioxidants to reduce oxidative stress (Coleman et al., 2020). For instance, Coleman et al., (2022), conducted a heat stress study in Holstein cows that showed supplementation of methionine helped cows maintain homeostasis in mTOR, and 1-carbon metabolism. Furthermore, there was also evidence that methionine supplementation helped maintain whole-blood antioxidant response during a time of heat stress (Coleman et al., 2022). More research is needed to investigate the effects of oxidative stress during gestation and developmental programming in beef heifers.

Vitamin B_{12}

Vitamin B_{12} (cobalamin) is synthesized in nature only by microorganisms unlike other vitamins and there are currently no requirements established for beef cattle because of microbial synthesis via cobalt (NASEM, 2016). It has always been considered that the microbial synthesis of B-vitamins was an adequate amount to meet needs of the animal (Schwab et al., 2006). However, modern animals are higher producers compared with animals from several decades ago and their requirements for B-vitamins should reflect this. Vitamin B_{12} is also another crucial metabolite in one-carbon metabolism. Vitamin B_{12} acts as a cofactor for enzymes in one-carbon metabolism and the propionate catabolic pathway. The folate and methionine cycle are linked by methionine synthase (MS), the enzyme that converts HCY to methionine, via 5-mTHF and B_{12} as

an essential cofactor (Lyon et al., 2020). If there is a vitamin B_{12} deficiency, folate will be trapped in 5-mTHF form and methionine production will be inhibited. This will also result in greater concentrations of homocysteine which can be toxic within the cell (Rush et al., 2014). Furthermore, deficiency of B_{12} can reduce cell proliferation due to blocks in cell cycling (Yang et al., 2016), decrease histone methylation (Mentch et al., 2015; Shiraki et al., 2014), and methylation level of proteins (Ghemrawi et al., 2019). Evidence is forming that shows a low maternal vitamin B_{12} status and protein intake are associated with increased risk of neural tube defect, low lean mass, increased insulin resistance, and impaired neurodevelopment in offspring (Green, 2017). Furthermore, a handful of studies showed that the offspring of mothers with a B_{12} deficiency had a lower birth weight compared to mothers who met requirement (Pepper and Black, 2011). If the fetus is experiencing a growth restriction, it can increase their risk of diabetes and insulin resistance later in life (Bavdekar et al., 1999). Additionally, B_{12} deficiency can also decrease activity of the propionate catabolic pathway through decreased methylmalonyl-CoA mutase (MUT) enzymatic activity which can lead to decreased myelin synthesis, increased cellular stress, and disrupted tricarboxylic acid (TCA) cycling. Therefore, vitamin B_{12} influences folate-dependent reactions and mitochondrial energy pathways (Rush et al. 2014).

In ruminants, cobalt is necessary for the production of vitamin B_{12} (Stangl et al., 2000) The supplementation of B_{12} may be necessary in diets with adequate cobalt to ruminants because there could be a deficiency of B_{12} due to factors such as seasonal changes, cobalt concentration in grasses, grazing habits, and more (McDowell, 2012). A study done on cattle induced a cobalt deficiency and investigated the effects on oxidative status and production of HCY. It was found the increased levels of HCY increase plasma carbonyl levels which resulted in degradation of plasma proteins (Stangl et al., 2000). As this information suggests, ruminant systems are in need

of a vitamin B_{12} requirement level to provide cattle. Additionally, the crucial roles vitamin B_{12} is involved in implies how it is imperative during gestation. More research is needed to confirm the effects of vitamin B_{12} supplementation during gestation.

Choline

Choline is an essential nutrient that serves as a component of structural lipoproteins, blood and membrane lipids, and as a precursor of neurotransmitter acetylcholine (Ueland, 2011). Choline can also be metabolized to betaine and used within the one-carbon cycle to remethylate homocysteine to methionine (Li et al., 2016). This interaction links choline and betaine to the folate-dependent one-carbon metabolism. Choline is an important source of one-carbon units especially during a folate deficiency. Choline can be synthesized in the body by methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) but this process is limited. Thus, dietary choline intake is necessary. In ruminants it has been found that rumen-protected choline helped increase milk yield in Holstein cows (Pinotti et al., 2003). Furthermore, in dairy cows it was found that feeding rumen-protected choline was associated with less endometritis, lower number of stillbirths, and more cyclic cows (Furken et al., 2014). It has also been observed that pre- and postnatal choline supply is crucial for neurodevelopment in rodents (Ueland, 2010).

In Figure 1B, it is depicted how all of these nutrients work together in the OCM pathway to keep it functioning. Folic acid and methionine are crucial nutrients and precursors to the pathway. Folic acid will be transformed to THF and requires the catalyzation by several enzymes to help transform homocysteine into methionine. The enzymes are catalyzed when they are in the presence of dietary micronutrients such as folate, choline, and B vitamins. Thus, if there is a folate deficiency the methionine cycle will suffer from incomplete cycling. The cycles are interconnected and require all nutrients described to function properly. Since all these nutrients

are necessary for optimal performance, adequate supply to beef heifers is crucial. The role one-carbon metabolites play in epigenetics, cell function, and fetal development should be investigated more in ruminant systems. Specifically, we can improve the next generation of beef cattle by ensuring adequate supply of these metabolites.

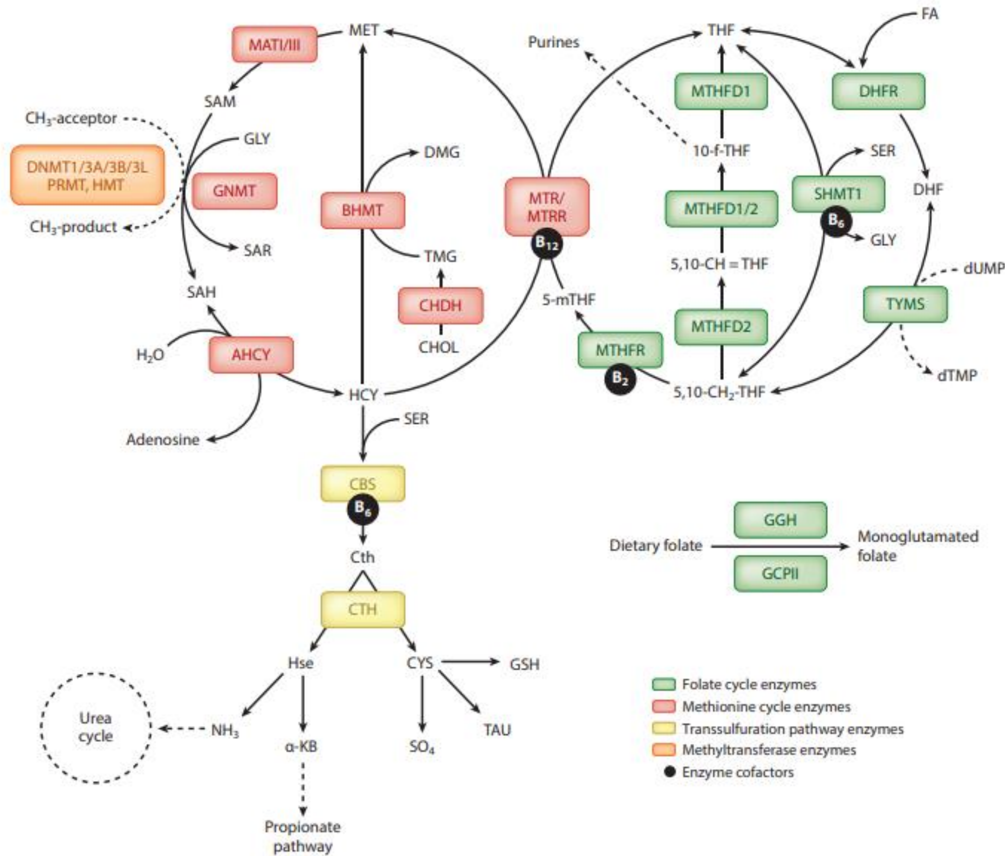

Figure 1.2 One-carbon Metabolism. The combination of the folate cycle and methionine cycle. (Clare et al., 2019).

Conclusion and Future Directions

In ruminant production systems, it is common for gestating cattle to undergo nutritional stressors such as undernutrition due to drought, low-quality forage, seasonal changes, and nutrient variation in feeds. In the past and recently, research in the area of developmental programming has been providing evidence that the dam's environment and nutrient status has lasting implications on the offspring's productive life. These implications can start as early on as

affecting embryo development, fetal organogenesis, and gene regulation that will be seen as pathologies postnatally. Nutritional management does not only affect the maintenance, growth, and production of the dam but also influences fetal growth and postnatal performance (Foroutan et al., 2021). Maternal nutrition is a major driver of developmental programming and improving the next generation of beef cattle. Recent research efforts have investigated measuring gene expression in ruminant fetal tissues and how nutrient availability will change it (Diniz et al., 2021 and Crouse et al., 2019). When looking into biochemical pathways, the OCM pathway is crucial to epigenetic regulation and plays an important role in developmental programming. As mentioned above, the metabolites methionine, choline, vitamin B_{12}, and folate are imperative in order to keep the OCM pathway cycling properly. Thus, it may be beneficial for producers in the ruminant industry to understand this concept and be able to strategically supplement these nutrients during early gestation to avoid dam malnutrition and maintain normal one-carbon metabolism. Currently, there is limited data on the effects of maternal nutrition and strategic supplementation during early gestation in beef heifers. Specifically, fetal organ weights, oxidative stress, and fetal gene expression are reasonable response variables to assess. Therefore, in the following chapters, these parameters will be investigated.

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CHAPTER 2. EFFECTS OF SUPPLEMENTING ONE-CARBON METABOLITES TO BEEF HEIFERS AT D 63 AND 161 OF GESTATION ON FETAL MORPHOMETRICS

Abstract

We hypothesized that a low maternal plane of nutrition would impair fetal growth in early gestation and that supplementation of one-carbon metabolites (OCM) would rescue growth restricted offspring at d 63 and 161 of gestation, Exp. 1, and Exp. 2, respectively. Both experiments had a 2 × 2 factorial arrangement of treatments with two levels of average daily gain (ADG), each with or without supplementation of OCM. In each experiment, seventy-two crossbred heifers were bred with female-sexed semen from a single sire. At breeding (d 0), beef heifers were individually fed using an electronic headgate system and randomly assigned to one of four treatments: Control (targeted: 0.45 kg/d ADG) without OCM (CON-OCM), CON with OCM (CON+OCM), Restricted (targeted: -0.23 kg/d ADG) without OCM (RES-OCM), or RES with OCM (RES+OCM). The basal diet given to all heifers for both experiments consisted of alfalfa-grass hay, corn silage, and ground corn. The OCM supplement consisted of ruminal-protected choline (44.4 g/d) and methionine (7.4 g/d) in a fine-ground corn carrier top-dressed daily, and weekly injections of folate (320 mg) and vitamin B_{12} (20 mg). The -OCM heifers received the corn carrier and saline injections. In Exp 1, heifers were slaughtered and fetal tissues were collected on d 63. In Exp. 2, heifers received treatment up to d 63 then were all placed on a common control diet with no supplement until slaughter and tissues were collected on d 161. Data were evaluated unadjusted and allometrically, where fetal organ weights were normalized to brain weight and fetal body weight and were analyzed using the MIXED procedures of SAS for main effects of gain, OCM, and their interaction. In Exp. 1 heifer data, there were pancreas gain × OCM interactions where CON+OCM weights were greater when

compared to each of the three remaining treatments ($P < 0.01$). Dam liver, live slaughter weight, and hot carcass weight had gain effects where CON heifers were heavier ($P < 0.01$). When looking at the fetus, fetal body weight tended ($P = 0.07$) to be lower and stomach complex weight was lower ($P = 0.026$) in RES compared with CON. When organ weights were normalized to total brain weight to assess allometric growth, OCM × gain interactions were observed in heart ($P = 0.04$), left longissimus dorsi ($P = 0.04$), and right hemisphere ($P = 0.01$) with CON+OCM greater than CON–OCM. The normalized left hemisphere tended to be greater in RES+OCM than RES-OCM ($P = 0.079$). In Exp. 2 heifer data, gain × OCM interactions were not present ($P > 0.05$). Live slaughter and hot carcass weight had gain effects where CON heifers were heavier than RES ($P < 0.01$). The 12th rib fat depth was larger in CON ($P = 0.03$) with a tendency ($P = 0.08$) for CON heifers to have a larger ribeye area. The +OCM heifers had an average yield grade of 1.69 while -OCM heifers had 2.02 ($P = 0.017$). In Exp. 2 fetal data, there was no differences in fetal body weight. However, trachea was heavier ($P = 0.04$) and spleen was lighter ($P = 0.05$) in fetuses from +OCM than -OCM. The right hindlimb muscle was influenced by a gain × OCM interaction ($P = 0.04$), where RES+OCM was heavier than CON+OCM. With allometric adjustments, right longissimus dorsi, rumen complex, and liver were heavier ($P \leq 0.05$) in RES compared with CON. Day 63 is within the period of peak primary myogenesis in cattle; therefore, differences in muscle growth during this time could have lasting implications in post-natal calf growth and performance. Furthermore, nutrient partitioning in liver and muscle tissue can affect many postnatal production traits such as lactation, reproduction, and carcass quality. These data suggest that providing OCM during early gestation may rescue skeletal muscle tissue growth in nutrient restricted offspring, and that restricting nutrients to dams results in prioritization of allometric growth of critical fetal organs such as liver and lungs.

Supplementation with OCM did not rescue all organ growth, but observed alterations in muscle and metabolic organ weights could have lasting postnatal implications.

Introduction

Developmental programming is the concept that maternal stressors affect fetal development and can program the increased risk of postnatal pathologies (Barker et al., 2004). Since the majority of fetal growth occurs during the last two months of gestation, the effect of maternal nutrition during early gestation may seem negligible (Robinson et al., 1977; Funston et al., 2010). However, prenatal growth trajectory responds to maternal nutrient intake from the early stages of embryonic life. For example, a maternal nutrient restriction during the first 50 days of gestation in beef heifers can alter transcript abundance of genes that influence tissue metabolism, accretion and function in fetal liver, muscle, and cerebrum tissues (Crouse et al., 2019).

Additionally, evidence suggests that in the early stages of fetal development, poor nutrition can influence placental growth, cell differentiation, and organ development. Furthermore, disrupted maternal nutrition during critical time windows of gestation may negatively affect fetal growth and development through epigenetic modifications such as changing patterns of DNA methylation and histone modification, impacting endocrine control, and ultimately leading to impaired growth and metabolism of the conceptus (Waterland and Jirtle, 2003 and 2004; MacLennan et al., 2004; McMillen and Robinson, 2005; Waterland et al., 2006; Meyer et al., 2012; Clare et al., 2019). Thus, inadequate nutrient supply in maternal diets may negatively affect fetal development impacting beef production by reducing the offspring’s feed efficiency and average daily gain.

In the industry, feed is one of the largest economic burdens for beef cattle producers. Because of maternal nutrition’s critical role in fetal development, it would be beneficial for

producers to target diets that supply nutrients designed for both parent and conceptus in order to improve the offspring's genetic potential, efficiency of growth, and productivity. Nutrients that are one-carbon metabolites (OCM) were used for strategic supplementation in this study included methionine, choline, folate and B_{12} and targeted the one-carbon metabolite pathway. This pathway has significance in fetal programming due to its role in providing methyl groups necessary for a normal epigenetic landscape.

Based off this information, the objective of this study was to investigate if OCM supplementation improved growth of fetal tissues which would aid in postnatal efficiency. We hypothesized that a low maternal plane of nutrition will impair fetal growth in early gestation and that supplementation of OCM will rescue the growth restriction.

Materials and Methods

Animals and Diets

Animal handling and care procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee (IACUC no. A21049). In both Exp. 1 and 2, seventy-two crossbred beef heifers were used. After pregnancy was diagnosed with ultrasound, the number of heifers per treatment for Exp. 1 was CON+OCM (n = 8), CON-OCM (n = 7), RES+OCM (n = 8), and RES-OCM (n = 9). In Exp. 2 number of heifers per treatment was CON+OCM (n = 8), CON-OCM (n = 7), RES+OCM (n = 7), and RES-OCM (n = 7). In Exp.1, heifers averaged body weight of 398 ± 32 kg and Exp. 2 heifers averaged 348 ± 19.5 kg. All heifers were subjected to a 7-day Select-Synch + CIDR estrus synchronization protocol (Lamb et al., 2010). After estrus synchronization, heifers were bred approximately 12 h after detected estrus with female sexed semen from a single sire. The basal diet was a total mixed ration and consisted of corn silage, ground corn, and an alfalfa/grass hay mix. The diet targeted a

crude protein amount of 13.5% and metabolizable energy amount of 2.56 Mcal/kg on a dry matter basis. Corn carrier for treatment delivery weighed 0.27 kg and was deducted from the percent corn in the total mixed ration. A vitamin/mineral premix (Trouw dairy VTS/Optimins, Trouw Nutrition USA, Highland, Illinois) was added to the corn carrier and provided based on averaged heifer body weight per label directions which resulted in an average of 10 g/heifer daily. The premix consisted of calcium (10 to 12%), magnesium (5%), potassium (5%), cobalt (180 mg/kg), copper (5,100 mg/kg), iodine (375 mg/kg), iron (1.2%), selenium (132 mg/kg), zinc (2.7%), vitamin A (2,775,775 IU/kg), vitamin D_3 (771,617 IU/kg), and vitamin E (13,228 IU/kg). Average daily gain was monitored via weekly weights and feed allotment adjusted accordingly to meet gain targets. In both experiments, the targeted ADG for control intake (CON) was 0.45 kg/d for restricted intake (RES) was -0.23 kg/d.

Experimental Design

At breeding (d 0), heifers in each experiment were assigned to one of four nutritional treatments in a 2 × 2 factorial arrangement: Control intake without OCM supplementation (CON-OCM), CON with OCM supplementation (CON+OCM), restricted intake without OCM supplementation (RES-OCM), or RES with OCM supplementation (RES+OCM). The OCM supplement consisted of rumen-protected choline (ReaShure, Balchem Inc., New Hampton, NY, 44.4 g/d) and methionine (Smartamine, Adisseo, China, 7.4 g/d) in a fine-ground corn carrier top-dressed daily, and weekly injections of folate (Spectrum Chemical Mfg. Corp., New Brunswick, NJ, 320 mg, 6 cc) and vitamin B_{12} (MWI Animal Health, Boise, ID, 20 mg, 4 cc). The -OCM heifers received the corn carrier and saline injections (one 6 cc and one 4 cc injection). Therefore, the experimental design had treatments arranged as a 2 × 2 factorial with

two levels of gain (achieved by altering dietary intake) and two levels of strategic OCM supplementation.

Tissue Sampling

In both experiments heifers were slaughtered in a federally inspected facility using captive bolt followed by exsanguination. The gravid uterus was removed first and taken for dissection of reproductive and fetal tissue. Gravid uterus consisted of fetus, fetal fluids (allantoic and amniotic), and fetal membranes (cotyledons, and intercotyledonary membranes). Fetal fluids amount was determined through subtraction of empty uterus, fetus, and fetal membrane weight. Maternal tissues were taken as they became available in the facility's process.

In Exp. 1, heifers were slaughtered, and fetal tissues collected on d 63 ± 2 of gestation. This day was chosen because it is a significant time point during organogenesis as critical to growth, health, and viability of offspring. In Exp. 2, heifers were slaughtered, and fetal tissues collected on d 161 ± 2 of gestation. This day was chosen to see how early supplementation of OCM effect secondary myogenesis and the second trimester of pregnancy.

Fetuses were removed from uterus and weighed, then underwent dissection which isolated all organs for individual weights to be measured. Fetal tissues were either stored by snap freezing or select tissues were placed in RNAlater (Ambion by Life Technologies Carlsbad, CA), Optimal Cutting Temperature Compound (OCT, Scigen Scientific Gardena, CA), or in 10% Neutral Buffered Formalin (NBF, VWR International Radnor, PA). In Exp. 1, due to its smaller size, tissues were placed on dry ice to be snap frozen. In Exp. 2, the larger tissues were first flash frozen in supercooled isopentane and then placed on dry ice until transport to the ultracold storage. All tissues were stored at -80 C until needed for analyses on collaborating projects.

In Exp. 1, due to size left and right kidneys were weighed together versus being weighed separately as in Exp. 2. Additionally, kidney capsules were not removed on d 63 but were removed when tissues were collected on d 161. Similarly, mammary tissue in phase 1 consisted of gelatinous skin attached and in Exp. 2 skin was able to be removed for the weight. The lung dissection did not consist of trachea portion attached. In Exp. 2 trachea was taken from the point of exiting the throat to the intersection of lung bifurcation.

Statistical Analysis

Data were evaluated unadjusted and allometrically, where organ weights were normalized to brain weight and fetal body weight and were analyzed using the MIXED procedures of SAS (Version 9.4, SAS Institute Inc., Cary, NC) for main effects of gain, OCM, and their interaction. Data are reported as LSMeans where significance was set at $P \leq 0.05$ and tendency was $0.05 < P \leq 0.10$. Individual heifer was the experimental unit and fixed effects were the four treatments. In the absence of interactions ($P > 0.05$) main effects of gain and OCM treatments will be presented in the results and in the presence of interactions, interactive means will be presented within the text and in Appendix Tables.

Results

Exp. 1 Heifer Data

The ADG achieved for each gain group was CON 0.60 kg/d and RES -0.23 kg/d. Main effects of gain and OCM are presented in Table 2A and interactive means, in the presence of interactions, are presented within the text and in appendix tables. In heifer data, there were no gain × OCM interaction ($P > 0.05$), except for pancreas ($P = 0.05$). Pancreas gain × OCM interactions included CON+OCM weights being greater when compared to CON-OCM, RES+OCM, and RES-OCM (324 vs. 259, 233, and 235 ± 18.58 g, respectively; $P < 0.01$;

Appendix Table 1). There was a tendency for pancreas in +OCM heifers to have a greater weight compared to -OCM unadjusted weights and normalized to hot carcass weight ($P = 0.07$ and 0.06, respectively). Maternal liver had a gain effect where CON heifers had a heavier weight ($P < 0.01$) and when normalized to hot carcass weight ($P = 0.01$). Live slaughter and hot carcass weight had gain effects with CON heifers being larger than RES ($P < 0.01$). Control heifers also had a larger ribeye area when compared to RES ($P = 0.038$). There were no effects in final yield grade or 12th rib fat depth. There was no significance when analyzing gravid uterus or fetal fluids.

Exp. 2 Heifer Data

The ADG achieved for each gain group was CON 0.67 kg/d and RES -0.16 kg/d. Main effect means are presented for gain and OCM (Table 2B). In heifer data, gain × OCM interactions were not present ($P > 0.05$). Live slaughter and hot carcass weight had gain effects where CON heifers were heavier than RES ($P < 0.01$). The 12th rib fat depth was greater in CON ($P = 0.03$). Control heifers tended ($P = 0.08$) to have a larger ribeye area compared with RES. The +OCM heifers had an average yield grade of 1.69 while -OCM heifers had 2.02 ($P = 0.017$). Liver and mammary gland weights were greater ($P < 0.01$) in CON compared with RES heifers. Gravid uterus, fetal fluids, CL ovary, pancreas, hypothalamus, and pituitary had no gain, OCM treatment, or interactions ($P \geq 0.05$).

Table 2.1 The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 63 of gestation (Exp. 1) on gain, carcass measurements, and selected organ weights in beef heifers.

	Treatments[2]					*P*-value[4]		
Item	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain × OCM**
Heifer Weights, kg								
d 0	404	394	394	404	10.31	0.45	0.45	0.49
d 63	441	379	408	412	10.10	<0.01	0.77	0.53
ADG, d 0 to 63	0.60	-0.23	0.23	0.14	0.04	<0.01	0.08	0.80
Live Slaughter Wt., kg	430	371	399	401	18.50	<0.01	0.86	0.57
Hot Carcass Wt., kg	244	211	228	227	9.74	<0.01	0.89	0.25
Ribeye Area (in.^{2})	10.44	9.76	10.29	9.91	0.16	0.03	0.23	0.17
12th Rib Fat Depth (in.)	0.169	0.161	0.157	0.172	0.01	0.71	0.51	0.38
Final Yield Grade	1.85	1.86	1.81	1.90	0.08	0.97	0.63	0.43
Liver, g	4,368	3,071	3,572	3,868	238.81	<0.01	0.36	0.34
g/kg Hot Carcass Wt.	18.13	14.58	15.81	16.91	0.97	0.01	0.40	0.22
Pancreas, g	291	234	279	247	12.66	<0.01	0.07	0.05
g/kg Hot Carcass Wt.	1.19	1.11	1.22	1.08	0.05	0.27	0.06	0.17
Gravid Uterus, g	830	801	786	845	37.00	0.56	0.24	0.65
g/kg Hot Carcass Wt.	3.42	3.81	3.47	3.76	0.17	0.12	0.24	0.99

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON = Control (0.60 kg/d); RES = Restricted (-0.23 kg/d); +OCM = one-carbon metabolite supplementation; -OCM = no one-carbon metabolite supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.

Table 2.2 The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 161 of gestation (Exp. 2) on gain, carcass measurements, and selected organ weights in beef heifers.

	Treatments[2]					*P*-value[4]		
Item	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Heifer Weights, kg								
d 0	354	348	350	352	4.00	0.51	0.82	0.35
d 63	396	338	365	369	7.29	<0.01	0.69	0.23
ADG, d 0 to 63	0.67	-0.16	0.24	0.27	0.08	<0.01	0.55	0.25
Live Slaughter Wt., kg	455	405	433	428	8.31	<0.01	0.73	0.64
Hot Carcass Wt., kg	246	217	232	232	6.15	<0.01	0.98	0.59
Ribeye Area (in.^{2})	10.28	9.41	10.19	9.50	0.25	0.08	0.16	0.87
12th Rib Fat Depth(in.)	0.153	0.115	0.133	0.135	0.009	0.03	0.92	0.09
Final Yield Grade	1.89	1.82	1.69	2.02	0.068	0.55	0.01	0.78
Liver, g	4,154	3,524	3,879	3,799	90.53	<0.01	0.58	0.76
g/kg Hot Carcass Wt.	16.93	16.21	16.72	16.43	0.33	0.13	0.54	0.23
Pancreas, g	316	292	311	296	11.24	0.29	0.52	0.28
g/kg Hot Carcass Wt.	1.28	1.35	1.34	1.29	0.07	0.46	0.57	0.42
Mammary, g	3,910	3,134	3,699	3,344	151.16	<0.01	0.19	0.55
g/kg Hot Carcass Wt.	15.91	14.35	15.78	14.48	0.61	0.08	0.14	0.73
Hypothalamus, g	3.64	2.96	3.53	3.08	0.29	0.27	0.46	0.74
mg/kg Hot Carcass Wt.	0.014	0.013	0.015	0.013	0.002	0.68	0.41	0.85
Pituitary, g	2.17	2.35	2.38	2.14	0.13	0.54	0.38	0.66
mg/kg Hot Carcass Wt.	0.008	0.01	0.01	0.009	0.0008	0.13	0.35	0.46
Gravid Uterus, g	13,514	14,006	13,768	13,752	330.48	0.48	0.98	0.96
g/kg Hot Carcass Wt.	55.00	64.70	60.13	59.58	2.13	<0.01	0.85	0.55
CL Ovary, g	7.42	6.89	7.32	6.99	0.42	0.54	0.70	0.13
mg/kg Hot Carcass Wt.	0.030	0.032	0.032	0.030	0.002	0.72	0.74	0.08

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON = Control (0.67 kg/d); RES = Restricted (-0.16 kg/d); +OCM = one-carbon metabolite supplementation; -OCM = no one-carbon metabolite supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.

Exp. 1 Fetal Tissues

Main effects of gain and OCM are presented in Table 2C. In the presence of interactions ($P \leq 0.05$), interactive means are presented in the text and in Appendix Table A3. Organ weights were normalized to total brain weight of the individual fetus and fetal body weight to assess allometric growth are described here (Table 2C and Appendix A3). After total brain weight normalization, OCM × gain interactions were observed in heart (0.25 vs. 0.21 ± 0.006 g, P = 0.04), right longissimus dorsi (0.21 vs. 0.15 ± 0.009 g, P = 0.04), and brain right hemisphere (0.22 vs. 0.18 ± 0.007 g, P = 0.01) with CON+OCM greater than CON–OCM. The normalized brain left hemisphere tended (0.22 vs. 0.18 ± 0.007 g, P = 0.079) to be greater in RES+OCM than RES-OCM. Organs were then normalized using fetal body weight. After body weight normalization, there was a significance with brain left hemisphere where R-OCM (8.67 ± 0.37 mg/g) weighed less than R+OCM (11.02 ± 0.37 mg/g, P = 0.02) and a tendency for C+OCM (15.42 ± 1.08 mg/g) intestines to be heavier than R+OCM (10.01 ± 1.08 mg/g, P = 0.09).

Exp. 2 Fetal Tissues

Main effects of gain and OCM supplementation are presented in Table 2D. Gain effects were seen in liver, lungs, rumen complex, right longissimus dorsi when normalized to total brain weight ($P \leq 0.05$). The RES fetuses were heavier in these organ weights compared with CON. Significant effects were seen in spleen unadjusted and normalized to both brain and body weight ($P \leq 0.05$). Within the spleen, -OCM fetuses had heavier organ weights compared to +OCM fetuses for both adjusted and unadjusted. Trachea data presented with an unadjusted mean treatment (P = 0.038) effect; as well as an adjusted to body weight tendency for both gain (P = 0.09) and OCM (P = 0.049) effects. The treatment group +OCM and gain group CON had heavier weights in trachea weights.

Interactive means before and after organ weights were normalized to total brain weight of the individual fetus and fetal body weight to assess allometric growth are presented in Appendix A4 and described herein. There was unadjusted gain × OCM interactions seen in mammary tissue (*P* = 0.02). Mammary tissue in C-OCM was heavier when compared to C+OCM and R-OCM (16.43, 13.61, and 14.10 ± 0.49 g, respectively). Similarly, when normalized to total brain weight, mammary tissue was heavier in C-OCM compared to C+OCM (*P* = 0.04) and there was a tendency for C+OCM to be smaller than R+OCM (*P* = 0.06). Gain × OCM interactions were seen in unadjusted right hindlimb muscle and normalized to both total brain weight and body weight (*P* ≤ 0.05). The right hindlimb muscle unadjusted weights showed that R+OCM and C-OCM were heavier compared to C+OCM (86.73, 83.38, and 68.92 ± 2.99 g, respectively). Similarly, when right hindlimb muscle was adjusted to total brain weight, R+OCM and C-OCM were heavier than C+OCM (1.53, 1.49, and 1.25 ± 0.05 g, respectively). The right hindlimb muscle values when adjusted to body weight were R+OCM and C-OCM which were heavier than C+OCM (21.53, 21.60, and 18.49 ± 0.65 mg/g, respectively). The lungs once normalized to brain and body weight showed gain × OCM interactions (*P* ≤ 0.05). Brain weight normalization, R-OCM (2.05 ± 0.04 g) were heavier compared to R+OCM (1.85 ± 0.04 g). Body weight normalization in lungs showed R-OCM (28.12 ± 0.46 mg/g) were heavier than R+OCM (25.24 ± 0.46 mg/g).

Table 2.3 The main effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 63 (Exp. 1) of gestation on fetal organ weight and allometric organ weight.

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**Trt**	**Gain×Trt**
Fetal Fluids, g	410	425	401	434	16.78	0.65	0.34	0.47
Fetal Body, g	19.57	18.02	18.52	19.07	0.42	0.07	0.51	0.85
Heart, g	0.22	0.19	0.21	0.20	0.006	0.11	0.69	0.42
g/g Brain Wt	0.233	0.228	0.234	0.227	0.006	0.71	0.55	0.04
mg/g Body Wt	11.16	10.79	11.25	10.70	0.25	0.46	0.28	0.40
Rumen Complex, g	0.38	0.32	0.34	0.36	0.014	0.02	0.53	0.85
g/g Brain Wt	0.41	0.37	0.38	0.39	0.01	0.08	0.51	0.19
mg/g Body Wt	19.64	17.73	18.53	18.83	0.51	0.07	0.77	0.85
Right Hemisphere, g	0.19	0.16	0.17	0.18	0.007	0.05	0.90	0.23
g/g Brain Wt	0.205	0.189	0.198	0.197	0.006	0.19	0.94	0.01
mg/g Body Wt	9.63	9.05	9.46	9.22	0.30	0.34	0.69	0.13
Left Hemisphere, g	0.196	0.179	0.190	0.185	0.009	0.33	0.80	0.08
g/g Brain Wt	0.209	0.205	0.213	0.202	0.007	0.81	0.42	0.07
mg/g Body Wt	9.98	9.84	10.23	9.59	0.37	0.83	0.36	0.02
Hindbrain, g	0.54	0.52	0.52	0.54	0.01	0.31	0.57	0.13
g/g Brain Wt	0.586	0.605	0.589	0.601	0.009	0.31	0.52	0.70
mg/g Body Wt	27.99	28.96	28.67	28.28	0.75	0.53	0.80	0.20
Right Longissimus Dorsi, g	0.17	0.14	0.16	0.15	0.009	0.13	0.58	0.13
g/g Brain Wt	0.18	0.16	0.18	0.17	0.01	0.26	0.44	0.04
mg/g Body Wt	8.83	7.78	8.65	7.96	0.47	0.27	0.47	0.15
Right Hindlimb, g	0.73	0.64	0.68	0.69	0.03	0.18	0.93	0.55
g/g Brain Wt	0.78	0.75	0.78	0.75	0.03	0.56	0.71	0.16
mg/g Body Wt	37.35	35.71	37.43	35.64	1.51	0.61	0.57	0.54
Intestine, g	0.26	0.20	0.24	0.23	0.02	0.21	0.75	0.08
g/g Brain Wt	0.28	0.24	0.27	0.25	0.03	0.55	0.68	0.07
mg/g Body Wt	12.96	11.42	12.72	11.67	1.08	0.49	0.63	0.09
Left Longissimus Dorsi, g	0.13	0.13	0.14	0.14	0.006	0.99	0.95	0.77
g/g Brain Wt	0.15	0.16	0.16	0.15	0.008	0.36	0.74	0.66
mg/g Body Wt	7.18	7.84	7.64	7.39	0.38	0.42	0.76	0.95
Left Hindlimb Muscle, g	0.48	0.46	0.46	0.48	0.02	0.49	0.67	0.87
g/g Brain Wt	0.52	0.53	0.52	0.53	0.02	0.72	0.73	0.38
mg/g Body Wt	24.28	25.22	25.03	24.47	0.80	0.59	0.75	0.98

Table 2.3 The main effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 63 (Exp. 1) of gestation on fetal organ weight and allometric organ weight. (Continued)

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Femur, g	0.049	0.043	0.045	0.047	0.002	0.23	0.65	0.92
g/g Brain Wt	0.052	0.050	0.050	0.052	0.002	0.71	0.69	0.58
mg/g Body Wt	2.49	2.39	2.41	2.48	0.09	0.63	0.74	0.65
Kidneys, g	0.204	0.188	0.194	0.198	0.007	0.33	0.77	0.73
g/g Brain Wt	0.21	0.22	0.22	0.21	0.007	0.68	0.87	0.16
mg/g Body Wt	10.23	10.43	10.49	10.18	0.32	0.77	0.66	0.67
Pancreas, g	0.06	0.04	0.05	0.05	0.004	0.15	0.45	0.55
g/g Brain Wt	0.06	0.05	0.05	0.06	0.005	0.32	0.53	0.32
mg/g Body Wt	3.10	2.55	2.60	3.04	0.21	0.21	0.31	0.88
Lungs, g	0.63	0.58	0.59	0.62	0.02	0.20	0.39	0.98
g/g Brain Wt	0.675	0.674	0.661	0.687	0.02	0.98	0.44	0.22
mg/g Body Wt	32.05	32.09	31.71	32.43	0.79	0.98	0.67	0.99
Liver, g	0.93	0.88	0.89	0.91	0.02	0.23	0.58	0.75
g/g Brain Wt	0.99	1.03	1.01	1.02	0.02	0.42	0.81	0.27
mg/g Body Wt	47.79	48.92	48.59	48.12	0.78	0.50	0.78	0.53
Uterus, g	0.026	0.019	0.022	0.023	0.003	0.31	0.92	0.59
g/g Brain Wt	0.028	0.023	0.026	0.025	0.003	0.40	0.95	0.97
mg/g Body Wt	1.36	1.09	1.25	1.21	0.16	0.43	0.91	0.85
Right Ovary, g	0.031	0.013	0.016	0.027	0.005	0.11	0.33	0.41
g/g Brain Wt	0.032	0.015	0.018	0.028	0.005	0.11	0.39	0.58
mg/g Body Wt	0.99	0.68	0.87	0.80	0.11	0.17	0.73	0.52
Left Ovary, g	0.0102	0.009	0.0104	0.009	0.0006	0.76	0.54	0.09
g/g Brain Wt	0.011	0.011	0.012	0.010	0.0006	0.95	0.34	0.15
mg/g Body Wt	0.49	0.54	0.57	0.47	0.03	0.54	0.13	0.14
Mammary, g	0.034	0.035	0.036	0.033	0.002	0.96	0.45	0.74
g/g Brain Wt	0.036	0.041	0.042	0.036	0.003	0.47	0.44	0.70
mg/g Body Wt	1.72	1.95	1.96	1.71	0.14	0.41	0.39	0.85

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON = Control gain (0.60 kg/d); RES = Restricted gain (-0.23 kg/d); +OCM = with one-carbon metabolite supplementation; -OCM = without one-carbon metabolite supplementation.
[3]Standard error of the mean. [4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain×OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation; Organs with significant interactions are described in the text.

Table 2.4 The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 161 of gestation (Exp. 2) on fetal organ weight and allometric organ weight.

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Fetal Fluids, g	5,724	6,089	5,978	5,836	268.53	0.52	0.80	0.65
Fetal Body, g	3,799	3,916	3,849	3,866	67.29	0.41	0.90	0.33
Mammary, g	15.02	14.97	14.72	15.26	0.49	0.95	0.57	0.02
g/g Brain Wt	0.27	0.28	0.27	0.28	0.008	0.61	0.45	0.03
mg/g Body Wt	3.97	3.84	3.83	3.97	0.13	0.62	0.60	0.11
Liver, g	124.01	130.02	125.31	128.71	2.92	0.33	0.58	0.79
g/g Brain Wt	2.24	2.42	2.29	2.37	0.04	0.03	0.32	0.68
mg/g Body Wt	32.59	33.19	32.49	33.30	0.45	0.49	0.36	0.39
Lungs, g	98.73	104.07	99.85	102.95	2.01	0.19	0.44	0.23
g/g Brain Wt	1.78	1.95	1.83	1.89	0.04	0.02	0.34	0.05
mg/g Body Wt	26.03	26.67	26.01	26.70	0.46	0.46	0.43	0.01
Spleen, g	10.72	11.19	10.17	11.74	0.38	0.53	0.04	0.77
g/g Brain Wt	0.19	0.21	0.19	0.22	0.006	0.20	0.01	0.37
mg/g Body Wt	2.81	2.86	2.65	3.02	0.08	0.69	0.01	0.28
Rumen Complex, g	54.06	56.83	56.07	54.82	1.31	0.31	0.64	0.30
g/g Brain Wt	0.97	1.06	1.03	1.01	0.02	0.04	0.18	0.60
mg/g Body Wt	14.24	14.54	14.58	14.19	0.26	0.58	0.48	0.75
Right Longissimus Dorsi, g	61.02	65.36	63.99	62.38	1.77	0.24	0.66	0.59
g/g Brain Wt	1.10	1.21	1.16	1.15	0.03	0.04	0.76	0.95
mg/g Body Wt	16.14	16.62	16.62	16.14	0.35	0.53	0.52	0.82
Right Hindlimb Muscle, g	76.16	81.25	77.83	79.58	2.99	0.38	0.76	0.03
g/g Brain Wt	1.38	1.48	1.39	1.46	0.05	0.25	0.43	0.09
mg/g Body Wt	20.05	20.54	20.01	20.57	0.63	0.69	0.65	0.04
Right Ovary, g	0.144	0.178	0.108	0.214	0.03	0.62	0.15	0.82
g/g Brain Wt	0.002	0.003	0.002	0.004	0.0006	0.54	0.14	0.74
mg/g Body Wt	0.038	0.046	0.028	0.056	0.009	0.69	0.15	0.78
Left Ovary, g	0.164	0.123	0.125	0.162	0.02	0.39	0.43	0.31
g/g Brain Wt	0.003	0.002	0.002	0.003	0.0003	0.46	0.45	0.33
mg/g Body Wt	0.044	0.031	0.033	0.042	0.006	0.35	0.45	0.39
Uterus, g	1.42	1.43	1.39	1.46	0.04	0.98	0.42	0.20
g/g Brain Wt	0.026	0.027	0.026	0.27	0.001	0.61	0.63	0.44
mg/g Body Wt	0.376	0.365	0.363	0.379	0.012	0.65	0.53	0.44

Table 2.4 The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 161 of gestation (Exp. 2) on fetal organ weight and allometric organ weight. (Continued)

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Heart, g	27.99	28.83	28.64	28.18	0.83	0.64	0.79	0.86
g/g Brain Wt	0.51	0.54	0.52	0.52	0.01	0.26	0.90	0.44
mg/g Body Wt	7.38	7.36	7.45	7.29	0.19	0.97	0.71	0.41
Trachea, g	1.45	1.29	1.50	1.24	0.06	0.18	0.03	0.88
g/g Brain Wt	0.026	0.024	0.027	0.023	0.001	0.27	0.11	0.81
mg/g Body Wt	0.387	0.327	0.393	0.321	0.019	0.09	0.04	0.79
Small Intestine, g	69.07	64.95	64.42	69.59	1.59	0.19	0.11	0.63
g/g Brain Wt	1.25	1.22	1.19	1.28	0.03	0.54	0.11	0.90
mg/g Body Wt	18.28	16.64	16.88	18.03	0.44	0.06	0.18	0.85
Large Intestine, g	16.92	16.01	16.31	16.64	0.49	0.37	0.75	0.31
g/g Brain Wt	0.31	0.30	0.30	0.31	0.007	0.63	0.61	0.55
mg/g Body Wt	4.47	4.09	4.24	4.32	0.12	0.12	0.74	0.68
Pancreas, g	3.03	2.85	2.99	2.89	0.17	0.62	0.78	0.90
g/g Brain Wt	0.054	0.053	0.054	0.053	0.05	0.83	0.75	0.85
mg/g Body Wt	0.79	0.72	0.77	0.75	0.04	0.37	0.78	0.88
Left Kidney, g	17.13	16.44	16.05	17.52	0.60	0.58	0.25	0.99
g/g Brain Wt	0.309	0.306	0.294	0.323	0.01	0.89	0.20	0.68
mg/g Body Wt	4.49	4.19	4.17	4.51	0.12	0.22	0.19	0.55
Right Kidney, g	16.89	16.26	16.29	16.86	0.45	0.51	0.55	0.75
g/g Brain Wt	0.305	0.303	0.299	0.310	0.007	0.88	0.47	0.88
mg/g Body Wt	4.44	4.15	4.25	4.35	0.09	0.15	0.60	0.72
Left Longissimus Dorsi, g	59.38	63.31	61.11	61.59	2.06	0.36	0.91	0.33
g/g Brain Wt	1.07	1.17	1.11	1.13	0.03	0.11	0.71	0.53
mg/g Body Wt	15.64	16.07	15.82	15.90	0.37	0.58	0.91	0.61
Left Hindlimb Muscle, g	70.72	79.97	74.14	76.55	3.14	0.15	0.70	0.35
g/g Brain Wt	1.28	1.46	1.33	1.41	0.05	0.11	0.48	0.71
mg/g Body Wt	18.55	20.22	19.15	19.63	0.58	0.17	0.69	0.49
Left Hemisphere Brain, g	25.45	24.29	25.09	24.65	0.56	0.32	0.71	0.47
g/g Brain Wt	0.46	0.45	0.46	0.45	0.006	0.68	0.67	0.76
mg/g Body Wt	6.72	6.23	6.53	6.41	0.14	0.09	0.65	0.95
Right Hemisphere Brain, g	25.50	24.77	25.16	25.11	0.48	0.48	0.96	0.58
g/g Brain Wt	0.46	0.46	0.46	0.46	0.005	0.88	0.87	0.82

Table 2.4 The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 161 of gestation (Exp. 2) on fetal organ weight and allometric organ weight. (Continued)

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Right Hemisphere Brain, g								
mg/g Body Wt	6.73	6.34	6.55	6.51	0.11	0.07	0.85	0.63
Hind Brain, g	4.48	4.55	4.43	4.59	0.23	0.89	0.74	0.88
g/g Brain Wt	0.081	0.085	0.081	0.085	0.004	0.71	0.69	0.89
mg/g Body Wt	1.19	1.17	1.17	1.20	0.07	0.88	0.83	0.85

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON = Control gain (0.67 kg/d); RES = Restricted gain (-0.16 kg/d); +OCM = with one-carbon metabolite supplementation; -OCM = without one-carbon metabolite supplementation.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain×OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation; Organs with significant interactions are described in the text.

Discussion

In Exp. 1 heifer data, as expected there were gain effects on maternal carcass and organs where CON gain heifers were greater when compared with RES gain heifers. Increased normalized organ weights of the fetal right and left hemisphere of the brain and heart were observed when heifers received OCM supplementation up to d 63. This data suggests in response to maternal undernutrition, the fetus was prioritizing organ and tissue development that is important for survival. The brain and muscle tissues have different priorities in nutrient partitioning when resources are limited, affecting their development and functionality. For example, the brain prioritizes its own glucose needs over other tissues (Diniz et al., 2021). Thus, when nutrients are restricted, more of the available glucose is allocated for the central nervous system function (Diniz et al., 2021). This can potentially impair muscle and other organ development by limiting nutrient availability. Therefore, OCM supplementation in theory should rescue possible impairments seen in muscle mass and development due to lower rates of gain.

It was also observed in Exp. 1 that left longissimus dorsi weights increased with OCM supplementation. Muscle fiber is especially vulnerable to nutrient availability because it is low priority in terms of nutrient partitioning during fetal development compared to brain, gut, and heart (Reynolds and Caton, 2012). If an animal is under a nutrient restriction, it will affect energy partitioning among metabolically active tissues (Hocquette et al., 2007). Additionally, skeletal muscle development in utero is imperative for efficient offspring performance regarding meat quality and carcass weight (Reynolds et al., 2019). Thus, the supply of OCM during gestation can help mitigate any negative effects that could be seen in skeletal tissue development due to maternal malnutrition, as seen in the improvement of the longissimus dorsi weights in Exp. 1. Because day 63 is within the period of peak primary myogenesis in cattle, differences in

muscle growth during this time could have lasting implications in post-natal calf growth and performance. For instance, altered fetal organ development can lead to structural defects in muscle tissue which is a prime example of structural defect because the number of muscle fibers is established before birth (Reynolds et al., 2019). Myofibers, adipocytes, and fibroblasts are all derived from a common pool of mesenchymal stem cells and there is evidence that suggests a nutrient-restriction can cause the cell differentiation to shift away from myogenesis (Blair et al., 2021). This phenomenon would result in depressed muscle fiber development and increased adipocyte formation (Du et al., 2010). Supporting this theory, it was observed in sheep that were nutrient restricted, there was a reduced number of myofibers in offspring (Zhu et al., 2006). Thus, factors affecting fetal muscle development can permanently make changes in muscle structure and growth potential of postnatal offspring. This concept is especially prevalent in beef fetuses since the maternal environment during development has high variation in feed quality or quantity.

In Exp. 2, in heifer data, it was observed as expected that CON diet increased certain carcass measurements such as live carcass weight, hot carcass weight, 12th rib fat depth, and ribeye area. Additionally, the USDA yield grade for +OCM heifers was 1.69 versus -OCM heifers at 2.02. Yield grade 1 denotes the highest yielding carcass. This data suggests that heifers receiving OCM treatment had a slightly improved yield grade since heifers receiving treatment were closer to Yield grade 1 than heifers who were not. Furthermore, since yield grade factors consist of carcass weight, 12th rib fat depth, and ribeye area this difference suggests that all of the factors analyzed in this project were improved due to treatment. There is evidence in literature that supplying nutrients that may not be met in the diet has improved beef carcass characteristics. For instance, Wang et al., 2018, injected vitamin A in Angus steer calves from birth to 1 month

of age. It was seen that vitamin A supplementation increased growth, amount of satellite cells, and the expression of myogenic markers. Furthermore, there was an increase in the latissimus dorsi muscle fiber size (Wang et al., 2018). A study done with angus-crossbred steers found that zinc supplementation increased hot carcass weight and a linear increase of yield grade (Messersmith et al., 2022).

When looking at fetal data, it is a common trend to see that the RES heifers had heavier masses across fetal organs such as liver, lungs, rumen complex, and right longissimus dorsi, which seems counter intuitive. However, since heifers only received RES intake resulting in lower gains from breeding until d 63 of gestation and were then placed on a control diet for the remainder of trial, observed increases could be attributed to compensatory growth following re-alimentation. There is much evidence supporting this idea. For example, a trial was done on cattle where they were placed on a nutrient restricted diet and then exhibited compensatory growth for 11 months following re-alimentation (Ryan et al., 1993). Ryan et al., (1993) accounted for the compensatory growth due to the increased intake. It has also been observed that re-alimentation following a restriction can improve efficiency in animals. Gonzalez et al., (2013) found that nutrient restricted pregnant beef cows were able to compensate and overcome the insults that occurred to fetal skeletal muscle development and return to normal muscle fiber size and muscle progenitor numbers before birth. Additionally, a study done on Yaks (Zou et al., 2019) following nutritional deprivation and refeeding of the same diet significantly increased the feed efficiency and nutrient digestibility. Specifically, nutrient digestibility was improved with rumen acetate, propionate, and microbial protein productions (Zou et al., 2019). Therefore, the heifers on the OCM study once being reintroduced to an adequate intake could have improved their efficiency and therefore had compensatory growth occurring in the fetus as well. Also, if

nutrient digestibility was improved, that would be seen in the fetal organ mass as well. In later chapters, the gene expression data will be discussed to further investigate this. Even though supplementation with OCM did not rescue all organ growth, alterations in muscle and metabolic organ weight observed, based upon published observations from the literature, could have lasting postnatal implications.

Conclusion

In conclusion, these data support the importance of nutrient supply during early gestation for fetal organ development and tissue energy partitioning. We hypothesized that a low maternal plane of nutrition would impair fetal growth in early gestation and that supplementation of OCM would rescue growth restricted offspring at d 63 and 161 of gestation, Exp. 1, and Exp. 2, respectively. In agreement with our hypothesis, the data shows that supplementation of OCM was able to increase fetal body, normalized brain tissue, and normalized muscle tissue weights. This shows the significance of providing an adequate supply of nutrients during early gestation as to not limit tissue partitioning and fetal growth. Therefore, improved fetal growth and enhanced muscle development could increase efficiency of industry animals resulting in more profit for producers.

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CHAPTER 3. EFFECTS OF MATERNAL RATE OF GAIN AND SUPPLEMENTING ONE-CARBON METABOLITES TO BEEF HEIFERS ON INDICES OF MATERNAL SERUM OXIDATIVE STRESS

Abstract

We hypothesized that low maternal plane of nutrition would impair antioxidant production, and that supplementation of one-carbon metabolites (OCM) in early gestation would rescue/increase total antioxidant capacity in beef heifers at d 63 and 161 of gestation, Exp. 1, and Exp. 2, respectively. Both experiments had a 2 × 2 factorial arrangement of treatments with two levels of maternal daily gain (Control, CON; targeted: 0.45 kg/d; vs. Restricted, RES; (targeted: -0.23 kg/d), each with or without supplementation of OCM. In each experiment, seventy-two crossbred heifers were bred with female-sexed semen from a single sire. At breeding (d 0), beef heifers were individually fed using an electronic headgate system and randomly assigned to one of four treatments (CON-OCM, CON+OCM, RES-OCM, and RES+OCM). The basal diet given to all heifers for both experiments consisted of alfalfa-grass hay, corn silage, and ground corn. The OCM supplement consisted of ruminal-protected choline (44.4 g/d) and methionine (7.4 g/d) in a fine-ground corn carrier top-dressed daily, and weekly injections of folate (320 mg) and vitamin B12 (20 mg). The -OCM heifers received the corn carrier and saline injections. In Exp 1, heifers were slaughtered on d 63. Maternal serum samples were collected on d -2 (prior to treatment), d 35 (middle of treatment period), and d 63 (prior to slaughter). In Exp. 2, heifers received treatment up to d 63 then were all placed on a common control diet with no supplement until slaughter on d 161. Maternal serum samples were collected on d 0 (prior to treatment), d 35 (middle of treatment period), d 63 (end of treatment period), and d 154 (prior to slaughter). The serum was analyzed for total antioxidant capacity (CRE) measured by copper reduction

equivalent and CRE change between days. Data were analyzed using the MIXED procedures of SAS for main effects of gain, OCM, and their interactions. In Exp. 1, when the percent change in CRE was calculated, there was a difference in change between the two gain levels ($P = 0.05$). CON heifers had a greater % change in CRE from d-2 to d63 than the RES heifers (7.2% vs 0.12%). In Exp. 2, heifers on CON treatments had a greater CRE compared with those receiving RES (1,971.2 vs 1,837.8 ± 17.9 µM, $P < 0.01$). When observing the data within specific days, there was a tendency for CRE in CON heifers to be greater ($P = 0.07$) at d 63 when compared with RES. At day 154 of gestation, CON heifers had a greater CRE capacity when compared with RES (2,080.2 vs. 1,898.6 ± 42.6 µM, $P = 0.02$). There were tendencies for +OCM heifers to have a greater total antioxidant capacity compared with controls when expressing the data as CRE change and percent change from d 0 until d 64 ($P = 0.09$ and 0.08, respectively). These data suggest that adequate amount of nutrients and the supply of OCM improved the overall antioxidant capacity in maternal serum.

Introduction

In ruminant production systems, inadequate maternal nutrition during gestation is a common stressor cattle undergo since many systems are designed for cattle to receive the majority of their nutrients from grazing. Specifically, without supplemental nutrients, overgrazing or low forage quality and/or quantity can cause undernutrition, which can have negative impacts on offspring growth efficiency and body composition (Wu et al., 2006; Caton et al., 2007; NASEM, 2016; Caton et al., 2019). Additionally, drought can lead to low quality forage or a forage shortage during the key window of early gestation and affect fetal development. In the beef industry, there is a wide variation among producer practices in regard to providing supplemental nutrients to their herds. Because of the large variation in

supplementation, it would be beneficial for the industry to better understand the impact supplementation of nutrients has during early gestation on fetal growth and development.

During gestation there is an increased demand for energy and other nutrients necessary to sustain fetal growth and lactation (Drackley, 1999; NASEM, 2016). Increased requirements and metabolic demands result in over production of reactive oxygen species (ROS) (Drackley, 1999). These ROS act as primary and secondary messengers to promote cell growth or death and have a key role in development by regulating transcription factors that have an impact on varying cell signaling pathways involved in differentiation, proliferation, and apoptosis (Sinenko et al., 2021). Oxidative stress occurs when oxygen radicals and ROS over accumulate, surpassing the antioxidant defense numbers (Sordillo and Aitkin, 2009). For instance, excessive ROS can inhibit critical pathways that are essential components of immunity, inflammatory response, and cellular adhesion. An example of this is the disfunction of NF-kB pathway which is associated with inflammation, arthritis, neurodegenerative diseases, and cancer (Zhang et al., 2016). Moreover, when ROS accumulate, they will cause damage to lipids, proteins, polysaccharides, DNA, and other macromolecules. The oxidized molecules will remove electrons from other molecules and, if not controlled, can cause damage to tissue, enzyme function, and muscle tone (Miller et al., 1993). Thus, if oxidative stress occurs, it can have a negative effect on embryonic development (Dennery, 2007). These effects can have a negative economic impact on beef production by reducing offspring feed efficiency, average daily gain, and carcass composition. For instance, there is an increase in the production of glucocorticoids in response to stress which will inhibit growth hormones. This inhibition can lead to reduced growth or carcass quality seen in production (Kumar et al., 2012). Suboptimal carcass values and decreased growth rate cost feedlot producers millions of dollars annually (Gardner et al., 1998). Finally, in cow-calf

operations, reproduction is a significant factor in profitable production. Environmental stress such as compromised maternal nutrition, have been reported to cause impaired oocytes, infertility, and reduced conception rates (Kumar et al., 2012).

Supplementation of one-carbon metabolites (OCM) has the potential to assist in the mitigation of negative effects related to maternal nutrition. These OCM are nutrients including B-vitamins (choline, folate, and vitamin B_{12}) and amino acids (methionine). These metabolites (nutrients) play key roles in a network of biochemical pathways where methyl groups are transferred from one compound to another for methylation processes (Mason, 2003). Moreover, one-carbon metabolism represents an interconnected route through which nutrients, including amino acids, can impact molecular events such as epigenetic regulation, energy metabolism, and antioxidant synthesis. In addition to being used for synthesis of proteins, amino acids can also directly or indirectly impact the immune system through their production of molecules such as the antioxidants glutathione (GSH) and histamine (Li et al., 2007). These antioxidants are key to the removal of ROS. Specifically, methionine is necessary for production of GSH, taurine, and methyl groups. These features are amplified by methionine's key role in one-carbon metabolism. Within these pathways, methionine produces S-adenosyl methionine (SAM), which can be utilized to methylate DNA, reducing inflammation and oxidative stress (Coleman et al., 2020). An OCM deficiency when there is a high demand for energy and nutrients to support gestation compromises milk production and contributes to systemic oxidative stress (McFadden et al., 2020). Therefore, the objectives of this chapter are to investigate the effects of OCM supplementation on maternal serum total antioxidant capacity.

Materials and Methods

Animals, Diet, and Experimental Design

Animal handling and care procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee (IACUC no. A21049). In both Exp. 1 and 2, seventy-two crossbred beef heifers were used. In Exp.1 heifers averaged body weight of 398 ± 32 kg and Exp. 2 heifers averaged 348 ± 20 kg. All heifers were subjected to a 7-day Select-Synch + CIDR estrus synchronization protocol (Lamb et al., 2010). After estrus synchronization, heifers were bred approximately 16 to 20 h after detected estrus with female sexed semen from a single sire (ST Genetics, Navasota, Texas). The basal diet consisted of corn silage, ground corn, and an alfalfa/grass hay mix. The diet targeted a crude protein amount of 13.48% and metabolizable energy amount of 2.56 Mcal/kg on a dry matter basis. Corn carrier for treatment delivery weighed 0.27 kg and was deducted from the percent corn in the total mixed ration. A vitamin/mineral premix (Trouw dairy VTS/Optimins, Trouw Nutrition USA, Highland, Illinois) was added to the corn carrier and provided based on averaged heifer body weight per label directions which resulted in an average of 10 g/heifer daily. The premix consisted of calcium (10 to 12%), magnesium (5%), potassium (5%), cobalt (180 mg/kg), copper (5,100 mg/kg), iodine (375 mg/kg), iron (1.2%), selenium (132 mg/kg), zinc (2.7%), vitamin A (2,775,775 IU/kg), vitamin D_3 (771,617 IU/kg), and vitamin E (13,228 IU/kg. Average daily gain was monitored via weekly weights and feed allotment adjusted accordingly to meet gain targets.

At breeding (d 0), heifers were assigned to one of four nutritional treatments in a 2 x 2 factorial arrangement: Control intake without OCM supplementation (CON-OCM), CON with OCM supplementation (CON+OCM), restricted intake without OCM supplementation (RES-OCM), or RES with OCM supplementation (RES+OCM). In both experiments, the targeted

ADG for control intake (CON) was 0.45 kg/d for restricted intake (RES) was -0.23 kg/d. The OCM supplement consisted of rumen-protected choline (ReaShure, Balchem Inc., New Hampton, NY, 44.4 g/d) and methionine (Smartamine, Adisseo, China, 7.4 g/d) in a fine-ground corn carrier top-dressed daily, and weekly injections of folate (Spectrum Chemical Mfg. Corp., New Brunswick, NJ, 320 mg, 6 cc) and vitamin B_{12} (MWI Animal Health, Boise, ID, 20 mg, 4 cc). The -OCM heifers received the corn carrier and saline injections (one 6 cc and one 4 cc injection). Therefore, the experimental design had treatments arranged as a 2×2 factorial with two levels of maternal gain (achieved by altering intake) and two levels of strategic OCM supplementation. After pregnancy was diagnosed with ultrasound, the number of heifers per treatment for Exp. 1 was CON+OCM (n = 8), CON-OCM (n = 7), RES+OCM (n = 8), and RES-OCM (n = 9). In Exp. 2 heifers per treatment was CON+OCM (n = 8), CON-OCM (n = 7), RES+OCM (n = 7), and RES-OCM (n = 7).

Serum Collection

In Exp. 1 maternal serum samples were collected via jugular venipuncture on days -2, 35, and 63 (day before slaughter) of gestation using 10 mL serum monoject corvac blood collection tubes (Cardinal Health, Dublin, OH). Samples for Exp. 2 were taken on days 0, 35, 63, and 154. Blood samples were allowed to clot for 20 minutes at room temperature before being centrifuged at 2,000 × g for 10 minutes. The serum was then separated from the rest of the contents and stored at -80˚C.

Antioxidant Capacity Assay

In this experiment the OxiSelect Total Antioxidant Capacity (TAC) Assay Kit from CellBioLabs was used on the hydrophilic fraction in bovine serum samples. This kit measures the total antioxidant capacity within a sample based on the reduction of copper (II) to copper (I)

by antioxidants such as uric acid. Upon reduction, the copper (I) ion further reacts with a coupling chromogenic reagent that produces a color with a maximum absorbance at 490 nm. This kit can be used for direct measurement of total antioxidant capacity from cell lysate, plasma, serum, urine, tissue homogenates and food extracts. Our data showed that bovine serum has a matrix effect in this assay. Absorbance values were not parallel to the standards. When serum samples were diluted (1x, 2x, 4x, 8x, 16x), the absorbance values did not change as expected. The more concentrated the sample, the more the matrix effect was observed, the curve was not linear. The less concentrated samples (dilutions 4x, 8x and 16x) presented a better linear curve, but still it was not parallel to the standards. Based on the data, the following modifications were proposed to the kit; Incubate with Copper Ion reagent at 10 min instead of 5min and dilute samples 8x (e.g., 20 uL sample + 140uL 18MΩ H2O for a total volume of 160 µL).

Samples were thawed and then 20 uL pipetted in duplicate in a 96-well microtiter plate (Greiner Bio-one). 180uL of 1X Reaction Buffer was then pipetted into each well. The plate was then placed into Synergy Gen5 3.11 microplate reader and initial absorbance was obtained at 490 nm. The microplate reader protocol is as follows: shake linear for 15 sec; read at 490 nm; remove plate, add in 50 uL of copper reagent; shake linearly for 15 sec; shake orbitally for 10 min; remove plate, add in 50 uL of Stop Solution; shake linearly for 15 sec; read at 490 nm.

Calculations

In the protocol of the microplate reader, the data were automatically calculated according to the manufacturer's protocol. The net absorbance was found by subtracting the initial absorbance readings for samples and standards from the final readings for each. The net absorbance was then plotted against the uric acid concentration for the uric acid standard curve.

We determined the "mM uric acid equivalents" (UAE) for samples by extrapolating the uric acid concentration from the sample's analogous uric acid OD490 nm value.

We then determined "uM Copper Reducing Equivalent" (CRE) for samples by multiplying the uric acid equivalence (UAE) concentration by 2,189 uM Cu^{++}/mM uric acid (1mM of uric acid=2,189 uM Copper Reducing Equivalents). The sample CRE values are proportional to the sample's total antioxidant capacity or total antioxidant power. We then determined the percent change in CRE using the following calculation: [(FinalDay – InitialDay)/FinalDay]*100.

Statistical Analysis

Data were analyzed using the MIXED procedures of SAS (Version 9.4, SAS Institute Inc., Cary, NC) for main effects of gain, OCM, day, and their interactions. Day was used as a repeated measure. In the presence of a significant day response and the absence of significant interactions involving day, the data were analyzed within day for main effects of gain, OCM, and their interactions. Data are reported as LSMeans where significance was set at $P \leq 0.05$ and tendency was $0.05 < P \leq 0.10$.

Results

Exp. 1 Copper Reduction Equivalent

As mentioned in materials and methods, the measurement of CRE measures total antioxidant capacity. Overall main effects are presented in Table 3A. There were no overall effects of Gain or OCM on CRE; however, there was a day effect (Table 3A; $P = 0.037$; d -2 = 1,552, d 35 = 1,606, d 63 = 1,617 ± 28.98). When assessing change in CRE (Table 3B), CON heifers exhibited an increase ($P \leq 0.05$) from d -2 to d 63, while RES did not. Heifers provided OCM presented with an increased ($P = 0.01$) in CRE from d -2 to d 35 of gestation when compared with those not receiving OCM. There were no three-way or two-way interactions

(Table 3A; $P \geq 0.06$); however, for clarity, the interactive means are presented in Figure 3A and briefly discussed herein. As expected, there were no differences at d -2 relative to treatment initiation. When CRE was analyzed using days as repeated measures there was a tendency in Gain × Day and OCM × Day interactions ($P = 0.07$ and 0.06, respectively). Specifically, there tended to be an increase in CRE in CON heifers compared to RES from d -2 to d 35 (1,532 vs. 1,611 µM; $P = 0.06$), and from d -2 to d 63 (1,532 vs. 1,664 µM; $P = 0.003$). The OCM × Day interactions tended ($P = 0.06$) to show an increase in CRE for +OCM heifers from d -2 to d 35 (1,517 vs. 1,636 µM; $P = 0.004$), as well as from d -2 to d 63 (1,517 vs. 1,622 µM; $P = 0.01$).

Exp. 2 Copper Reduction Equivalent

There were no two-way or three-way interactions ($P > 0.05$) in Exp. 2 for copper reduction equivalents; therefore, main effect means are presented in Tables 3C and 3D. Heifers on CON treatments had a greater CRE compared with those receiving RES (1,971.2 vs 1,837.8 ± 17.94 µm; $P < 0.01$). When observing the data within specific days, there was a tendency for CRE in CON heifers to be greater ($P = 0.07$) at d 63 when compared with RES. At day 154 of gestation, CON heifers had a greater CRE capacity when compared with RES (2,080.2 vs. 1,898.6 ± 42.61 µm, $P = 0.02$; d0 = 1,840, d35 = 1,871, d63 = 1,921, d154 = 1,986 ± 39.95). There were tendencies for +OCM heifers to have a greater CRE capacity compared with controls when expressing the data as CRE change and percent change from d 0 until d 64 ($P = 0.09$ and 0.08, respectively).

Table 3.1 Exp. 1: The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] on total antioxidant capacity (CRE) in maternal serum

	Treatments[2]					*P*-value[4]		
Item	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**Day**	**OCM**
CRE (µm)	1,602.4	1,581.15	1,592.2	1,591.3	14.11	0.53	0.037	0.98

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON = Control (0.60 kg/d); RES = Restricted (-0.23 kg/d); +OCM = one-carbon metabolite supplementation; -OCM = without one-carbon metabolite supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of CON vs RES; OCM = Main effect of one-carbon metabolite supplementation.
Interactive effects *P*-values: Gain × Day = 0.07; OCM × Day = 0.06; Gain × OCM = 0.12; Gain × OCM × Day = 0.83.

Table 3.2 Exp. 1: The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] on total antioxidant capacity (CRE) and percent change.

	Treatments[2]					*P*-value[4]		
Item	**CON**	**RES**	**+OCM**	**-OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
CRE (µm)								
d -2	1,531.9	1,577.6	1,519.2	1,590.2	21.79	0.29	0.11	0.41
d 35	1,611.0	1,611.9	1,646.8	1,576.1	23.24	0.98	0.13	0.19
d 63	1,665.4	1,572.8	1,632.3	1,605.9	29.14	0.12	0.65	0.29
CRE Change[5]								
D63 – D-2 (µm)	125.3	11.9	95.7	41.5	29.44	0.05	0.34	0.53
D63 – D-2 (%)	7.21	-0.88	4.62	1.34	1.85	0.03	0.36	0.98
D63 – D35 (µm)	76.0	-39.0	-8.49	45.5	36.38	0.13	0.47	0.89
D63 – D35 (%)	3.91	-4.12	-2.67	1.15	2.34	0.09	0.42	0.55
D35 – D-2 (µm)	79.1	37.4	127.5	-11.01	28.77	0.44	0.01	0.67
D35 – D-2 (%)	4.45	1.64	7.95	-1.21	1.83	0.41	0.01	0.99

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON = Control gain (0.60 kg/d); RES = Restricted gain (-0.23 kg/d); +OCM = with one-carbon metabolite supplementation; -OCM = without one-carbon metabolite supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.
[5]Percent change in copper reduction equivalent equation described in materials and methods.

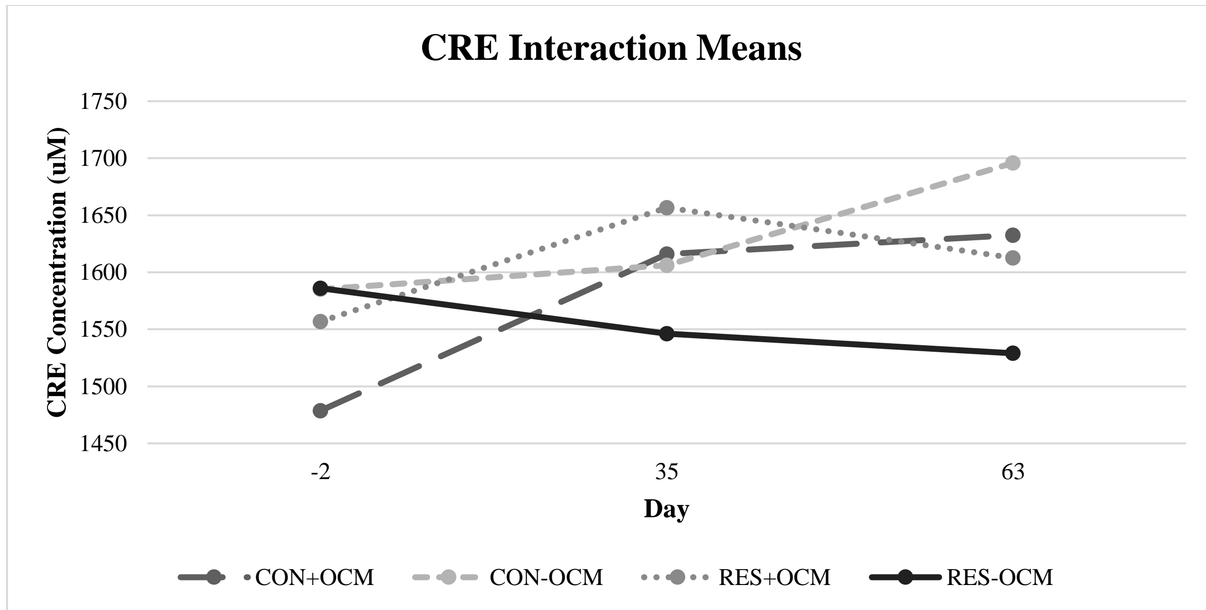

Figure 3.1 Copper Reduction Equivalent (CRE; total antioxidant capacity). Interaction Means of maternal serum on d -2, 35, and 63 of gestation (Exp. 1). *P*-values: Gain × Day = 0.07; OCM × Day = 0.06; Gain × OCM = 0.12; Gain × OCM × Day = 0.83. Standard Error of the Mean (SEM) = 14.11.

Table 3.3 Exp. 2: The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] on total antioxidant capacity (CRE) in maternal serum

	Treatments[2]					*P*-value[4]		
Item	CON	RES	+OCM	-OCM	SEM[3]	Gain	Day	OCM
CRE (μm)	1971.2	1837.8	1931.4	1877.6	17.94	<0.01	<0.01	0.24

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON = Control gain (0.60 kg/d); RES = Restricted gain (-0.23 kg/d); +OCM = with one-carbon metabolite supplementation; -OCM = without one-carbon metabolite supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.
Interactive effects *P*-values: Gain × Day = 0.62; OCM × Day = 0.23; Gain × OCM = 0.78; Gain × OCM × Day = 0.23.

Table 3.4 Exp. 2: The effect of maternal plane of nutrition and supplementation with one-carbon metabolites[1] on total antioxidant capacity (CRE) and percent change.

	Treatments[2]					*P*-value[4]		
Item	CON	RES	+OCM	-OCM	SEM[3]	Gain	OCM	Gain × OCM
CRE (µm)								
d 0	1892.0	1785.5	1842.8	1834.6	29.77	0.08	0.89	0.56
d 35	1924.6	1836.6	1892.7	1868.5	32.91	0.20	0.72	0.65
d 63	1987.9	1874.0	1981.6	1880.3	32.56	0.07	0.11	0.82
d 154	2080.2	1898.6	2051.9	1926.8	42.61	0.02	0.11	0.17
CRE Change[5]								
D154 – D0 (µm)	188.12	113.16	209.09	92.18	42.07	0.38	0.17	0.41
D154 – D0 (%)	8.58	4.55	9.29	4.13	2.08	0.34	0.22	0.49
D154 – D35 (µm)	155.49	62.05	159.19	58.34	44.69	0.29	0.25	0.13
D154 – D35 (%)	6.80	1.78	6.73	2.22	2.32	0.28	0.33	0.14
D154 – D63 (µm)	92.22	24.65	70.35	46.52	47.13	0.49	0.81	0.22
D154 – D63 (%)	3.60	-0.25	2.04	1.58	2.40	0.44	0.92	0.26
D63 – D0 (µm)	95.89	88.50	138.74	45.66	26.92	0.89	0.09	0.36
D63 – D0 (%)	4.74	4.44	6.94	2.26	1.34	0.91	0.08	0.36
D63 – D35 (µm)	63.26	37.39	88.83	11.82	37.52	0.74	0.33	0.83
D63 – D35 (%)	3.02	1.09	3.89	0.36	1.97	0.64	0.39	0.99
D35 – D0 (µm)	32.64	51.11	49.90	33.84	40.65	0.83	0.85	0.45
D35 – D0 (%)	1.39	1.63	1.59	1.40	2.42	0.96	0.96	0.31

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON = Control gain (0.60 kg/d); RES = Restricted gain (-0.23 kg/d); +OCM = with one-carbon metabolite supplementation; -OCM = without one-carbon metabolite supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.
[5]Percent change in copper reduction equivalent: [(Final – Initial)/Final]*100.

Discussion

In beef production systems, the wide variety of supplementation strategies and chances of compromised forage quality and quantity during gestation can result in ROS overaccumulation. For instance, weather conditions can decrease nutrient concentration in forage such as vitamins and trace elements. This deficiency can cause a reduction in removal of ROS leading to an overaccumulation (Aurousseau et al., 2006). This overaccumulation will result in oxidative stress that can cause significant amounts of damage on the cellular and organ level throughout the animal's body. For instance, ROS can cause damage to tissues that include mitochondrial/nuclear DNA, lipid peroxidation, apoptosis/proliferation, liver function, and enzyme function (Su et al., 2019). The OCM supplemented during this study have the capability

to counteract the damage that can occur from ROS production. In Exp. 1 +OCM heifers had a positive increase and percent change in total antioxidant capacity from d -2 to d 35 ($P = 0.01$). This shows that the animals receiving the OCM supplementation would likely have better antioxidant defenses in the case of oxidative stress.

From this study, we can see that the heifers receiving a control level of gain had a greater antioxidant capacity than the heifers on a nutrient restriction. Additionally, the +OCM heifers appeared to increase their antioxidant capacity throughout the trial, the longer they received supplementation. The OCM supplementation in Exp. 2 tended to provide heifers with a greater increase in CRE from d 0 to 63, when supplementation ended ($P = 0.08$; Table 3D). Additionally, there was a gain tendency and difference for CON heifers having a greater capacity compared to RES heifers on d 63 and 154 ($P = 0.07$, 0.02, respectively; Table 3D). The data reported suggests that the adequate nutrient intake and supplementation of OCM did enhance total antioxidant capacity. The enhancement of total antioxidant capacity could have come from multiple pathways. For example, methionine is a precursor of succinyl-CoA, s-adenosyl-methionine, homocysteine, cysteine, creatine, and carnitine. Methionine directly influences the function of the immune system via the production of these metabolites. Cysteine production can be used in the synthesis of the antioxidant glutathione and taurine (Martinez et al., 2017). With more production of these antioxidants, cellular systems can easily eliminate low concentrations of ROS. Additionally, there is evidence that methionine can chelate lead and remove it from tissues which decreases oxidative stress (Patra et al., 2001). When looking at the one-carbon metabolite pathway, it is seen how these precursors lead contribute to the transsulfuration pathway. When methionine intake is increased, the transmethylation pathway decreases and flux of the transsulfuration pathway increases resulting in more antioxidant production. If a redox

challenge does occur due to high levels of ROS this can cause negative feedback on enzymes that catalyze the regeneration of methionine from homocysteine (Dalto and Matte, 2017). Furthermore, the OCM pathway is catalyzed when in the presence of the metabolites (folate, vitamin B_{12}, and choline) that were given during this study (Suh et al., 2016). In this study, CRE was improved due to a greater gain level as well as heifers receiving OCM supplementation. Further research is needed to investigate specific oxidative stress parameters such as protein, lipid, and DNA damage.

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CHAPTER 4. EFFECTS OF SUPPLEMENTING ONE-CARBON METABOLITES TO BEEF HEIFERS FROM D 0 TO 63 OF GESTATION ON FETAL LIVER AND MUSCLE TISSUE GENE EXPRESSION

Abstract

We hypothesized a low maternal plane of nutrition will cause differential expression of fetal genes in early gestation and that supplementation of one-carbon metabolites (OCM) will mitigate differential expression in offspring at d 63. The experimental design is a 2 × 2 factorial arrangement of treatments with two levels of maternal average daily gain (ADG), each with or without supplementation of OCM. Seventy-two crossbred beef heifers were bred with female-sexed semen from a single sire. At breeding (d 0), the heifers were individually fed using an electronic headgate system and assigned to a treatment: Control intake (targeted: 0.45 kg/d ADG) without OCM (CON-OCM), CON with OCM (CON+OCM), Restricted intake (targeted: -0.23 kg/d ADG) without OCM (RES-OCM), or RES with OCM (RES+OCM). The basal diet consisted of corn silage, ground corn, and an alfalfa/grass hay mix. The OCM supplement consisted of ruminal-protected choline (44.4 g/d) and methionine (7.4 g/d) in a fine-ground corn carrier top-dressed daily, and weekly injections of folate (320 mg) and vitamin B12 (20 mg). The -OCM heifers received the corn carrier and saline injections. Heifers were slaughtered and fetal tissues were collected on d 63. Tissues were immediately placed in RNAlater and stored in -80 C until analysis. Extraction of RNA and quality checks were performed at North Dakota State University and eukaryotic mRNA-seq was performed on samples with data output of 6 Gb/20M PE150 read-pairs per sample via Novogene. A total of 9 genes were downregulated in fetal muscle tissue from CON heifers and 1 gene was upregulated when compared with RES. Additionally, in fetal muscle, 4 genes were downregulated in fetuses from heifers not receiving

OCM supplementation when compared with provided OCM. In fetal liver tissue, 5 genes were downregulated in CON compared with RES. Two genes were upregulated and three downregulated in liver tissue +OCM fetuses compared to -OCM. The genes mentioned are involved in pathways such as mTOR signaling, ROS production, eukaryotic translation initiation factor 4 (eIF4) and serine/threonine protein kinase (p70S6K) signaling, and more. Thus, OCM supplementation and maternal rate of gain did influence transcript abundance of genes associated with cellular processes such as cell death, growth, free radical production, and calcium signaling.

Introduction

There is evidence that maternal metabolic state, physiological traits, or environmental factors can influence fetal growth and development that will "program" permanent changes postnatally (Barker and Clark 1997). During early gestation, fetal organogenesis and differentiation of uteroplacental tissues occur, which are critical events for normal fetal development (Funston et al., 2010). Dams that undergo stress during early, but not late gestation, are likely to produce normal birth weight offspring. However, the offspring 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 stressors can induce epigenetic changes that will result in phenotypic changes due to altered gene expression. Specifically, altered gene expression can impact the offspring's production potential by "programming" susceptibilities to metabolic issues in specific tissues, such as liver and muscle, and reduce performance (Waterland and Jirtle, 2004). Furthermore, undernutrition can affect energy partitioning within these metabolically active tissues which can affect economic traits such as lactation, reproduction, meat quality, body composition, and carcass weight (Greenwood et al., 2004; Chavatte-Palmer et al., 2016; Reynolds and Vonnahme 2016). If offspring are

metabolically compromised, efficiency can be reduced in livestock production systems (Reynolds and Caton, 2012; Caton et al., 2019). Liver and muscle are key tissues for energy balance as well as beef products for harvest and sale. Since skeletal muscle, which has economic importance to producers, develops during the intrauterine stage, manipulating fetal muscle development during gestation could improve production efficiency. Muscles have a lower priority in nutrient partitioning compared to other vital tissues (Diniz et al., 2021). Thus, maternal nutrient supply is crucial and can affect muscle composition and therefore the final meat quality (Radunz et al., 2012). Radunz et al. (2012), found that maternal dietary energy source can alter fetal adipose tissue development and progeny's intramuscular fat deposition. The liver is a key metabolic organ that connects various tissues metabolically such as skeletal muscle and adipose tissue (Rui, 2014). In addition, the metabolic pathways in the liver are regulated by epigenetic modifications of gene promoters (Pinney and Simmons, 2010). One-carbon metabolites (OCM) have the potential to rescue epigenetic changes caused by stressors in these tissues because of their role in gene regulation through the one-carbon metabolic pathway. Specifically, the goal of one-carbon metabolism is to manage the provision of methyl groups for methylation reactions such as DNA and histone proteins (Friso et al., 2017). Methylation at specific sites on the DNA sequence and histone tails are a crucial epigenetic feature for regulating gene expression (Lee et al., 2020). The one-carbon metabolism pathway is dependent on several nutrients such as folate, vitamin B_{12}, choline, and methionine. The dietary supply of these methyl donors is necessary for normal growth, development, and physiological function (Rush et al., 2014). Therefore, we hypothesized that differing rates of maternal body weight gain resulting from changes in intake during early gestation would cause differential gene expression

in fetal liver and muscle tissue and that OCM supplementation will counteract unfavorable gene expression patterns.

Materials and Methods

Animals, Diet, and Experimental Design

Animal handling and care procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee (IACUC no. A21049). Seventy-two crossbred beef heifers were used and had an averaged body weight of 398 ± 32 kg at the start of trial. Heifers were then subjected to a 7-day Select-Synch + CIDR estrus synchronization protocol (Lamb et al., 2010). After estrus synchronization, heifers were bred approximately 16 to 20 h after detected estrus with female sexed semen from a single sire (ST Genetics, Navasota, Texas). The basal diet was a total mixed ration and consisted of corn silage, ground corn, and an alfalfa/grass hay mix. The diet targeted a crude protein amount of 13.5% and metabolizable energy amount of 2.56 Mcal/kg on a dry matter basis. The daily top-dressed corn carrier for treatment delivery weighed 0.27 kg and was deducted from the percent corn in the total mixed ration. A vitamin/mineral premix (Trouw dairy VTS/Optimins, Trouw Nutrition USA, Highland, Illinois) was added to the corn carrier and provided based on averaged dry matter provided per label directions which and averaged 10 g/heifer daily. The premix consisted of calcium (10 to 12%), magnesium (5%), potassium (5%), cobalt (180 mg/kg), copper (5,100 mg/kg), iodine (375 mg/kg), iron (1.2%), selenium (132 mg/kg), zinc (2.7%), vitamin A (2,775,775 IU/kg), vitamin D_3 (771,617 IU/kg), and vitamin E (13,228 IU/kg). Average daily gain was monitored via weekly weights and feed allotment adjusted accordingly to meet gain targets. The targeted ADG for control intake (CON) was 0.45 kg/d for restricted intake (RES) was -0.23 kg/d. Actual ADG for control intake was 0.60 kg/d and -0.23 kg/d for restricted intake.

At breeding (d 0), heifers were assigned to one of four nutritional treatments in a 2 × 2 factorial arrangement: Control intake without OCM supplementation (CON - OCM), CON with OCM supplementation (CON + OCM), restricted intake without OCM supplementation (RES - OCM), or RES with OCM supplementation (RES + OCM). The OCM supplement consisted of rumen-protected choline (ReaShure, Balchem Inc., New Hampton, NY, 44.4 g/d) and methionine (Smartamine, Adisseo, China, 7.4 g/d) in a fine-ground corn carrier top-dressed daily, and weekly injections of folate (Spectrum Chemical Mfg. Corp., New Brunswick, NJ, 320 mg, 6 cc) and vitamin B12 (MWI Animal Health, Boise, ID, 20 mg, 4 cc). The -OCM heifers received the corn carrier and saline injections (one 6 cc and one 4 cc injection). Therefore, the experimental design had treatments arranged as a 2 × 2 factorial with two levels of feed intake (planes of nutrition) and two levels of strategic OCM supplementation. After pregnancy was diagnosed with ultrasound, the number of heifers per treatment was CON+OCM (n = 8), CON-OCM (n =7), RES+OCM (n = 8), and RES-OCM (n = 9).

Tissue Sampling

Heifers were slaughtered in a federally inspected facility using captive bolt stunning followed by exsanguination., and fetal tissues collected on d 63±2 of gestation. This day was chosen because it is a significant time point during organogenesis as critical to growth, health, and viability of offspring. Specifically, organogenesis is complete and peak primary myogenesis is occurring. The gravid uterus was removed first and taken for dissection of reproductive and fetal tissue.

Fetuses were removed from uterus and weighed, then underwent dissection which isolated all organs and select ones were individually weighed. Fetal liver and muscle tissues were

placed in RNAlater (Ambion by Life Technologies Carlsbad, CA). All tissues were stored at -80 C until needed for analyses on collaborating projects.

RNA Extraction and Sequencing

In order to extract RNA from fetal liver and muscle tissues, the RNeasy Plus Universal Mini Kit (Qiagen) was used for lysis and subsequent purification of high-quality total RNA. Tissues were removed from -80 C freezer and ≤ 50 mg of each tissue was lysed and homogenized. The remaining steps were followed per manufacturer's protocol. The quality of RNA was measured using a Quibit 4. Then the RNA was stored on dry ice and sent to Novogene where Eukaryotic mRNA-seq was performed on samples with data output of 6 Gb/20M PE150 read-pairs per sample. The library preparation kit used was New England BioLabs Ultra II mRNA Library Prep Kit for Illumina. Bioinformatics was analyzed using Qiagen CLC Genomics Workbench versions 9.5.3. Pathway analysis was determined using Qiagen IPA (Ingenuity Pathway Analysis) that has a knowledge base of over 7.8 million literature findings to compare results to a pre-computed library of over 90,000 curated gene expression datasets (Kramer et al., 2014). The Fisher's Exact Test was used to calculate a statistical significance of overlap of the dataset molecules with various sets of molecules that represent annotations such as canonical pathways and upstream regulators. Data are reported as a differential expression analysis for all group pairwise comparisons between main effects and treatment groups. Significance is declared at a P-value ≤ 0.05 and tendency 0.05 ≥ 0.1.

Results

Fetal Muscle Tissue

When analyzed for gain differences in fetal longissimus dorsi, tissue fetuses from CON heifers had 9 genes that were downregulated and 1 was upregulated (P = <0.001; Table 4A). The pathways that are associated with these genes include reactive oxygen species (ROS) production,

mTOR (mammalian target of rapamycin) signaling, LXR/RXR (liver X receptor/ retinoid X receptor) activation, and p70S6K (p70 ribosomal s6 kinase) signaling (Table 4B). Heifers not receiving OCM supplementation compared with supplemented heifers had fetuses with 4 downregulated genes in their longissimus dorsi muscles (Table 4C). When comparing fetuses from RES-OCM and RES+OCM treated heifers, 43 genes were downregulated in RES-OCM heifers (Table 4D). These genes are associated with pathways such as calcium signaling, oxytocin signaling, PAK (p-21 activated kinase) signaling, SNARE (soluble N-ethylmaleimide-sensitive factor activating protein receptor) signaling, apelin cardiomyocyte signaling, RHOA (Ras homolog family member A) signaling, and regulation of actin-based motility by Rho proteins (Table 4E). There were 4 downregulated genes and 1 upregulated gene in fetal muscle tissue from dams fed CON+OCM when compared with RES+OCM fed heifers (Table 4F). Furthermore, there were 14 downregulated and 6 upregulated genes in fetal muscle from heifers fed CON-OCM compared with those from heifers fed RES+OCM (Table 4H). Only 1 gene was upregulated in fetuses from RES-OCM compared to CON+OCM fed heifers (Table 4J). Pathways associated with the genes in Table 4J consisted of mTOR signaling, EIF2 (eukaryotic initiation factor 2) signaling, and regulation of eIF4 (eukaryotic translation initiation factor 4) and p70S6K signaling (Tables 4G, 4I and 4K). No differences were seen in the comparison between CON-OCM and CON+OCM. When transcript abundance in fetal muscle from heifers fed CON-OCM and RES-OCM were compared, 34 genes were downregulated in CON-OCM (Table 4L). The pathways involved with these genes were GP6 (glycoprotein VI platelet) signaling, hepatic stellate cell activation, osteoarthritis pathway, wound healing signaling pathway, role of osteoclases in Rheumatoid arthritis signaling, pulmonary fibrosis idiopathic

signaling, pathogen induced cytokine storm signaling, neutrophil extracellular trap signaling, and more (Table 4M).

Fetal Liver Tissue

All genes described have a significant *P*-value < 0.01. When looking at main effects, 5 genes were downregulated in CON vs. RES gain levels (Table 4N). Additionally, 2 genes were upregulated, and 3 genes were downregulated in +OCM compared to -OCM fetal liver tissue (Table 4P). Pathways associated with these genes included mTOR signaling, regulation of eIF4 and p70S6K signaling, tight junction signaling, macrophages and monocytes, and more (Table 4O and 4Q). One gene was upregulated in RES+OCM vs. RES-OCM and 1 gene was downregulated (Table 4R). In CON-OCM compared to RES-OCM, 3 genes were downregulated and were associated with tight junction signaling, ILK (Integrin linked kinase) signaling, calcium signaling, and more pathways (Table 4T). Similarly, 3 genes were downregulated in CON+OCM vs. RES-OCM with 1 gene upregulated (Table 4U). Two genes were downregulated in CON-OCM vs. RES+OCM and CON+OCM vs. RES+OCM (Tables 4W and 4X). Pathways associated with these genes are listed in Table 4Y. Finally, one gene was upregulated in CON+OCM vs. CON-OCM (Table 4Z).

Table 4.1 Differentially expressed genes due to heifer rate of gain[1] in fetal longissimus dorsi muscle tissue: CON vs. RES

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
CCDC80	-1.29	0.05	< 0.001	protein_coding
CD44	-1.31	0.09	< 0.001	protein_coding
CLU	-1.39	0.05	< 0.001	protein_coding
ENSBTAG00000006383	-173.19	< 0.01	< 0.001	protein_coding
ENSBTAG00000031205	-10.54	0.02	< 0.001	protein_coding
ENSBTAG00000045568	-10.09	0.05	< 0.001	protein_coding
ENSBTAG00000053974	6.92	0.02	< 0.001	protein_coding
EPYC	-9.56	0.05	< 0.001	protein_coding
PAMR1	-1.46	0.03	< 0.001	protein_coding
SCARA5	-1.39	0.02	< 0.001	protein_coding

[1]Heifer gain levels: CON = 0.60 kg/d; RES = -0.23 kg/d
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of gain levels.
[4]A gene or transcript classification.

Table 4.2 Canonical pathways in fetal longissimus dorsi muscle tissue due to gain[1]: CON vs. RES

Pathway	Overlapping genes	*P*-value[2]	-log10 of *p*-value
LXR/RXR Activation	1	0.03	1.59
FXR/RXR Activation	1	0.03	1.58
Atherosclerosis Signaling	1	0.03	1.56
Role of PKR in Interferon Induction and Antiviral Response	1	0.03	1.55
DHCR24 Signaling Pathway	1	0.03	1.54
Regulation of eIF4 and p70S6K Signaling	1	0.04	1.43
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1	0.04	1.40
Coronavirus Pathogenesis Pathway	1	0.04	1.37
Clathrin-mediated Endocytosis Signaling	1	0.04	1.37
mTOR Signaling	1	0.04	1.35

[1]Gain levels: CON = 0.60 kg/d; RES = -0.23 kg/d
[2]Main effect of gain levels

Table 4.3 Differentially expressed genes in fetal longissimus dorsi muscle tissue due to treatment[1]: -OCM vs. +OCM

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
DMP1	-5.72	0.05	< 0.001	protein_coding
ENSBTAG00000027962	-101.78	< 0.01	< 0.001	protein_coding
ENSBTAG00000031205	-13.39	< 0.01	< 0.001	protein_coding
ENSBTAG00000045568	-10.15	0.07	< 0.001	protein_coding

[1]Treatment levels: -OCM = saline injections and no OCM; +OCM = 44.4 g/d choline, 7.4 g/d methionine, 320 mg vitamin B_{12}, and folate
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation levels.
[4]A gene or transcript classification.

Table 4.4 Differentially expressed genes in fetal longissimus dorsi muscle tissue: RES-OCM vs. RES+OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ALDOA	-1.36	0.08	< 0.01	protein_coding
ATP5F1D	-1.47	0.08	< 0.01	protein_coding
CDKN1A	-1.32	0.05	< 0.001	protein_coding
CKB	-1.59	0.03	< 0.001	protein_coding
CRYAB	-1.37	0.09	< 0.01	protein_coding
CSDC2	-1.34	0.09	< 0.01	protein_coding
DRAP1	-1.49	0.08	< 0.01	protein_coding
EIF3F	-1.33	0.09	< 0.01	protein_coding
ENO3	-1.39	0.09	< 0.01	protein_coding
ENSBTAG00000048931	-1.53	0.03	< 0.001	protein_coding
ENSBTAG00000052720	-1.55	0.08	< 0.01	protein_coding
HRC	-1.43	0.04	< 0.001	protein_coding
HSPB1	-1.44	0.08	< 0.01	protein_coding
HSPB2	-1.55	0.04	< 0.001	protein_coding
ISYNA1	-1.41	0.08	< 0.01	protein_coding
LSP1	-1.69	0.02	< 0.001	protein_coding
MDH1	-1.31	0.05	< 0.001	protein_coding
MYL2	-1.55	0.04	< 0.001	protein_coding
MYL3	-1.83	0.04	< 0.001	protein_coding
MYL4	-1.64	0.05	< 0.001	protein_coding
MYL6B	-1.50	0.08	< 0.01	protein_coding
MYL7	-2.09	0.03	< 0.001	protein_coding
MYL9	-1.47	0.08	< 0.01	protein_coding
MYLPF	-1.46	0.05	< 0.001	protein_coding
PPDPF	-1.32	0.09	< 0.01	protein_coding
PRR5	-1.50	0.08	< 0.01	protein_coding
PRXL2B	-1.57	0.08	< 0.01	protein_coding
RALY	-1.44	0.08	< 0.01	protein_coding
RPS28	-1.95	0.05	< 0.001	protein_coding
RPS29_2	-1.98	0.04	< 0.001	protein_coding
SLN	-1.41	0.08	< 0.01	protein_coding
SMTN	-1.29	0.09	< 0.01	protein_coding
TNNC1	-1.63	0.03	< 0.001	protein_coding
TNNC2	-1.74	0.05	< 0.001	protein_coding
TNNI1	-1.53	0.04	< 0.001	protein_coding
TNNI2	-1.81	0.04	< 0.001	protein_coding
TNNT1	-1.69	0.03	< 0.001	protein_coding
TNNT2	-1.69	0.02	< 0.001	protein_coding
TNNT3	-1.57	0.08	< 0.01	protein_coding
TPM2	-1.47	0.08	< 0.01	protein_coding
TUBA4A	-1.53	0.05	< 0.001	protein_coding
TUBB2B	-1.46	0.09	< 0.01	protein_coding

[1]RES-OCM = Restricted (-0.23 kg/d) without supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.5 Canonical pathways in fetal longissimus dorsi muscle tissue: RES-OCM vs. RES+OCM[1]

Pathway	Overlapping genes	*P*-value[2]	-log10 of *p*-value
Dilated Cardiomyopathy Signaling Pathway	9	< 0.01	15.85
Calcium Signaling	9	< 0.01	14.33
Protein Kinase A Signaling	5	< 0.01	5.24
Oxytocin Signaling Pathway	4	< 0.01	4.49
Apelin Cardiomyocyte Signaling Pathway	3	< 0.01	4.42
Regulation of Actin-based Motility by Rho	3	< 0.01	4.22
PAK Signaling	3	< 0.01	4.20
RHOA Signaling	3	< 0.01	4.13
Gα12/13 Signaling	3	< 0.01	4.04
SNARE Signaling Pathway	3	< 0.01	4.01

[1]RES-OCM = Restricted (-0.23 kg/d) without supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]Main effect of one-carbon supplementation and gain levels.

Table 4.6 Differentially expressed genes in fetal longissimus dorsi muscle tissue: CON+OCM vs. RES+OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ENSBTAG00000006383	-281.53	≤ 0.001	≤ 0.001	protein_coding
ENSBTAG00000031205	-19.32	0.01	≤ 0.001	protein_coding
ENSBTAG00000045568	-19.90	0.05	≤ 0.001	protein_coding
HOXD11	49.13	0.02	≤ 0.001	protein_coding
IFI27	-13.02	0.03	≤ 0.001	protein_coding

[1]CON+OCM = Control (0.45 kg/d) with one-carbon metabolite supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.7 Canonical pathways in fetal longissimus dorsi muscle tissue: CON+OCM vs. RES+OCM[1]

Pathway	Overlapping Genes	*P*-value[2]	-log10 of *p*-value
Regulation of eIF4 and p70S6K Signaling	1	0.02	1.64
Coronavirus Pathogenesis Pathway	1	0.03	1.59
mTOR Signaling	1	0.03	1.57
EIF2 Signaling	1	0.03	1.54

[1]CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]Main effect of one-carbon supplementation levels.

Table 4.8 Differentially expressed genes fetal longissimus dorsi muscle tissue: CON-OCM vs RES+OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ANXA2	-1.41	0.09	≤ 0.001	protein_coding
CLU	-1.55	0.08	≤ 0.001	protein_coding
DNAAF6	-6.89	0.09	≤ 0.001	protein_coding
ENSBTAG00000006383	-155.37	< 0.01	≤ 0.001	protein_coding
ENSBTAG00000031205	-24.62	< 0.01	≤ 0.001	protein_coding
ENSBTAG00000033515	1.57	0.04	≤ 0.001	protein_coding
ENSBTAG00000045568	-20.77	0.03	≤ 0.001	protein_coding
ENSBTAG00000052033	16.63	0.04	≤ 0.001	protein_coding
ENSBTAG00000053626	3.72	0.04	≤ 0.001	protein_coding
ENSBTAG00000053974	15.77	< 0.01	≤ 0.001	protein_coding
ENSBTAG00000054051	16.27	0.04	≤ 0.001	protein_coding
FSTL3	-1.92	< 0.01	≤ 0.001	protein_coding
H2BC8	4.28	0.04	≤ 0.001	protein_coding
IBSP	-16.12	0.08	≤ 0.001	protein_coding
IFI27	-8.95	0.09	≤ 0.001	protein_coding
MFAP5	-1.60	0.04	≤ 0.001	protein_coding
MMP9	-23.18	0.08	≤ 0.001	protein_coding
NUDT18	-2.79	0.08	≤ 0.001	protein_coding
PADI2	-2.27	0.09	≤ 0.001	protein_coding
SCARA5	-1.59	< 0.01	≤ 0.001	protein_coding

[1]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.9 Canonical pathways in fetal longissimus dorsi muscle tissue: CON-OCM vs. RES+OCM[1]

Pathway	Overlapping Genes	*P*-value[2]	-log10 of *p*-value
Role of PKR in Interferon Induction and Antiviral Response	1	0.02	1.64
Regulation of eIF4 and p70S6K Signaling	1	0.03	1.52
Coronavirus Pathogenesis Pathway	1	0.03	1.47
mTOR Signaling	1	0.04	1.45
Activin Inhibin Signaling Pathway	1	0.04	1.44
EIF2 Signaling	1	0.04	1.42

[1]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]Main effect of one-carbon supplementation and gain levels.

Table 4.10 Differentially expressed genes in fetal longissimus dorsi muscle tissue: RES-OCM vs. CON+OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ENSBTAG00000006383	211.76	≤ 0.001	≤ 0.001	protein_coding

[1]CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation levels.
[4]A gene or transcript classification.

Table 4.11 Canonical pathways in fetal longissimus dorsi muscle tissue: RES-OCM vs. CON+OCM[1]

Pathway	Overlapping Genes	*P*-value[2]	-log10 of *p*-value
Regulation of eIF4 and p70S6K Signaling	1	< 0.01	2.12
Coronavirus Pathogenesis Pathway	1	< 0.01	2.06
mTOR Signaling	1	< 0.01	2.04
EIF2 Signaling	1	< 0.01	2.02

[1]CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation.
[2]Main effect of one-carbon supplementation levels.

Table 4.12 Differentially expressed genes in fetal longissimus dorsi muscle tissue: CON-OCM vs. RES-OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ACAN	-2.45	0.02	≤ 0.001	protein_coding
ATP1A3	-102.95	0.05	≤ 0.001	protein_coding
CCDC80	-1.46	0.008	≤ 0.001	protein_coding
CHADL	-4.46	0.09	≤ 0.001	protein_coding
CILP2	-1.91	0.05	≤ 0.001	protein_coding
CLU	-1.49	0.08	≤ 0.001	protein_coding
CNMD	-16.96	0.01	≤ 0.001	protein_coding
CNTN2	-49.34	0.003	≤ 0.001	protein_coding
COL2A1	-2.13	0.007	≤ 0.001	protein_coding
COL8A2	-1.88	0.03	≤ 0.001	protein_coding
COL9A1	-3.99	0.04	≤ 0.001	protein_coding
COL9A2	-3.36	0.02	≤ 0.001	lncRNA
COL9A3	-2.87	0.09	≤ 0.001	protein_coding
COMP	-3.28	0.08	≤ 0.001	protein_coding
ELAVL4	-80.81	0.09	≤ 0.001	protein_coding
ENSBTAG00000006383	-116.86	0.003	≤ 0.001	protein_coding
EPYC	-26.42	0.02	≤ 0.001	protein_coding

Table 4.12 Differentially expressed genes in fetal longissimus dorsi muscle tissue: CON-OCM vs. RES-OCM[1] (Continued)

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
FGFR3	-2.82	0.09	≤ 0.001	protein_coding
ITGA11	-1.71	0.09	≤ 0.001	protein_coding
MATN1	-27.44	0.01	≤ 0.001	protein_coding
MATN4	-6.75	0.02	≤ 0.001	protein_coding
MPPED2	-1.68	0.04	≤ 0.001	protein_coding
NCAN	-10.77	0.01	≤ 0.001	protein_coding
NEFM	-9.33	0.006	≤ 0.001	protein_coding
P4HA3	-1.44	0.02	≤ 0.001	protein_coding
PAMR1	-1.63	0.02	≤ 0.001	protein_coding
PCSK1N	-7.72	0.04	≤ 0.001	protein_coding
PRPH	-43.08	0.003	≤ 0.001	protein_coding
RTN1	-15.49	0.01	≤ 0.001	protein_coding
SCARA5	-1.59	0.003	≤ 0.001	protein_coding
STMN3	-13.96	0.04	≤ 0.001	protein_coding
SYT5	-7.06	0.08	≤ 0.001	protein_coding
THBS2	-1.56	0.05	≤ 0.001	protein_coding
TUBB4A	-48.42	0.01	≤ 0.001	protein_coding

[1]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d).
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.13 Canonical pathways in fetal longissimus dorsi muscle tissues: CON-OCM vs. RES-OCM[1]

Pathway	Overlapping Genes	*P*-value[2]	-log10 of *p*-value
GP6 Signaling Pathway	3	< 0.01	3.73
Hepatic Fibrosis / Hepatic Stellate Cell Activation	3	< 0.01	3.19
Osteoarthritis Pathway	3	< 0.01	2.94
Wound Healing Signaling Pathway	3	< 0.01	2.86
Role Of Osteoclasts In Rheumatoid Arthritis Signaling Pathway	3	< 0.01	2.61
Pulmonary Fibrosis Idiopathic Signaling Pathway	3	< 0.01	2.54
Pathogen Induced Cytokine Storm Signaling Pathway	3	< 0.01	2.38
Amyotrophic Lateral Sclerosis Signaling	2	< 0.01	2.33
Neutrophil Extracellular Trap Signaling Pathway	3	< 0.01	2.30
Semaphorin Neuronal Repulsive Signaling Pathway	2	< 0.01	2.11

[1]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation.
[2]Main effect of one-carbon supplementation and gain levels.

Table 4.14 Differentially expressed genes due to gain[1] in fetal liver tissue: CON vs. RES

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ENSBTAG00000006383	-35.73	< 0.01	≤ 0.001	protein_coding
ENSBTAG00000031205	-22.37	< 0.01	≤ 0.001	protein_coding
ENSBTAG00000054580	-163.09	< 0.01	≤ 0.001	protein_coding
MYH3	-11.00	0.06	≤ 0.001	protein_coding
MYH8	-24.01	0.03	≤ 0.001	protein_coding

[1]Gain levels: CON = 0.60 kg/d; RES = -0.23 kg/d
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of gain levels.
[4]A gene or transcript classification.

Table 4.15 Canonical pathways in fetal liver tissue due to gain[1]: CON vs. RES

Pathway	Overlapping genes	*P*-value[2]	-log10 of *p*-value
Regulation of eIF4 and p70S6K Signaling	1	0.02	1.81
Coronavirus Pathogenesis Pathway	1	0.02	1.77
mTOR Signaling	1	0.02	1.75
SNARE Signaling Pathway	1	0.01	1.95
Dilated Cardiomyopathy Signaling Pathway	1	0.01	1.91
Cellular Effects of Sildenafil (Viagra)	1	0.01	1.91
Tight Junction Signaling	1	0.01	1.83
Hepatic Fibrosis / Hepatic Stellate Cell Activation	1	0.02	1.79
ILK Signaling	1	0.02	1.77
Agranulocyte Adhesion and Diapedesis	1	0.02	1.76

[1]Gain levels: CON = 0.60 kg/d; RES = -0.23 kg/d.
[2]Main effect of gain levels.

Table 4.16 Differentially expressed genes in fetal liver tissue due to treatment[1]: +OCM vs. -OCM

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ACTA1	-49.95	< 0.01	≤ 0.001	protein_coding
ENSBTAG00000027962	49.61	< 0.01	≤ 0.001	protein_coding
ENSBTAG00000031205	17.49	< 0.01	≤ 0.001	protein_coding
ENSBTAG00000054580	-29.16	< 0.01	≤ 0.001	protein_coding
MYH3	-10.55	0.06	≤ 0.001	protein_coding

[1]Treatment levels: -OCM = saline injections; +OCM = 44.4 g/d choline, 7.4 g/d methionine, 320 mg/week vitamin B_{12}, and 20 mg/week folate
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation levels.
[4]A gene or transcript classification.

Table 4.17 Canonical pathways in fetal liver tissue due to treatment[1]: +OCM vs. -OCM

Pathway	Overlapping genes	*P*-value[2]	-log10 of *p*-value
Mechanisms of Viral Exit from Host Cells	1	≤ 0.01	2.76
MSP-RON Signaling Pathway	1	≤ 0.01	2.62
Remodeling of Epithelial Adherens Junctions	1	≤ 0.01	2.55
Agrin Interactions at Neuromuscular Junction	1	≤ 0.01	2.54
Caveolar-mediated Endocytosis Signaling	1	≤ 0.01	2.51
Crosstalk between Dendritic Cells and Natural Killer Cells	1	≤ 0.01	2.42
ABRA Signaling Pathway	1	≤ 0.01	2.41
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	1	≤ 0.01	2.40
Death Receptor Signaling	1	≤ 0.01	2.39
VEGF Signaling	1	≤ 0.01	2.38

[1]Treatment levels: -OCM = saline injections; +OCM = 44.4 g/d choline, 7.4 g/d methionine, 320 mg/week vitamin B_{12}, and 20 mg/week folate
[2]Main effect of one-carbon supplementation levels.

Table 4.18 Differentially expressed genes in fetal liver tissue: RES+OCM vs. RES-OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ENSBTAG00000031205	29.18	0.01	≤ 0.001	protein_coding
ENSBTAG00000054580	-29.80	0.06	≤ 0.001	protein_coding

[1]CON-OCM = Control (0.45 kg/d) without one-carbon metabolite supplementation; CON+OCM = Control (0.45 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.19 Differentially expressed genes in fetal liver tissue: CON-OCM vs. RES-OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ACTC1	-69.43	0.03	≤ 0.001	protein_coding
ENSBTAG00000054580	-295.75	0.03	≤ 0.001	protein_coding
MYH8	-429.93	0.03	≤ 0.001	protein_coding

[1]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.20 Canonical pathways in fetal liver tissue: CON-OCM vs. RES-OCM[1]

Pathway	Overlapping Genes	*P*-value[2]	-log10 of *p*-value
Dilated Cardiomyopathy Signaling Pathway	2	< 0.01	4.40
Cellular Effects of Sildenafil (Viagra)	2	< 0.01	4.40
Tight Junction Signaling	2	< 0.01	4.24
ILK Signaling	2	< 0.01	4.14
Agranulocyte Adhesion and Diapedesis	2	< 0.01	4.10
RHOGDI Signaling	2	< 0.01	4.06
Calcium Signaling	2	< 0.01	4.06
Actin Cytoskeleton Signaling	2	< 0.01	3.97
Mechanisms of Viral Exit from Host Cells	1	< 0.01	2.46
MSP-RON Signaling Pathway	1	< 0.01	2.31

[1]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation.
[2]Main effect of one-carbon supplementation and gain levels.

Table 4.21 Differentially expressed genes in fetal liver tissue: CON+OCM vs. RES-OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ENSBTAG00000006383	-166.55	0.001	≤ 0.001	protein_coding
ENSBTAG00000027962	42.23	0.06	≤ 0.001	protein_coding
ENSBTAG00000054580	-302.37	0.05	≤ 0.001	protein_coding
MYH3	-49.51	0.005	≤ 0.001	protein_coding

[1]CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.22 Canonical pathways in fetal liver tissue: CON+OCM vs. RES-OCM[1]

Pathway	Overlapping Genes	*P*-value[2]	-log10 of *p*-value
SNARE Signaling Pathway	1	0.01	1.94
Dilated Cardiomyopathy Signaling Pathway	1	0.01	1.90
Cellular Effects of Sildenafil (Viagra)	1	0.01	1.90
Tight Junction Signaling	1	0.02	1.82
Regulation of eIF4 and p70S6K Signaling	1	0.02	1.81
Hepatic Fibrosis / Hepatic Stellate Cell Activation	1	0.02	1.78
ILK Signaling	1	0.02	1.77
Coronavirus Pathogenesis Pathway	1	0.02	1.76
Agranulocyte Adhesion and Diapedesis	1	0.02	1.75
mTOR Signaling	1	0.02	1.74

[1]CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation.
[2]Main effect of one-carbon supplementation and gain levels.

Table 4.23 Differentially expressed genes in fetal liver tissue: CON-OCM vs. RES+OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ENSBTAG00000027962	-87.95	0.09	≤ 0.001	protein_coding
ENSBTAG00000031205	-37.37	0.01	≤ 0.001	protein_coding

[1]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.24 Differentially expressed genes in fetal liver tissue: CON+OCM vs. RES+OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ENSBTAG00000006383	-126.68	0.004	≤ 0.001	protein_coding
ENSBTAG00000031205	-59.11	0.001	≤ 0.001	protein_coding

[1]CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Table 4.25 Canonical pathways in fetal liver tissue: CON+OCM vs. RES+OCM[1]

Pathway	Overlapping Genes	*P*-value[2]	-log10 of *p*-value
Regulation of eIF4 and p70S6K Signaling	1	< 0.01	2.11
Coronavirus Pathogenesis Pathway	1	< 0.01	2.06
mTOR Signaling	1	< 0.01	2.04
EIF2 Signaling	1	< 0.01	2.02

[1]CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[2]Main effect of one-carbon supplementation and gain levels..

Table 4.26 Differentially expressed genes in fetal liver tissue: CON+OCM vs. CON-OCM[1]

Name	Fold Change	FDR[2] *p*-value	*P*-value[3]	Biotype[4]
ENSBTAG00000027962	134.97	0.048	≤ 0.001	protein_coding

[1]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation.
[2]False Discovery Rate (FDR) is the rate that features called significant are truly null. FDR = expected (# false predictions/ # total predictions).
[3]Main effect of one-carbon supplementation and gain levels.
[4]A gene or transcript classification.

Discussion and Conclusion

When investigating differentially expressed genes using Qiagen IPA, many pathways were associated with the genes listed above. In heifers receiving the CON rate of gain versus RES, genes were downregulated that are associated with ROS production. Since ROS production is directly related to the animal's immune response it is important to maintain normal levels of ROS as to not over accumulate and surpass antioxidant levels. Free radicals are highly reactive and can interact with more stable nonradicals (macromolecules, lipids, proteins, nucleic acids, and carbohydrates) causing damage resulting in oxidative stress. Thus, heifers receiving CON intake were more capable of reducing their ROS production versus RES heifers. This implies that a low maternal gain resulting from low nutrient intake has the capability of resulting in oxidative stress due to overproduction of ROS. Another pathway mentioned is mTOR signaling. If oxidative stress occurs, it will begin transcriptional alterations of cellular proteins that impairs the mTOR network signaling. ROS will inhibit mTORC1 by direct phosphorylation of TSC2 (Tuverous sclerosis complex 2). Genetic data from Drosophila show that TSC1 and TSC2 negatively regulate cell growth and cell size (Potter et al., 2001). Furthermore, oxidants are known to reduce the phosphorylation of mTOR substrates which will hinder mRNA translation (Zhang et al., 2009). Therefore, gene expression affected by OCM supplementation can assist in mTOR signaling to reduce the number of negative effects on cell growth and size due to TSC1 and TSC2 activation. Available ROS can trigger cellular pathways involving items such as death and survival signals. Each organ has its own critical threshold towards ROS that is regulated by an intricate signaling system (Chatterjee et al., 2016). Therefore, the pathways affected by gain can have downstream effects on cellular death and oxidative stress levels. The serine/threonine kinase, p70S6K, participates in the control of protein synthesis by phosphorylating the 40S

ribosomal protein S6, which is involved in the translation of certain mRNAs (Dennis et al., 2012). Fetuses from CON heifers also showed a downregulation in genes associated with p70s6K signaling which is known for its regulatory role in protein synthesis and cell growth. However, enhanced expression or activation of p70s6k has been associated with cancer types and suggests that it serves as a biomarker for disease monitoring (Artemenko et al., 2022). Thus, fetuses from CON heifers experienced a downregulation in this disease biomarker compared to fetuses from heifers receiving RES. When looking at the heifers not receiving OCM supplementation, 4 genes were downregulated compared to +OCM heifers. One of these genes is dentin matrix protein 1 (DMP1) which is critical for mineralized tissue formation. It achieves this by initiating nucleation and modulation of mineral phase morphology (Martinez et al., 2009). The normal expression of this gene is crucial for normal development of the fetus. Another pathway association is the eukaryotic translation initiation factor 4 and p70S6K signaling. These two play critical roles in translational regulation and regulate cell growth by inducing protein synthesis components. The recruitment of mRNAs to ribosomes to initiate translation is mediated by the initiation factors of eIF4. Other pathways listed in genes affected are ILK and calcium signaling. ILK is a crucial signaling protein that interacts with cytoplasmic domains of integrin chains and is an adaptor for signal transduction from extracellular sites to intracellular in the liver (Martucci et al., 2021). Calcium signaling being affected can cause dysregulation and can lead to acute and chronic liver diseases. This is because calcium is a versatile secondary messenger that controls many hepatic functions. For example, lipid and carbohydrate metabolism are regulated by calcium (Oliva-Vilarnau et al., 2018).

In conclusion, multiple genes in both fetal muscle and liver tissue were differentially expressed due to either rate of gain or OCM supplementation. These genes were associated with

many critical pathways in cellular processes. For instance, signaling of certain proteins that have an influence on cellular growth. Therefore, this differential expression could lead to reduced fetal growth and result in less efficient offspring. Furthermore, the amount of ROS production could result in fetal or maternal oxidative stress causing lipid and protein damage to the body. The one-carbon metabolites can aid in this because of the impact they have on epigenetic regulation, protein synthesis via mTOR, energy metabolism, and antioxidant synthesis (Coleman et al., 2020). The supplementation of these metabolites during a time of undernutrition can help mitigate these differentially expressed genes.

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CONCLUSIONS

In this dissertation, beef heifers were subjected to two levels of rate of gain and supplemented one-carbon metabolites or not during early gestation. The analysis of data consisted of heifer carcass measurements, heifer ADG, heifer organ weights, fetal organ weights, fetal allometric growth, heifer serum oxidative stress levels, and fetal tissue gene expression. The overall hypothesis was that heifers receiving a restricted level of gain would be negatively affected and OCM supplementation would be able to rescue or reduce the negative effects. The goal is to investigate developmental programming, fetal development, and their association with the one-carbon metabolite pathway in beef cattle.

In chapter 2, the data suggests that, as expected, heifers receiving RES gain did have lower body and organ weights. Their fetuses were also affected by this level of gain and critical organs such as heart, brain, and skeletal muscle. After weights were taken it was seen that OCM supplementation helped increase the weights of those organs. This not only displayed how providing those key nutrients helped with fetal development, but also how the nutrient partitioning was prioritized to critical organs for sustaining life.

In chapter 3, maternal serum was analyzed for total antioxidant capacity the day of breeding, halfway through the trial, and day 63 of gestation. In Exp. 1, the data suggests that there was an interaction between day and heifers receiving OCM supplementation as well as heifers on CON gain level. The heifers receiving OCM supplementation and adequate intake had an increased total antioxidant capacity throughout early gestation. This implies that in ruminant systems where pregnant cows may undergo environmental stressors, if key nutrients are supplied, they would have a higher capacity to battle reactive oxygen species. This higher capacity would help reduce the risk of oxidative stress.

In chapter 4, fetal liver and muscle tissue was analyzed for gene expression using RNA. The data presented shows many genes affected by level of gain and OCM supplementation. For example, fetuses from CON heifers had a downregulation of genes related to reactive oxygen species production and p70S6K signaling. The downregulation of ROS production would assist in cell health but overall reducing the chance of oxidative stress. Additionally, p70S6K has been identified as a disease biomarker. Thus, fetuses from CON heifers were not experiencing an enhancement in that biomarker. Other pathways were upregulated in fetuses receiving OCM supplementation associated with calcium signaling and mTOR signaling. Therefore, level of gain and OCM supplementation did have an effect on gene expression and supplying those key nutrients were able to assist in mitigation of negative effects that can be seen during nutritional insults.

The data presented herein provides a strong starting point to further investigate implementation of strategic supplementation of OCM into ruminant production systems and why it is important for heifers in early gestation. Future directions should include investigation of the re-alimentation effect when heifers return to a normal diet and are no longer suffering from a restricted intake. Additionally, oxidative stress levels should be evaluated in more depth. Specifically, measures of protein, DNA, and lipid damage would give a more detailed insight into the stress levels heifers face during gestation. Finally, RNA analysis further into gestation would be a beneficial look into how the genes are expressed throughout the second trimester as well.

APPENDIX. CHAPTER 2 TABLES

Table A1. The interactive mean effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 63 of gestation (Exp. 1) on gain, carcass measurements, and selected organ weights in beef heifers.

	Treatments[2]					*P*-value[4]		
Item	**CON-OCM**	**CON+OCM**	**RES-OCM**	**RES+OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain × OCM**
Heifer Weights, kg								
d 0	405	404	404	384	15.13	0.45	0.45	0.49
d 63	439	444	386	373	14.81	<0.01	0.77	0.53
ADG, d 0 to 63	0.54	0.63	-0.29	-0.17	0.06	<0.01	0.08	0.80
Live Slaughter Wt., kg	427	432	375	365	8.32	<0.01	0.86	0.57
Hot Carcass Wt., kg	240	248	214	207	4.38	<0.01	0.89	0.25
Ribeye Area (in.^{2})	10.03	10.84	9.79	9.74	0.16	0.03	0.23	0.17
12th Rib Fat Depth (in.)	0.16	0.17	0.18	0.14	0.01	0.71	0.51	0.38
Final Yield Grade	1.96	1.74	1.83	1.89	0.08	0.97	0.63	0.43
Liver, g	4,670	4,066	3,065	3,077	350.48	<0.01	0.36	0.34
g/kg Hot Carcass Wt.	19.49	16.77	14.32	14.85	1.32	0.01	0.40	0.22
Pancreas, g	259	324	235	233	18.58	<0.01	0.07	0.05
g/kg Hot Carcass Wt.	1.07	1.32	1.09	1.13	0.08	0.27	0.06	0.17
Gravid Uterus, g	848	811	842	760	54.31	0.56	0.24	0.65
g/kg Hot Carcass Wt.	3.57	3.28	3.95	3.67	0.26	0.12	0.24	0.99

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.

Table A2. The interactive mean effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 161 of gestation (Exp. 2) on gain, carcass measurements, and selected organ weights in beef heifers.

	Treatments[2]					*P*-value[4]		
Item	**CON-OCM**	**CON+OCM**	**RES-OCM**	**RES+OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain × OCM**
Heifer Weights, kg								
d 0	358	349	345	351	8.91	0.51	0.82	0.35
d 63	404	388	334	342	10.42	<0.01	0.69	0.23
ADG, d 0 to 63	0.72	0.62	-0.18	-0.14	0.05	<0.01	0.55	0.25
Live Slaughter Wt., kg	456	455	399	411	15.44	<0.01	0.73	0.64
Hot Carcass Wt., kg	244	248	219	215	9.02	<0.01	0.98	0.59
Ribeye Area (in.^{2})	9.90	10.66	9.10	9.72	0.48	0.08	0.16	0.87
12th Rib Fat Depth (in.)	0.14	0.16	0.13	0.10	0.16	0.03	0.92	0.09
Final Yield Grade	2.04	1.75	2.00	1.64	0.12	0.55	0.01	0.78
Liver, g	4,135	4,173	3,462	3,586	154.25	<0.01	0.58	0.76
g/kg Hot Carcass Wt.	17.07	16.79	15.79	16.63	0.49	0.13	0.54	0.23
Pancreas, g	296	336	296	287	24.38	0.29	0.52	0.28
g/kg Hot Carcass Wt.	1.22	1.34	1.36	1.34	0.09	0.46	0.57	0.42
Mammary, g	3,652	4,166	3,037	3,232	282.65	<0.01	0.19	0.55
g/kg Hot Carcass Wt.	15.11	16.71	13.85	14.85	0.90	0.08	0.14	0.73
Hypothalamus, g	3.32	3.97	2.83	3.09	0.64	0.27	0.46	0.74
mg/kg Hot Carcass Wt.	0.013	0.016	0.013	0.014	0.003	0.68	0.41	0.85
Pituitary, g	2.11	2.24	2.16	2.53	0.29	0.54	0.38	0.66
mg/kg Hot Carcass Wt.	0.008	0.009	0.009	0.012	0.001	0.13	0.35	0.46
Gravid Uterus, g	13,522	13,506	13,982	14,030	749.40	0.48	0.98	0.96
g/kg Hot Carcass Wt.	55.60	54.41	63.56	65.85	3.13	<0.01	0.85	0.55
CL Ovary, g	7.91	6.93	6.08	7.72	0.92	0.54	0.70	0.13
mg/kg Hot Carcass Wt.	0.033	0.027	0.028	0.035	0.003	0.72	0.74	0.08

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.

Table A3. The interactive mean effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 63 of gestation on fetal organ weight and allometric organ weight.

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON-OCM**	**CON+OCM**	**RES-OCM**	**RES+OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Heart, g	0.21	0.22	0.20	0.19	0.007	0.11	0.69	0.42
g/g Brain Wt	0.21	0.25	0.23	0.21	0.007	0.71	0.55	0.04
mg/g Body Wt	10.67	11.66	10.73	10.85	0.25	0.47	0.28	0.40
Right Hemisphere, g	0.18	0.20	0.17	0.15	0.007	0.05	0.90	0.23
g/g Brain Wt	0.18	0.22	0.20	0.17	0.007	0.19	0.94	0.01
mg/g Body Wt	9.04	10.22	9.40	8.69	0.30	0.34	0.69	0.13
Left Hemisphere, g	0.21	0.18	0.16	0.19	0.009	0.34	0.81	0.08
g/g Brain Wt	0.21	0.20	0.18	0.22	0.007	0.81	0.42	0.07
mg/g Body Wt	10.52	9.45	8.67	11.02	0.37	0.84	0.36	0.02
Right Longissimus Dorsi, g	0.15	0.18	0.15	0.13	0.009	0.13	0.58	0.13
g/g Brain Wt	0.15	0.21	0.17	0.14	0.011	0.26	0.44	0.04
mg/g Body Wt	7.79	9.86	8.12	7.44	0.47	0.27	0.47	0.15
Intestine, g	0.21	0.31	0.24	0.17	0.022	0.22	0.75	0.08
g/g Brain Wt	0.21	0.34	0.28	0.20	0.028	0.55	0.68	0.07
mg/g Body Wt	10.51	15.42	12.83	10.01	1.08	0.48	0.64	0.09
Hindbrain, g	0.57	0.52	0.51	0.53	0.012	0.32	0.57	0.13
g/g Brain Wt	0.59	0.57	0.60	0.60	0.008	0.31	0.52	0.70
mg/g Body Wt	28.81	27.17	27.75	30.17	0.75	0.53	0.80	0.20
Right Hindlimb, g	0.71	0.74	0.66	0.62	0.028	0.18	0.93	0.55
g/g Brain Wt	0.73	0.84	0.78	0.71	0.030	0.56	0.71	0.16
mg/g Body Wt	35.47	39.24	35.79	35.62	1.51	0.61	0.57	0.54
Left Hindlimb Muscle, g	0.49	0.47	0.46	0.45	0.019	0.49	0.68	0.88
g/g Brain Wt	0.50	0.52	0.55	0.50	0.016	0.72	0.73	0.38
mg/g Body Wt	24.03	24.54	24.92	25.52	0.80	0.58	0.75	0.97
Left Longissimus Dorsi, g	0.14	0.14	0.14	0.14	0.007	0.99	0.95	0.77
g/g Brain Wt	0.14	0.15	0.16	0.16	0.008	0.36	0.74	0.66
mg/g Body Wt	7.04	7.33	7.75	7.94	0.38	0.42	0.76	0.95
Femur, g	0.050	0.048	0.044	0.042	0.002	0.23	0.65	0.92
g/g Brain Wt	0.051	0.052	0.052	0.048	0.001	0.71	0.69	0.58
mg/g Body Wt	2.58	2.42	2.38	2.41	0.09	0.63	0.74	0.65

Table A3. The interactive mean effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 63 of gestation on fetal organ weight and allometric organ weight. (Continued)

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON-OCM**	**CON+OCM**	**RES-OCM**	**RES+OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Kidneys, g	0.20	0.20	0.19	0.18	0.007	0.33	0.77	0.73
g/g Brain Wt	0.20	0.22	0.22	0.21	0.007	0.68	0.87	0.16
mg/g Body Wt	9.93	10.53	10.43	10.44	0.32	0.77	0.66	0.67
Pancreas, g	0.058	0.056	0.051	0.040	0.003	0.16	0.46	0.55
g/g Brain Wt	0.065	0.064	0.061	0.046	0.005	0.32	0.53	0.32
mg/g Body Wt	3.29	2.91	2.81	2.29	0.21	0.21	0.31	0.88
Stomach Complex, g	0.39	0.38	0.33	0.31	0.014	0.02	0.54	0.86
g/g Brain Wt	0.40	0.41	0.39	0.35	0.011	0.08	0.51	0.19
mg/g Body Wt	19.69	19.58	17.97	17.48	0.51	0.07	0.77	0.86
Lungs, g	0.64	0.61	0.59	0.56	0.019	0.20	0.39	0.98
g/g Brain Wt	0.66	0.68	0.70	0.63	0.016	0.98	0.44	0.22
mg/g Body Wt	32.43	31.68	32.44	31.73	0.79	0.98	0.67	0.99
Liver, g	0.95	0.91	0.88	0.87	0.019	0.23	0.59	0.75
g/g Brain Wt	0.97	1.01	1.06	1.00	0.021	0.42	0.81	0.27
mg/g Body Wt	48.08	47.50	48.16	49.67	0.78	0.50	0.78	0.53
Uterus, g	0.028	0.024	0.018	0.021	0.003	0.31	0.93	0.59
g/g Brain Wt	0.027	0.028	0.022	0.022	0.003	0.40	0.95	0.97
mg/g Body Wt	1.38	1.35	1.05	1.15	0.16	0.43	0.92	0.85
Right Ovary, g	0.041	0.021	0.014	0.012	0.005	0.11	0.33	0.41
g/g Brain Wt	0.039	0.024	0.016	0.013	0.005	0.11	0.39	0.58
mg/g Body Wt	0.88	1.09	0.72	0.65	0.10	0.17	0.73	0.52
Left Ovary, g	0.011	0.009	0.008	0.011	0.0	0.76	0.55	0.09
g/g Brain Wt	0.011	0.011	0.009	0.012	0.0	0.95	0.34	0.15
mg/g Body Wt	0.49	0.50	0.44	0.64	0.03	0.54	0.13	0.14
Mammary, g	0.032	0.038	0.033	0.036	0.002	0.96	0.45	0.74
g/g Brain Wt	0.033	0.040	0.400	0.042	0.003	0.47	0.44	0.70
mg/g Body Wt	1.56	1.87	1.86	2.05	0.14	0.41	0.39	0.85

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[3]Standard error of the mean. [4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.

Table A4. The interactive mean effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 161 of gestation on fetal organ weight and allometric organ weight.

	Treatments[2]					*P*-value[4]		
Organ Weight	CON-OCM	CON+OCM	RES-OCM	RES+OCM	SEM[3]	Gain	OCM	Gain×OCM
Mammary, g	16.43	13.61	14.10	15.84	0.49	0.95	0.57	0.02
g/g Brain Wt	0.29	0.25	0.26	0.29	0.008	0.61	0.45	0.03
mg/g Body Wt	4.25	3.69	3.69	3.99	0.12	0.62	0.60	0.11
Liver, g	126.50	121.51	130.92	129.11	2.92	0.33	0.58	0.79
g/g Brain Wt	2.26	2.21	2.48	2.36	0.04	0.03	0.32	0.68
mg/g Body Wt	32.63	32.56	33.97	32.42	0.40	0.49	0.36	0.39
Lungs, g	97.85	99.61	108.05	100.09	2.01	0.19	0.44	0.23
g/g Brain Wt	1.74	1.82	2.04	1.84	0.04	0.02	0.34	0.05
mg/g Body Wt	25.28	26.78	28.12	25.24	0.48	0.46	0.43	0.01
Spleen, g	11.40	10.04	12.08	10.29	0.38	0.53	0.04	0.77
g/g Brain Wt	0.20	0.18	0.23	0.19	0.006	0.20	0.01	0.37
mg/g Body Wt	2.92	2.69	3.13	2.59	0.07	0.69	0.01	0.28
Rumen Complex, g	54.83	53.28	54.80	58.86	1.31	0.31	0.64	0.30
g/g Brain Wt	0.98	0.97	1.04	1.07	0.02	0.04	0.18	0.60
mg/g Body Wt	14.13	14.34	14.26	14.83	0.26	0.58	0.48	0.75
Right Longissimus Dorsi, g	61.18	60.85	63.58	67.14	1.77	0.24	0.66	0.59
g/g Brain Wt	1.09	1.11	1.20	1.22	0.03	0.04	0.76	0.95
mg/g Body Wt	15.82	16.47	16.46	16.77	0.34	0.53	0.53	0.82
Right Hindlimb Muscle, g	83.38	68.92	75.77	86.73	2.99	0.38	0.76	0.03
g/g Brain Wt	1.49	1.25	1.44	1.53	0.05	0.25	0.43	0.09
mg/g Body Wt	21.60	18.49	19.55	21.53	0.65	0.69	0.65	0.04
Right Ovary, g	0.18	0.09	0.24	0.12	0.03	0.62	0.15	0.82
g/g Brain Wt	0.003	0.002	0.004	0.002	0.0006	0.54	0.14	0.74
mg/g Body Wt	0.05	0.03	0.06	0.03	0.008	0.69	0.15	0.78
Left Ovary, g	0.21	0.12	0.12	0.13	0.02	0.39	0.43	0.32
g/g Brain Wt	0.004	0.002	0.002	0.002	0.0004	0.46	0.45	0.33
mg/g Body Wt	0.054	0.033	0.031	0.032	0.006	0.35	0.42	0.38
Uterus, g	1.52	1.32	1.40	1.45	0.04	0.98	0.42	0.20
g/g Brain Wt	0.03	0.02	0.03	0.03	0.001	0.61	0.63	0.44
mg/g Body Wt	0.39	0.35	0.36	0.37	0.012	0.65	0.53	0.44

Table A4. The interactive mean effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 161 of gestation on fetal organ weight and allometric organ weight. (Continued)

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON-OCM**	**CON+OCM**	**RES-OCM**	**RES+OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Heart, g	27.61	28.37	28.76	28.89	0.83	0.64	0.79	0.86
g/g Brain Wt	0.49	0.52	0.55	0.53	0.01	0.26	0.90	0.44
mg/g Body Wt	7.13	7.63	7.46	7.27	0.19	0.97	0.71	0.41
Trachea, g	1.33	1.58	1.14	1.43	0.06	0.19	0.04	0.88
g/g Brain Wt	0.02	0.03	0.02	0.03	0.001	0.27	0.11	0.81
mg/g Body Wt	0.35	0.43	0.29	0.36	0.02	0.09	0.05	0.79
Small Intestine, g	72.41	65.73	66.77	63.12	1.59	0.19	0.10	0.63
g/g Brain Wt	1.29	1.21	1.26	1.17	0.03	0.54	0.11	0.90
mg/g Body Wt	18.77	17.78	17.29	15.98	0.44	0.06	0.18	0.84
Large Intestine, g	17.61	16.25	15.66	16.37	0.49	0.37	0.75	0.31
g/g Brain Wt	0.32	0.29	0.29	0.30	0.007	0.63	0.61	0.55
mg/g Body Wt	4.55	4.38	4.08	4.10	0.11	0.12	0.74	0.68
Pancreas, g	3.00	3.06	2.77	2.92	0.17	0.62	0.78	0.91
g/g Brain Wt	0.053	0.055	0.052	0.052	0.003	0.83	0.75	0.85
mg/g Body Wt	0.78	0.81	0.72	0.73	0.04	0.38	0.78	0.88
Left Kidney, g	17.86	16.39	17.18	15.71	0.60	0.59	0.25	0.99
g/g Brain Wt	0.32	0.30	0.33	0.29	0.01	0.89	0.20	0.68
mg/g Body Wt	4.59	4.40	4.43	3.95	0.12	0.22	0.18	0.55
Right Kidney, g	17.33	16.46	16.39	16.13	0.45	0.51	0.56	0.75
g/g Brain Wt	0.31	0.30	0.31	0.29	0.007	0.88	0.47	0.88
mg/g Body Wt	4.46	4.43	4.24	4.07	0.09	0.15	0.60	0.72
Left Longissimus Dorsi, g	61.72	57.04	61.45	65.17	2.06	0.36	0.91	0.33
g/g Brain Wt	1.10	1.04	1.17	1.18	0.03	0.11	0.71	0.53
mg/g Body Wt	15.88	15.39	15.92	16.24	0.36	0.58	0.91	0.61
Left Hindlimb Muscle, g	74.94	66.50	78.16	81.78	3.14	0.15	0.71	0.35
g/g Brain Wt	1.34	1.22	1.48	1.44	0.05	0.11	0.48	0.71
mg/g Body Wt	19.20	17.90	20.05	20.39	0.58	0.17	0.69	0.49
Left Hemisphere Brain, g	25.65	25.25	23.65	24.92	0.56	0.32	0.71	0.47
g/g Brain Wt	0.457	0.459	0.448	0.457	0.005	0.68	0.67	0.76
mg/g Body Wt	6.64	6.79	6.17	6.28	0.13	0.09	0.65	0.96
Right Hemisphere Brain, g	25.75	25.25	24.47	25.07	0.48	0.48	0.96	0.58

Table A4. The interactive mean effects of maternal plane of nutrition and supplementation with one-carbon metabolites[1] from day 0 to 161 of gestation on fetal organ weight and allometric organ weight. (Continued)

	Treatments[2]					*P*-value[4]		
Organ Weight	**CON-OCM**	**CON+OCM**	**RES-OCM**	**RES+OCM**	**SEM[3]**	**Gain**	**OCM**	**Gain×OCM**
Right Hemisphere Brain								
g/g Brain Wt	0.459	0.46	0.464	0.459	0.005	0.88	0.87	0.82
mg/g Body Wt	6.66	6.80	6.37	6.31	0.10	0.07	0.85	0.63
Hind Brain, g	4.60	4.36	4.59	4.50	0.23	0.89	0.74	0.88
g/g Brain Wt	0.082	0.080	0.087	0.83	0.004	0.71	0.69	0.89
mg/g Body Wt	1.20	1.19	1.21	1.14	0.07	0.88	0.83	0.86

[1]Methionine (7.4 g/d), choline (44.4 g/d), folate (320 mg/week), vitamin B_{12} (20 mg/week).
[2]CON-OCM = Control (0.60 kg/d) without one-carbon metabolite supplementation; CON+OCM = Control (0.60 kg/d) with one-carbon metabolite supplementation; RES-OCM = Restricted (-0.23 kg/d) without supplementation; RES+OCM = Restricted (-0.23 kg/d) with supplementation.
[3]Standard error of the mean.
[4]Gain = Main effect of feed intake levels; OCM = Main effect of one-carbon metabolite supplementation; Gain × OCM = Main effect of feed intake levels interaction with one-carbon metabolite supplementation.