DIETARY IMPACTS ON MITOCHONDRIAL RESPIRATION OF GESTATING HEIFERS AND FETAL OFFSPRING

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Kathryn Rose Margaret Slavick

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Carl Dahlen
Chair

Dr. Kendall Swanson

Dr. Joel Caton

Dr. Danielle Condry

Approved:

April 5, 2024
Date

Dr. Guillermo Scaglia
Department Chair

ABSTRACT

Two projects were conducted to evaluate dietary impacts on mitochondrial respiration of gestating heifers and fetal offspring. Our first hypothesis was that maternal vitamin and mineral supplementation during gestation improves the liver, muscle, and jejunum mitochondrial function of F1 and F2 offspring. In project 1, vitamin and mineral supplementation was provided to the F0 generation of dams from breeding to calving to isolate the effects of nutrition during pregnancy on future generations. Our second hypothesis was that altering limit-fed diets' forage: concentrate ratio would influence mitochondrial respiration in maternal and fetal jejunum and liver. In project 2, replacement heifers were fed high-concentrate and high-forage diets to evaluate the dietary impacts on fetal cellular metabolism of key metabolic organs. In both project 1 and 2, modulations of metabolism occurred in key metabolic organs of gestating heifers.

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DEDICATION

To my mom, Kathleen, who taught me the value of education. Thank you for always encouraging me to grow my curious mind.

To my dad, Robert, for passing on your love of the land and the livestock to me. Thank you for teaching me to appreciate the simple things in life.

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LIST OF ABBREVIATIONS

Acetyl-coA.....Acetyl coenzyme A

ADP.....Adenosine diphosphate

AI .....Artificial insemination

ATP .....Adenosine triphosphate

BCRC.....Beef Cattle Research Complex

CON .....Control

COX3 .....Cytochrome c oxidase subunit III

CS.....Citrate synthase

Cu.....Copper

DM .....Dry matter

E .....Electron transfer capacity

ETC.....Electron transport chain

F0 .....First parent or origin

F1 .....First filial generation

F2 .....Second filial generation

g.....Grams

g•heifer^{-}1•d^{-}.....Grams per heifer per day

GIT.....Gastrointestinal tract

IU .....International unit

IUGR.....Intrauterine growth restriction

K.....Potassium

kg.....Kilogram

L .....Leak respiration

mg .....Milligram

mL .....Milliliter

Na.....Sodium

NADH.....Dihydronicotinamide adenine dinucleotide

ND2.....NADH:ubiquinone oxidoreductase core subunit II

NDSU.....North Dakota State University

ng.....Nanogram

NRC .....National Research Council

O_2 .....Oxygen

P .....Phosphorus

P .....Oxidative phosphorylation capacity

PI.....NADH-linked oxidative phosphorylation capacity

pmol $O_2 \cdot s^{-1}$ .....Picomole of oxygen per second

ROS.....Reactive oxygen species

TCA.....Tricarboxylic acid

TMR.....Total mixed ration

uL .....Microliter

VFA.....Volatile fatty acid

VTM.....Vitamin and trace mineral

Zn .....Zinc

CHAPTER 1: LITERATURE REVIEW

Introduction to Beef Cattle Feeding Strategies

A variety of factors, such as feed costs, foodstuff availability, and producer objectives, influence beef cattle feeding strategies within various beef operations. Beef cattle spend most of their productive life grazing pasture and consuming a predominantly forage-based diet (Terry et al., 2021). High-forage diets have a forage inclusion rate of 50% or more (Chase and Grant, 2013). Forages are high-fiber feed components containing structural carbohydrates, cellulose, hemicellulose, and lignin, commonly found in forage feedstuffs such as grass hay, corn silage, or legumes like alfalfa (Corson et al., 1999).

High-concentrate diets are commonly fed in intensive beef operations to support rapidly growing cattle on finishing feedlot diets and dairy operations to support lactation demands post-parturition. Rations comprising 70% concentrate feeds or more are considered high-concentrate diets (Terry et al., 2021). High-concentrate diets characteristically include low fiber, high energy feed components containing greater amounts of "easily digestible" non-structural carbohydrates, starches, and sugars, commonly found in cereal grains such as corn, wheat, barley, oats, and rye (NASEM, 2016).

Ad Libitum and Limit-Feeding Strategies

Ad libitum conditions allow for cattle to consume their diet based on individual appetite, which can result in consumption of energy above maintenance requirements, fluctuations in intake (Fulton et al., 1979), and increased feed waste (Cunningham et al., 2005). Many methods exist to reduce producers' feed waste. However, ad libitum feeding still allows for 10 – 35% hay waste, depending on the methods implemented (Lechtenberg et al., 1974). Limit-feeding strategies have been explored as a method to reduce waste and increase feed efficiency in beef

production systems. Limit-feeding is largely researched and utilized in intensive beef feedlot and dairy systems; therefore, further research is needed to better understand the energy metabolism and fetal programming impacts of adopting these methods to feed beef cows and replacement heifers (Loerch, 1996).

With regards to beef cattle, evaluations comparing *ad libitum* feeding of silage versus limit-feeding high grain diets targeting similar rates of gain during the growing phase in steers showed that the limit-fed steers had increased shrunk, empty body, and liver weights compared to ad libitum steers; while producing similar results of Choice graded carcasses after 45 days in the feedlot (Coleman et al., 1995). Several *ad libitum* models have evaluated dry matter intake, passage rate, and dry matter digestibility. Increased inclusion of forage has been reported to increase passage rate with *ad libitum* feeding (Ledoux et al., 1985). Results from a high-concentrate diet experiment suggest that rapid passage rate may be due to enhanced concentrate digestibility, reduced gut fill, and increased dry matter intake (Davis et al., 2014). Overall, it has been reported that increased passage rates reduce digestibility (Ledoux et al., 1985). However, limit-feeding may mitigate this response to high-forage and high-concentrate diets, as intake restriction often results in decreased passage rate of digesta and greater digestibility (Mertens, 1987). Limit-feeding may also increase rumen retention due to lower intake and reduced ruminal fill (Trubenbach et al., 2019). Increased ruminal retention time results in reduced pH fluctuations and improvements in fiber digestion, which may be key to increased digestibility and feed utilization (Mould and Ørskov, 1983).

The improved feed utilization in limit-feeding scenarios may be a beneficial feed management strategy to implement for cow-calf producers, especially for the use of nutritional maintenance of cows and heifers, as they do not need to grow aggressively in the way feeder

cattle require. In addition, this method may be utilized to conserve feedstuffs and mitigate feed waste and feed costs. Having evaluated the various feed types' impacts on the rumen, it is important to consider the balance between forage and concentrate inclusion to ensure adequate nutrition while also avoiding digestive upset and metabolic disorders.

High-forage Diets

Diets comprised of 50% or more forage are typically defined as high-forage diets. The large majority of US cattle are fed forage-based diets comprised of 90% to 100% forages before entering feedlots for finishing (Galyean and Goetsch, 2015). Forages have increased fiber carbohydrates, which comprise the plant cell wall and are distinguished as neutral detergent fibers (hemicellulose) and acid detergent fibers (cellulose and lignin; NASEM, 2016). In addition to fiber, nonstructural carbohydrates are also present in forages, but total nonstructural carbohydrates vary depending on the source of forage. For example, alfalfa and cereal hays, in which grain is present, may contain increased concentrations of starch compared to forages that do not contain heads of grain (Roberts et al., 2015).

Common forage feedstuffs fed in the beef cattle industry include pasture, hay, native grass species, and silages. Hay and silage varieties include legumes, grasses, and crops such as alfalfa, ryegrass, sudangrass, timothy, millet, oats, wheat, corn, sorghum, cereal grain straw, and many other regional forages (Roberts et al., 2015). Forage quality has a major impact on the overall diet quality, and supplementation is required in diets largely consisting of low-quality hay (Vanzant and Cochran, 1994). Fiber consumption increases chewing and rumination behaviors (Mertens, 1997), which in turn stimulates saliva production. Saliva is an important contributor to the rumen buffer, bicarbonate, which helps to balance ruminal pH (Owens et al., 1998).

In the rumen, a limiting factor of high-forage diets includes the quality of the forages, with low-quality forages having reduced digestibility and palatability, which can make meeting cattle nutrition requirements difficult. Consumption of low-quality, high-forage diets can be limited by rumen fill as cattle may not be able to increase dry matter intake or meet their nutritional requirements due to rumen capacity limitations. Mertens reported that in addition to rumen capacity limitations, the distension of the rumen itself signals to the satiety centers of the brain to stop consuming feed (Mertens, 1994). The high inclusion rate of forages is an important component to consider while limit-feeding, especially in regards to forage quality due to limitations in rumen capacity. Forages, such as minimally processed hays, are characterized as buoyant long-fiber particles that "make up" the ruminal mat and denser smaller particles are more likely found in the rumen liquid layer. Particles collected on the mat are processed further through rumination; the size and formation of the ruminal mat are key to stimulating reticulorumen contractions and promoting digesta passage (Hooper and Welch, 1985).

Consumption of forages and increasing size of the ruminal mat places pressure on the ruminal wall, stimulating the vagus nerve to induce rumination. In concentrate diets, this stimulation is lacking, potentially reducing rumination (Van Soest, 1994). It has been observed that as the penetration resistance value of the ruminal mat increases, rumination activity increases as well (Izumi and Unno, 2010). Additionally, the inclusion of non-forage fibers such as beet pulp decreases the ruminal penetration resistance value (Izumi and Unno, 2010). The ruminal mat plays an important role in the fermentation process and digestibility. The lack of a sufficiently formed ruminal mat may result in an escape of food particles from the rumen and shift fiber digestion to the hindgut rather than the rumen (Zebeli et al., 2007).

The rumen of cattle fed high-forage diets creates a niche environment for fiber digestors. Structural carbohydrate fermenters include the following key bacterial species: *Rumino coccocus albus, R. flavafaciens, Fibrobacter succinogenes, Butyrivibrio fibrisolvens,* and *Eubacterium cellosolvens* (Van Soest, 1994). These primary bacterial species begin the conversion of carbohydrates to volatile fatty acids (VFAs). Secondary fermenters such as methanogens convert the VFAs to CO_2 and CH_4. It is well known that grass-fed cattle produce more methane due to the increased requirement for ruminal fermentation (Benchaar et al., 2001; Aryee et al., 2023). Overall, methane emission and the presence of methanogenic bacteria are dependent on the forage quantity and quality provided (Sakamoto et al., 2020). Forage inclusion is an important factor for rumination and salivation, as well as ruminal pH and fermentation.

High-concentrate Diets

High-concentrate diets are predominately comprised of concentrate feeds that are characteristically high in starch and low in fiber (Vasconcelos and Galyean, 2007). Concentrate feeds are described to be non-fiber or non-structural carbohydrates. Many concentrates are energy-dense grains comprised of water-soluble carbohydrates such as monosaccharides and starches and soluble fibers such as beta-glucans (NASEM, 2016). Concentrate feedstuffs include energy-rich cereal grains such as corn, soybean meal, wheat, and oats. Increasing the level of concentrate feeds in the diets of cattle results in increased dry matter intake and body weight gain (Chen et al., 2021). Additionally, processing of grain to reduce particle size and expose the endosperm of cereal grains has been shown to improve microbial digestion of starch in the rumen (McAllister et al., 1994). For example, steam-flaked corn and high-moisture corn have been observed to have 33% greater digestibility compared to dry-rolled corn in situ (Cooper et al., 2002).

Beef cattle are typically introduced to high-concentrate diets when entering the feedlot for finishing. Increased concentrate level in cattle diets impacts various functions of the gastrointestinal tract. It has been observed that increasing dietary concentrate inclusion in ruminants results in a reduction in rumination behavior, which can reduce saliva production and buffering capacity within the rumen (Desnoyers et al., 2008). In addition to the reduction of bicarbonate production, the nature of the high-concentrate diet allows for rapid rumen fermentation, resulting in decreased pH and altered VFA levels. When feeding high-concentrate diets, there is a transition from predominately acetate production to an increase in propionate production (Chen et al., 2021).

The combination of rapid fermentation and high levels of propionate reduces rumen pH and increases the risk of acidosis. Overall, as VFA concentration increases, ruminal pH decreases (Penner et al., 2009). Typically, acetate is utilized in peripheral tissues and oxidized to produce ATP or incorporated into triglycerides. Propionate is utilized by the liver for the process of gluconeogenesis (Lin et al., 2012) or as an energy source. Propionate is a critical volatile fatty acid for ruminants as almost no glucose reaches the small intestine for absorption (Lin et al., 2012).

Feeding of high-concentrate diets impacts the intestinal microbiome; it has been observed that feeding a high-concentrate diet results in a decrease in the relative abundance of microbes at phyla and genera levels. The major modulations observed include a decreased abundance of *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* (Zhang et al., 2022). These bacterial species are major cellulolytic degraders that may be reduced due to the lack of fiber content inclusion within the diet and their sensitivity to low pH. Additionally, the relative abundance of *Methanobrevibacter*, *Methanosacrina,* and *Methanosphaera* decreases due to the presence of

low pH in the rumen (Zhang et al., 2022). Not only do compositional changes in the microbial ecology exhibit dysbiosis, but changes in the function of commensal bacteria occur, including inhibition of methane, lipid, and amino acid metabolism (Zhang et al., 2022).

During the state of dysbiosis, the proliferation and inhibition of differing microbe populations may result in the accumulation of lipopolysaccharides and other toxins (Sanz-Fernandez et al., 2020). These significant changes to the rumen environment not only occur prior to the digesta reaching the gastric stomach, but other changes in the microbial microbiome are seen as well. As dietary starch consumption increases, the amount of post-ruminal starch that escapes rumen digestion increases (Sanz-Fernandez et al., 2020). In typical cattle digestion, starches are fermented and utilized by ruminal microbes as the small intestine of ruminants has a limited capacity to digest starch (Owens et al., 1986). An increased incidence of starch entering the large intestine results in fermentation, accumulating VFAs and reducing digesta pH – similar to what is observed in the rumen (Sanz-Fernandez et al., 2020). Additionally, starch digestion within the rumen decreases as dietary starch inclusion increases, resulting in greater starch flow to the small intestine and greater levels of unabsorbed starch in the feces (Sanz-Fernandez et al., 2020).

The effects of subacute ruminal acidosis (SARA) within the hindgut may have more detrimental effects compared to that of the rumen due to the lack of its buffering capacity and the nature of the single layer of columnar epithelium within the hindgut versus that of the rumen's stratified squamous lining (Sanz-Fernandez et al., 2020). Due to these disadvantages, during hindgut acidosis, we see increased sloughing of epithelium and lesions of the intestine due to mucosal damage. Qiu et al. (2019) has observed changes in the microbial community when feeding high-concentrate diets, as *Firmicutes:Bacteroides* ratio decreases.

Common Supplementation Practices of Beef Producers

Limited survey data highlighting the common nutritional management strategies and supplementation practices of North Dakota producers and of the Northern Great Plains exists. Due to this lack of knowledge, we can deduce the needs of cattle based on nutritional analysis of grazed pastures and feed. Range grasses and forages have a dynamic range of mineral concentrations due to fluctuations in soil type and environmental conditions. In the northern plains' region, it has been reported that the nutrients P, K, Na, Zn, and Cu fall below the recommended nutrient requirements for lactating cows and optimal animal performance (Grings et al., 1996). In other regional studies, it has been noted that forage concentrations of Cu and Zn were marginal to deficient and unlikely to support breeding cattle needs (McCarthy et al., 2023). Therefore, it is important for producers to evaluate grazing pastures, especially those stocked with breeding, lactating, and gestating cattle.

Overview of Fetal Programming of Metabolic Organs

The Dutch Hunger Study first introduced the idea of developmental programming, suggesting that maternal nutrition during gestation can have lasting impacts on offspring, especially in terms of metabolic disorders (Barker, 1990). A special focus on dietary nutrient supply before and during pregnancy is warranted as it has been reported that imbalances in nutrition, such as nutrient restriction or over-abundance, during the periconceptual period have the potential to alter metabolic outcomes of offspring (George et al., 2012). Early gestation is a critical period due to the nature of organogenesis and embryonic development that occurs during the first 50 days (Caton et al., 2020). During organogenesis, changes in nutrient supply alter the development of tissues, including the liver, skeletal muscle, and gastrointestinal tract (GIT), which play key roles in energy metabolism and maintenance. These high nutrient-demanding

tissues have a large influence on whole-animal energetics; thus, it is important to explore the imprint of fetal adaptations post-parturition and beyond.

It has been reported that together, the liver and gastrointestinal tract represent 45 – 50% of the animal's total basal energy requirement (Johnson et al., 1990). The liver itself comprises approximately 22% of basal energy requirements, while accounting for less than 2% of body weight in cattle (Caton et al., 2000; Montanholi et al., 2017). Conversely, muscle accounts for approximately 20% of energy use, whereas it comprises about 40% of the animal's overall body weight (Caton et al., 2000). Changes in the diet of the animal may influence cellular metabolism and whole animal energetics in multiple ways, such as metabolic organ cross-talk, availability of nutrients to the fetus, microbiota changes, and mitochondrial adaptations.

Potential Nutritional Influences

Maternal dietary changes influence embryonic nourishing fluids, such as histotrophic and allantoic fluids. Menezes et al. (2021) reported that glutamine, cystine, arginine, and aspartate concentrations were increased in heifers with a moderate rate of gain compared to heifers with a low rate of gain. The abundance of amino acids and metabolites, such as arginine and methionine, are critical for a successful pregnancy due to their roles in rapid cellular growth, metabolism, and production of metabolites utilized in one-carbon metabolism and regulation of epigenetic modifications (Crouse et al., 2020).

Ovine intrauterine growth restriction (IUGR) models showcase the fetal adaptions that may take place as dietary modulations occur. Placental insufficiency and low fetal nutrient concentrations often elicit stunted growth and adaptations of metabolic organs, such as reductions in mitochondrial substrate utilization and oxidative phosphorylation capacities; in some instances, these adaptations may become permanent and follow offspring into adulthood,

predisposing them to metabolic disorders and disease (Pendleton et al., 2021). Undernutrition and overnutrition can result in states of IUGR. First parity, over-nourished ewes can exhibit IUGR in fetuses, with fetal weights being reduced by 30-60% at 0.9 gestation (Pendleton et al., 2021; Carr et al., 2012). Fetal adaptations to the IUGR environment include slowing skeletal muscle growth while increasing hepatic glucose production, as glucose is a major energy source for the developing fetus. Low lean mass and primed glucose metabolism are then carried into adulthood, and offspring exhibit greater adiposity (Pendleton et al., 2021; Camacho et al., 2017).

In a normal gestation, resulting in no IUGR, fetal glucose is derived and utilized from the dam by way of placental transfer (Teng et al., 2002). However, during extreme nutrient restriction, early activation of fetal endogenous glucose production is observed to meet the fetal glucose demand (Houin et al., 2015). Alongside modulations in gluconeogenesis, livers from IUGR fetuses can have low growth rates, modifications in rates of protein synthesis, low mitochondrial redox states, and low TCA intermediates (Pendleton et al., 2021). The lower mitochondrial activity is also associated with lower amino acid degradation of arginine, histidine, proline, and tryptophan. Interestingly, arginine, histidine, and proline converge at α-ketoglutarate, a rate-limiting step of the TCA cycle (Pendleton et al., 2021). To meet the gluconeogenesis needs of the fetus, skeletal muscle must provide the liver with amino acids, such as alanine and glutamine, a process that is also known as skeletal muscle:liver crosstalk. *In utero*, modifications to muscle and liver metabolism are of benefit for fetal survival; however, priming these key metabolic organs may be a link to postnatal challenges (Hales and Barker, 2001).

Research has indicated that there is a link between the host microbiome and fetal metabolism, as well as fetal colonization in utero. The in-utero colonization hypothesis has been introduced recently, and it is thought that the maternal gut microbiome is transmitted from

mothers to the offspring residing in the uterus prior to birth (Amat et al., 2022). A potential mechanism of maternal influence on fetal offspring derives from the dam's intestinal microbiome, as the species present during digestion supply varying metabolites to the host and are dependent on the available digesta substrates (Li, 2018). An example of this phenomenon is Bifidobacterium, which produces folate and butyrate. Additional metabolites that can be produced by bacteria include biotin, acetate, and acetyl-CoA, which are utilized in regulating energy metabolism and epigenetics (Li, 2018). These bioactive metabolites can be utilized by the host for cellular biochemical pathways.

Mitochondrial Respiration

Mitochondria generate energy in the forms of ATP and heat. Generally, mitochondrial energy output is about 80% ATP and 20% heat (Rolfe et al., 1999). The electron transport chain, which resides on the inner mitochondrial membrane, is responsible for the conversion of nutrients into electrical potential in the form of H+ protons. These protons then either enter ATP synthase to aid in the conversion of ADP into usable ATP, or they enter proton leak, aided by uncoupler proteins, where the energy is dissipated as heat (Bertholet and Kirichok, 2020). Evaluating mitochondrial metabolism and in vitro liver, muscle, and gastrointestinal tract oxygen consumption allows for a better understanding of whole animal energy utilization and nutrient requirements. The role of mitochondrial proton leak in skeletal muscle and liver respiration contributes to the standard metabolic rate of the animal. For example, it is estimated that proton leak within liver and skeletal muscle mitochondria accounts for approximately 20% of the standard metabolic rate in rats (Rolfe and Brand, 1996).

Hepatic Mitochondria Adaptations

The liver is a vital organ and important for many major metabolic mitochondrial processes, is highly adaptable, and is influenced by dietary changes. Hepatocytes comprise up to 85% of the liver, and within them hepatic mitochondria are the functioning powerhouses working to regulate gluconeogenesis, lipogenesis, ureagenesis, B-oxidation, ETC, ketogenesis, one carbon metabolism, epigenetic factors, ROS, and fission/fusion (Morio et al., 2021).

In terms of caloric nutrient requirements, several studies have been completed exploring restricted maternal diets impacts on the dam and offspring's epigenetic profile, metabolite status, and hepatic energy utilization. Restricting ewes to 60% of NRC requirements (NRC, 2007) at d 50 of gestation resulted in decreased weight, O_2 consumption, and citrate synthase activity in the fetal liver. In this study, a compensatory effect between key metabolic organs may have been observed as O_2 consumption and citrate synthase activity increased within intestinal tissue (Prezotto et al., 2014). Being that the liver is a highly metabolic organ and abundant with mitochondria responsible for a variety of vital processes, macronutrients and micronutrients are essential for proper function.

Nutrient availability influences hepatic mitochondrial metabolism. Diets of nutrient excess have the potential to elicit oxidative stress, leading to mitochondrial dysfunction. Micronutrients working as cofactors or antioxidants are vital for optimal mitochondrial function. Hepatic mitochondrial health is especially important as the abundant mitochondria are responsible for several metabolic pathways, such as fatty acid oxidation, TCA cyclicity, ATP production, and the synthesis of lipids and cholesterol (Rodríguez-Cano et al., 2020). Distressed mitochondria are a source of reactive oxygen species, which can lead to inhibitory actions on the

electron transport chain and epigenetic alterations that impact mitochondrial metabolism (Rodríguez-Cano et al., 2020).

The ability of hepatic mitochondria to balance fission and fusion during periods of energy demand and deficit reflects its capacity to adapt to the nutritional environment of the animal (Morio et al., 2021). Nutrients that are deficient at essential periods of development can result in conditioning the fetal liver to certain nutritional environments that may not be reflective of what is introduced postnatally (Christian and Stewart, 2010). The thrifty phenotype hypothesis proposes that maternal undernutrition during gestation and fetal growth restriction result in altered metabolism, such as obesity and insulin resistance in adult offspring (Hales and Barker, 2001). It is thought that during the undernourished gestational development, energy and nutrients are favorably shunted to critical organs, metabolically priming organs to function in a state of undernourishment (George et al., 2012). However, when an animal is fed a diet of adequacy or abundance, there can be increased insulin resistance or adiposity (George et al., 2012).

In previous studies conducted by Ford et al. (2007), it has been observed that ewes that were subjected to 50% nutrient restriction from day 28 to 78 of gestation had a 30% restriction in fetal growth at the point of mid-gestation (Ford et al., 2007). However, ewes returned to 100% NRC requirements after day 78 for the remainder of gestation had lambs of both sexes born at similar size and weight when compared to controls. Interestingly, male lambs born to nutrient-restricted ewes demonstrated “thriftiness” as they gained weight quickly and exhibited greater body weights at four months of age. Additionally, this cohort of male lambs restricted during early gestation had greater glucose resistance, increased visceral fat mass, and decreased lean mass at eight months of age (Ford et al., 2007).

Fetal programming effects were observed in "long-term" studies conducted by George et al. (2012). Two cohorts of 6-year-old ewes were fed during an 11-week ad libitum feeding trial to evaluate the long-term impacts of gestational nutrition restriction. The first cohort of ewes was from gestationally nutrient-restricted dams during early gestation, whereas the second cohort was from dams fed 100% of the NRC recommendations (NRC, 2007). Results showed that offspring from dams that were nutrient-restricted during gestation had increased daily feed intake and feed efficiency. Additionally, insulin sensitivity was lower, and glucose sensitivity was greater in ewes from nutrient-restricted dams than in ewes from control dams. Liver weights were not different between cohorts, but a greater hepatic lipid percentage was noted in ewes from nutrient-restricted dams. Overall, this study showed that 50% nutrient restriction during early fetal development leads to long-term metabolic changes suggesting the presence of the thrifty phenotype in male offspring, increased insulin secretion, and alterations in glucose metabolism (Ford et al., 2007; George et al., 2012).

On the opposite end of dietary energy intake spectrum is overconsumption, often studied in high fat diet animal models. High consumption of fat or changes in lipid metabolism results in the increased presence of lipids in hepatocytes and impaired mitochondrial function (Teodoro et al., 2008). However, vitamin and mineral supplementation has the potential to help overcome these effects as mice fed a high fat, choline deficient diet initially and then supplemented with choline had a slight improvement in mitochondrial function, however their impaired mitochondrial respiration was still evident (Teodoro et al., 2008). Supplementation of niacin to rats with pre-existing hepatic steatosis was observed to have improved mitochondrial function and reduced the severity of steatosis (Ganji et al., 2014). Collectively, these studies suggest that the liver is highly adaptable to nutritional insults and nutrient supplementation.

Skeletal Muscle Mitochondria Adaptation

Muscle mitochondria have the ability to adapt to challenges in a manner similar to the liver, and innate differences in animals could potentially be used to select and breed efficient livestock (Casal et al., 2018). Breed has been observed to influence mitochondrial functionality and oxidative phosphorylation capacity. Brahma and Angus cattle breeds exhibit differing skeletal muscle mitochondria energetics that influence muscular traits and influence heat tolerance and meat quality factors such as tenderness (Ramos et al., 2020). Breed effect is important to consider when designing research focused on energy metabolism.

Modifying dietary feeding or supplementation strategies can influence skeletal muscle energetics (Vestergaard et al., 2000). Maternal methionine supplementation during gestation induced DNA methylation and mitochondrial gene expression in beef calves, which could result in changes in mitochondrial function and assembly of electron transfer chain (ETC) proteins (Amorín et al., 2023). Varying dietary levels of selenium and vitamin E can influence skeletal muscle mitochondrial respiratory capacity in horses (Owen et al., 2022). In the absence of vitamin E supplementation, it was observed that selenium supplementation decreased leak respiration, therefore improving mitochondrial efficiency (Owen et al., 2022). In addition to respirometry parameters, mitochondrial density has been shown to be influenced by dietary changes (Du et al., 2018).

Mitochondrial density is an important parameter to quantify as mitochondria density is correlated with maximal oxygen consumption in muscle tissue (Hoppeler et al., 1987). Interestingly, grass-fed Angus steers have been observed to have greater mtDNA copy numbers in skeletal muscle and liver when compared to grain-fed steers (Bai et al., 2020). In rat models, maternal consumption of low protein diets during gestation resulted in female offspring

experiencing mitochondrial dysfunction in skeletal muscle, highlighting the importance of adequate nutrition during gestation and the plasticity in programming skeletal muscle mitochondria (Vidyadharan et al., 2022).

Jejunal Mitochondrial Adaptation

Initial findings from our lab group showed a greater capacity for ATP synthesis in the jejunum of calves from dams supplemented with vitamins and minerals throughout gestation having greater mitochondrial respiration (Menezes et al., 2022), encouraging us to further explore vitamin and mineral supplementation impacts on cellular metabolism. Epithelial cells in the small intestine turn over every 3 to 5 days, as a protective mechanism and adaptation to dietary changes, stressors, or microbiome alterations (Rath et al., 2018) so changes in mitochondrial function could dramatically affect function and energy use by the small intestine.

Diet composition and inclusion of feedstuffs such as a casein-corn starch combination, a barley-canola meal combination, or alfalfa impact liver and intestine size in pigs, subsequently impacting oxygen consumption of these tissues (Nyachoti et al., 2000). Feeding of a casein-corn starch combination resulted in greater cecal tissue oxygen consumption in pigs compared to the inclusion of a barley-canola meal combination or alfalfa (Nyachoti et al., 2000). Stress events and disease states are commonly associated with increased reactive oxygen species, mitochondrial dysfunction, and impaired ATP synthesis (Chernyavskij et al., 2023). In humans, consumption of high-fat diets impairs colonocyte mitochondrial function, resulting in decreased ATP synthesis (Guerbette et al., 2022).

In cattle, the liver and gastrointestinal tract utilize 45 – 50% of the basal energy requirement (Johnson et al., 1990). Therefore, metabolic changes in these tissues can impact whole animal energetics and nutrient requirements. In cows restricted to 60% of their nutritional

requirements, fetal jejunal oxygen consumption is decreased at approximately day 85 of gestation; however, following re-alimentation, a greater oxygen consumption is observed (Prezotto et al., 2016). Additionally, gestation serves as a factor influencing intestinal oxygen consumption; pregnant heifer small intestinal tissue oxygen consumption was observed to increase linearly throughout gestation (Scheaffer et al., 2003). However, decreased oxygen consumption and cellular turnover were observed in the pregnant heifer's ileum throughout pregnancy compared to those in non-pregnant heifers – a potential route of energy conservation due to the increased energy demands of gestation (Scheaffer et al., 2003).

There has been significant research on the effects of diet on the microbiome. Volatile fatty acids are produced by microbes in the gastrointestinal tract and influence the function of the intestine. Butyrate, in particular, can be utilized by colonocytes, in the form of acetyl-CoA and NADH, for ATP synthesis (Mafra et al., 2019). Diet can affect the microbiome in the GIT and, subsequently, fermentation patterns. High-concentrate inclusion in diets increases concentrations of propionate and butyrate (Chen et al., 2021). Sodium butyrate has been shown to alleviate mitochondrial dysfunction and increase mitochondrial DNA copy number (Li et al., 2022). Propionate, when supplied to hepatocytes with increased free fatty acid levels, alleviated the incidence of mitochondrial dysfunction (Wang et al., 2022). Microbiome alterations and fermentation shifts are additional components to consider when evaluating dietary impacts on the gastrointestinal tract.

Summary and Study Aim

Much research exists evaluating the impacts of nutritional insults during gestation on the dam and fetal offspring mitochondrial function and oxygen consumption. However, through the evaluation of maternal gestational nutrition and its influence on the programming of fetal

metabolism and mitochondrial respiration, we can better understand the implications of common feeding strategies and establish the data required to guide producers to make confident cattle selections and production decisions in the future. By exploring the diet modulations between cattle-fed high-forage diets and high-concentrate diets, as well as the easily adoptable practice of vitamin and trace mineral supplementation, we can better understand the impacts of dietary modulations on mitochondria function and the multigenerational impacts of maternal nutrition influence on fetal development.

The objectives of project one were to evaluate the effects of maternal VTM supplementation during gestation on mitochondrial respiration in the jejunum, liver, and skeletal muscle of F1 and F2 offspring. Additionally, mitochondrial density was assessed by evaluating mitochondrial citrate synthase activity and copy number of tissues of the F1 and F2 generations. The objective of project two was to evaluate the influence of feeding high-concentrate and high-forage diets in a limit-fed setting on mitochondrial respiration in the jejunum and liver tissue of replacement heifers and their fetal offspring. Collectively, the goal of the research was to evaluate the impacts of gestational nutrition on metabolism at the tissue level to increase our understanding of how tissue-level responses may influence whole-animal energetics.

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CHAPTER 2: MULTIGENERATIONAL EFFECTS OF MATERNAL VITAMIN AND MINERAL SUPPLMENTATION THROUGHOUT GESTATION IN BEEF HEIFERS – EFFICIENCY OF ENERGY UTILIZATION IN KEY METABOLIC TISSUES[1]

Abstract

The objective of this study was to evaluate the effects of maternal vitamin and mineral supplementation during pregnancy on F1 offspring liver and muscle energy utilization throughout pregnancy, as well as F2 offspring jejunum, liver, and muscle mitochondrial respiration and mitochondrial quantity of the aforementioned tissues at d 247 ± 3 of gestation. Thirty-one crossbred Angus heifers (F0 generation) were blocked by BW, bred via artificial insemination (AI) with female-sexed semen, and randomly assigned to one of two dietary treatments: 1) basal diet without the addition of a vitamin and mineral supplement (CON, n = 14), or 2) basal diet with the addition of a vitamin and mineral supplement (VTM, n =17). Treatments were applied from breeding through calving. Both cohorts of F0 dams were fed a common diet post-calving, which included vitamin and mineral supplementation. All F1 heifer calves were reared by their dams under common management conditions through weaning. After weaning, F1 heifers were managed as a single group throughout breeding and pregnancy attainment. Sixteen F1 heifers pregnant with F2 female fetuses (7 from CON and nine from VTM dams) were then used to evaluate jejunum, liver, and muscle cellular metabolism during pregnancy. At d 179 and d 247 ± 3 of gestation, liver and muscle biopsies were collected to

[1] The material in this chapter was co-authored by K. R. M. Slavick, A. C. B. Menezes, J. L. Hurlbert, K. S. Safain, K. Bochantin-Winders, J. D. Kirsch, K. C. Swanson, and C. R. Dahlen. J. L. Hurlbert had primary responsibility for collecting liver and muscle biopsies and K. R. M. Slavick was the primary developer of the conclusions presented here. K. R. M. Slavick also drafted and revised all versions of this chapter, and C. R. Dahlen and K. C. Swanson assisted in revisions, statistical analysis, and other manuscript preparations.

assess tissue mitochondrial respiration via high-resolution respirometry. Additionally, maternal and fetal jejunum were collected to assess oxygen consumption at the time of harvest at d 252 ± 3 of gestation. The listed tissue types collected from the third trimester were evaluated for protein concentration, mitochondrial DNA copy number, and citrate synthase. Grandmaternal dietary treatment did not influence fetal jejunum, liver, and muscle oxygen consumption in F2 heifers ($P \geq 0.17$). Maternal liver and jejunum mitochondrial respiration in F1 heifers were not influenced by maternal dietary treatment ($P \geq 0.70$) or stage of gestation ($P \geq 0.27$). Maternal muscle mitochondrial LEAK respiration (L) was greater ($P = 0.05$) in CON compared with VTM heifers. Additionally, L was greater in muscle during the third trimester compared to the second trimester ($P = 0.02$). Increased L may indicate decreased energetic efficiency as energy is not utilized for functional purposes but is rather dissipated as heat. Protein concentration of all tissue types collected from both F1 heifers and F2 fetuses, were not impacted by F0 VTM supplementation ($P \geq 0.39$; Figure 1). Additionally, CS activity ($P \geq 0.13$; Figure 2) and mitochondrial DNA (mtDNA) copy number of the genes ND2 ($P \geq 0.18$; Figure 3) and COX3 ($P \geq 0.24$; Figure 3) were not impacted by VTM supplementation. These data provide insight regarding fetal programming effects on heifer cellular energy consumption in key metabolic organs, which may be beneficial for better-characterizing energy requirements and tissue function during growth, development, and pregnancy.

Introduction

Many maternal nutrition experiments have focused on the last trimester of gestation without evaluating multigenerational gestation effects on live offspring postnatally. Imbalances in nutrition during the periconceptual period, such as nutrient restriction or over-abundance, have been shown to alter the metabolic and energetic outcomes of offspring (George et al., 2012).

The liver, skeletal muscle, and gastrointestinal tract (GIT) play key roles in energy metabolism and maintenance. These organs are developed within the first 50 days of gestation, suggesting that maternal nutritional insults during this window of time may affect their metabolism and function (Prezotto et al., 2014).

These high energy-demanding organs greatly influence whole-animal energetics; thus, it is important to explore fetal adaptations of these tissues post-parturition and beyond. Together, the liver and gastrointestinal tract represent 45 – 50% of the animal's total basal energy requirement (Johnson et al., 1990). The high rate of cellular turnover and absorption of nutrients within the GIT requires many ATP-demanding processes (Rath et al., 2018). In cattle, the liver itself comprises approximately 22% of basal energy requirements, while accounting for less than 2% of body weight (Caton et al., 2000; Montanholi et al., 2017).

Hepatocytes comprise up to 85% of liver tissue; hepatic mitochondria are abundant in hepatocytes, and are important in many metabolic functions, including gluconeogenesis, lipogenesis, ureagenesis, B-oxidation, electron-transfer chain (ETC), ketogenesis, one-carbon metabolism, epigenetic factors, reactive oxygen species (ROS), and fission/fusion (Morio et al., 2021). Muscle accounts for approximately 20% of energy use, whereas it comprises about 40% of the animal's overall body weight (Caton et al., 2000). Micronutrients, such as B vitamins, zinc, selenium, and many others, are vital for mitochondrial processes (Rodríguez-Cano et al., 2020). Evaluation of vitamin and mineral supplementation throughout gestation is needed to better understand how this easily adoptable production practice influences offspring energetic and metabolic traits.

Our group developed a research model to evaluate the effects of maternal vitamin and mineral supplementation during the first trimester of gestation on maternal and fetal outcomes.

These supplementation strategies have resulted in altered concentrations of amino acids in fetal fluids (Menezes et al., 2021) and minerals in liver and muscle (McCarthy et al., 2022), and fetal hepatic energetic (Crouse et al., 2022) and lipidomic (Menezes et al., 2023) profiles. Our more recent experiments have examined the effects on fetal physiology and metabolic programming in response to vitamin and mineral supplementation for the whole gestational period (Hurlbert et al., 2024).

Preliminary data indicated greater efficiency of ATP synthesis in small intestine of neonatal calves from supplemented dams (Menezes et al., 2022), which may indicate a greater capacity of nutrient utilization. (Casal et al., 2018). The current study aims to further evaluate initial findings reported by Hurlbert et al. (2024) and Menezes et al. (2021) and explore whether the factors altering 30-h neonatal calves and growth performance to weaning are present in mitochondrial mechanisms of key metabolic organs in F1 heifers during gestation and F2 fetuses. We hypothesized that maternal vitamin and mineral supplementation during gestation influences liver, muscle, and jejunum oxygen consumption and improves mitochondrial function of F1 and F2 offspring.

An additional objective included the evaluation of mitochondrial density parameters and their use in high-resolution respirometry data normalization strategies. To effectively evaluate mitochondria respiration levels, it is important to consider mitochondrial density. Commonly used mitochondria biomarkers include citrate synthase activity (CS) and mitochondrial DNA (mtDNA) (Carvalho et al., 2020; Longchamps et al., 2020). Additionally, protein concentration per tissue (Prezotto et al., 2014) is also used as a method of normalizing data. A wide variety of biomarkers are utilized to quantify mitochondria in this field of study, and some research regarding biomarker evaluation of skeletal muscle mitochondria has taken place (Larsen et al.,

2012). However, gaps in knowledge exist regarding quantification of liver and jejunum mitochondria quantification.

Materials and Methods

All animal care, management practices, and experimental procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee (Protocol #A21047).

Animals and Sampling

Thirty-one crossbred Angus heifers (F0 generation) were housed and individually fed (American Calan, Northwood, NH) at the NDSU Animal Nutrition and Physiology Center (ANPC). Dietary treatments, procedures applied to F0 heifers, and F1 offspring growth performance are as described by Hurlbert et al. (2024). Briefly, F0 heifers were estrus synchronized via a 7-day Select-Synch + CIDR protocol, and AI bred with female sexed semen from a single sire. Heifers were then randomly assigned to receive either a basal diet (CON; n = 14) or a basal diet with addition of a vitamin and mineral supplement (VTM, n =17). The VTM supplement used was a loose product (113 g•heifer^{-1}•d^{-1}, Purina Wind & Rain Storm All-Season 7.5 Complete, Land O'Lakes, Inc., Arden Hills, MN; Table 2.1) that was individually added to the F0 heifers' daily diet allotment. Briefly, the basal diet offered consisted of 60% grass hay, 30% corn silage, and 10% DDGS premix, which Hurlbert et al. (2024) described.

Heifers were moved to the NDSU Beef Cattle Research Complex (BCRC; Fargo, ND, USA) for the last trimester of gestation, where they were group-housed and individually-fed via the Insentec system (Hokofarm Group B.V., the Netherlands). Heifers continued to receive their respective treatments, and the vitamin and mineral supplement was incorporated into the total mixed ration (TMR) for VTM heifers. Treatments were applied from breeding to calving. Post-

calving, both cohorts of F0 dams were fed a common TMR, which included vitamin and mineral supplementation. The F1 heifer calves of differing gestational backgrounds (CON, n = 14 vs. VTM, n = 17) were then reared by their dams. At approximately two months of age, F1 heifer calves were placed on pasture with their F0 dams and were later weaned at approximately eight months of age. At weaning, F1 heifers were fed a common TMR and transported at d 50 post-weaning to the BCRC for heifer development. At the BCRC, F1 heifers were fed for *ad libitium* intake, a TMR comprised of 70% winter wheat hay, 20% corn silage, and 10% DDGS premix with vitamin and mineral supplement (DM basis).

Table 2.1. Composition of vitamin and trace mineral supplement[1] provided to VTM treatment F0 dams throughout gestation and to all F1 heifer offspring throughout growth and gestation; company guaranteed analysis.

Item	Guaranteed Analysis	
Minerals	Min	Max
Ca, g/kg of DM	135.0	162.0
P, g/kg of DM	75.0	-
NaCl, g/kg of DM	180.0	216.0
Mg, g/kg of DM	10.0	-
K, g/kg of DM	10.0	-
Mn, mg/kg of DM	3,600.0	-
Co, mg/kg of DM	12.0	-
Cu, mg/kg of DM	1,200.00	-
I, mg/kg of DM	60.0	-
Se, mg/kg of DM	27.0	-
Zn, mg/kg of DM	3,600.0	-
Vitamins		
A, IU/kg of DM	661,500.0	-
D, IU/kg of DM	66,150.0	-
E, IU/kg of DM	661.5	-

[1] Purina Wind and Rain Storm All Season 7.5 Complete Mineral (Land O' Lakes, Inc., Arden Hills, MN); ingredients: dicalcium phosphate, monocalcium phosphate, processed grain by-products, plant protein products, calcium carbonate, molasses products, sodium chloride, mineral oil, potassium chloride, magnesium oxide, ferric oxide, vitamin E supplement, vitamin A supplement, lignin sulfonate, cobalt carbonate, manganese sulfate, ethylenediamine dihydroiodide, zinc sulfate, copper chloride, vitamin D3 supplement, natural and artificial flavors, and sodium selenite.

[2] VTM supplement provided at a rate of 113 g•heifer^{-1}•d^{-1}.

Heifers of the F1 generation (n = 31; BW = 412.5 ± 53.68 kg; 14 mo) were split into two breed groups approximately were estrus-synchronized with a 7-d Select Synch + CIDR protocol and timed-AI bred with female sexed semen from a single sire, approximately two weeks apart to facilitate ease of collections and harvests. Pregnancies of F1 heifers were confirmed using transrectal ultrasound at d 35 of gestation, and fetal sex was evaluated at d 65 to confirm the presence of female fetuses. Sixteen F1 heifers gestating female fetuses (7 from CON dams and 9 from VTM dams) were selected for this study. Heifers were then transported to the ANPC at approximately d 55 to 62 of gestation, where they were individually fed to meet nutrient requirements (NASEM, 2016) for gestating replacement heifers at approximately 0.80 kg gain per day. The total mixed ration consisting of 70% winter wheat hay, 20% corn silage, and 10% DDGS premix containing the vitamin and mineral supplement (DM basis).

Liver and Muscle Biopsy Collection

Liver and muscle biopsies were collected on d 179 and d 247 ± 3 of gestation to evaluate cellular energetics during the second and third trimester of pregnancy. Heifers were restrained in a squeeze chute, and the biopsy sites were clipped and scrubbed three times with betadine and 70% ethanol. Flunixin meglumine (Banamine, Merck Animal Health; Madison, NJ) was dosed at 1.1 – 2.2 mg/kg BW and administered intravenously. A local anesthetic (3-mL Lidocaine Injectable – 2%; MWI, Boise, ID) was administered subcutaneously at both biopsy sites. For liver biopsies, a 1-cm incision was made at the biopsy site between the 10^{th} and 12^{th} ribs. Liver samples (*20 mg*) were collected using a 14-gauge Tru-Cut biopsy trochar (Merit Medical, South Jordan, UT; McCarthy et al., 2022). For muscle biopsies, a 2-cm incision was made on the back above the longissimus dorsi muscle between the 12^{th} and 13^{th} rib. Muscle samples (*20 mg*) were collected using a Bergstrom muscle biopsy needle. The biopsy site was closed with surgical

staples, and an aerosol protective barrier (Aluspray, Neogen; Lexington, KY) was topically applied. Liver and muscle samples were placed into ice-cold preservation media (BIOPS; 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$-$6H_2O$, 20 mM taurine, 15 mM Na_2phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, and 50 mM MES; pH 7.1; (Veksler et al., 1987) and transported to the laboratory for immediate performance of high-resolution respirometry assays, as described below.

Tissue Collection

Heifers of the F1 generation were slaughtered in a federally inspected facility at d 252 (± 3 d) of gestation via captive bolt and exsanguination. Internal organs, including the gravid uterus, were removed, weighed, and sampled. Fetuses were removed and weighed, and fetal muscle (*20 mg)* was collected from the longissimus dorsi muscle. Fetal liver (*20 mg)* was collected from the right anterior section of the right lobe, to mimic the site at which biopsies were taken in the maternal counterpart. Fetal skeletal muscle and liver were transported and processed similarly to maternal muscle and liver tissues, as described above. Jejunum samples were collected from F1 heifers and F2 fetuses. Briefly, the small intestine was removed and measured from the cranial and caudal ends, respectively. The maternal jejunum was located by identifying the portal vein and mesenteric vein to demarcate the sections of the small intestine (Caton et al., 2009). The fetal duodenum denoted the one-meter section measured from the pyloric junction, and the fetal ileum denoted the one-meter section measured from the ileocecal junction. Weights were recorded for the respective sections of the small intestine. A one-meter section of jejunum was transversely cut from the middle of the remaining segment of small intestine. The jejunum section was then cut longitudinally to expose the inner mucosa, and rinsed with distilled water to remove digesta contents. Mucosa was then scraped utilizing a glass microscope slide, similar to

Trotta et al. (2020), and 0.5 g of scraped epithelial mucosa was placed in a microtube containing respiration media (MIR05; 0.5 mM EGTA, 3 mM $MgCl_2$-$6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM d-sucrose, and 1 g/L BSA essentially fatty acid free, pH 7.1) and transported to the laboratory for high-resolution respirometry analysis, as described below.

High-Resolution Respirometry

Liver and Muscle Tissue Permeabilization

Fresh liver samples were placed in a 2-mL microtube containing a saponin solution (0.1 ug/mL BIOPS) and incubated for 20 min at 4ºC. For fresh muscle samples, visual fat was removed from the tissue, and bundled muscle fibers were separated before being placed in saponin solution and incubated for 30 minutes at 4ºC. After permeabilization, both liver and muscle samples were placed in a microtube containing respiration media (MIR05; 0.5 mM EGTA, 3 mM $MgCl_2$-$6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM d-sucrose, and 1 g/L BSA essentially fatty acid free, pH 7.1) and incubated for 10 min at 4 ºC. Samples were then blotted dry. Samples (approximately 5 mg) were placed in chambers of the Oroboros O2k Fluorespirometer (Oroboros Instruments, Innsbruck, Austria) to assess tissue oxygen consumption and mitochondrial function utilizing a substrate-inhibitor-uncoupler protocol (Gnaiger E., 2020).

Jejunum Preparation

Jejunum was immediately transferred to the laboratory for analysis. Jejunum epithelial mucosa was blotted dry. Samples (approximately 20 mg) were then placed in chambers of the Oroboros O2k Fluorespirometer (Oroboros Instruments, Innsbruck, Austria).

Respirometry Analysis

High-resolution respirometry was performed using the Oroboros O2k Fluorespirometer. Each respirometer contains two 2.4-mL chambers (Chambers A and B). To keep consistency, on biopsy days, chamber A was used for liver samples, and chamber B was used for muscle samples. On tissue collection days, chamber A was used for the maternal jejunum and chamber B for the fetal jejunum. The instrument was calibrated before (background calibration) and on the day of collection (air calibration) with MiR05 buffer at 39 ºC. Both calibrations followed manufacturers recommendations according to the type of tissue that was processed. Briefly, for liver samples, the calibrations were performed at air saturation, and for muscle and intestinal samples, hyperoxic conditions were created by adding O_2 gas to the chambers (400 – 600 uM O_2 and 250 – 300 uM O_2 for muscle and intestines, respectively; (Kuznetsov et al., 2008). Mitochondrial respiration (MiR05) media for muscle samples was added with 6 mg creatine to facilitate ADP transport (Walsh et al., 2001).

A substrate-inhibitor-uncoupler protocol (SUIT) was utilized to assess oxygen consumption focused on complex-1 of the electron transport chain, which is responsible for ATP production. Specifically, SUIT – 012 (Oroboros Instruments) was utilized to assess coupling control and NADH-linked substrates (Gnaiger E., 2020). Assay substrates were administered to chambers as follows: 1) pyruvate (5 mM) immediately followed by malate (2 mM) to exhibit the resting oxygen flux compensating for proton leak (LEAK) during dissipative respiration (L); 2) ADP (2.5 mM) to determine oxidative phosphorylation capacity (P) 3) addition of cytochrome C (10 uM) utilized for quality control and indication of mitochondrial outer membrane integrity; 4) glutamate (10 mM) to exhibit nicotinamide adenine dinucleotide (NADH)-linked oxidative phosphorylation respiration and capacity (PI); 5) uncoupler (UCCP) titration, administering 1uL

amount until maximum uncoupled respiration was achieved to exhibit electron transfer capacity (E); 6) Antimycin A (2.5 uM) to inhibit complex III and examine baseline residual oxygen consumption of mitochondrial respiration. The following states are evaluated and calculated as follows: Dissipative respiration (LEAK) describes the oxygen consumption utilized to compensate for proton leak, greater LEAK respiration indicates greater proton leak and heat production. The capacity of OXPHOS respiration (P) indicates the respiratory capacity of mitochondria to produce ATP when abundant substrates are available. Cytochrome C is a protein associated with the inner mitochondrial membrane, respiratory values at this stage are not utilized for calculation but rather to test the integrity of the mitochondrial outer membrane (Gnaiger E., 2020). Samples exhibiting an increase in respiration greater than 15% in response to cytochrome c addition indicate damage to the mitochondrial outer membrane and are therefore excluded from analysis. Respiration of NADH-linked OXPHOS (PI) evaluates the NADH electron transfer-pathway and respiration through glutamate dehydrogenase. Electron transfer capacity (E) measures oxygen consumption in a noncoupled state. Antimycin A administration inhibits the transfer of electrons, subsequently ending the cascade of electron transfer in the respirometry chamber and allows for the determination of residual oxygen consumption (ROX). Oxygen concentrations within the MiR05 buffer were recorded every 2 to 4 s and O_2 fluxes were calculated and corrected for calibration background oxygen flux (DatLab 4 analysis software; Oroboros Instruments). The following calculations are then used to report the integrative (*pmol O2 per mg tissue)* mitochondrial respiratory capacities:

1.) LEAK respiration = (Oxygen consumption rate after pyruvate and malate addition) – (non-mitochondrial respiration or ROX)
2.) OXPHOS capacity = (Oxygen consumption rate after addition of ADP) – (ROX)

3.) NADH – linked OXPHOS capacity = (Oxygen consumption rate after addition of cytochrome C and glutamate) – (ROX)

4.) ET capacity = (Oxygen consumption rate after titration of uncoupler substrate) – (ROX)

Integrative reporting methods, account for whole cell or multicellular tissue respiratory values; whereas intrinsic reporting methods, account for mitochondrial phenotype and density parameters (Divakaruni and Jastroch, 2022).

Protein Concentration

Protein concentration in jejunum, liver, and muscle was assayed using the bicinchoninic acid (BCA) procedure and a Pierce BCA Protein Assay Kit (ThermoScientific, #23227, Rockford, IL, USA), using a microplate reader (SPECTRAmaxTM 340; Molecular Devices Corporation, Sunnyvale, CA, USA), according to manufacturer's directions. Muscle, jejunum, and liver were homogenized and diluted for analysis and concentrations of protein were calculated utilizing manufacturer methods and reported as mg protein/g tissue.

Citrate Synthase

Citrate synthase (CS) activity was analyzed using a commercially available citrate synthase assay kit (CS0720, Sigma-Aldrich, St. Louis, MO, USA), using a microplate reader (SPECTRAmaxTM 340; Molecular Devices Corporation, Sunnyvale, CA, USA), according to manufacturer's directions. Muscle, jejunum, and liver were homogenized for analysis of tissue extracts and activity was normalized to mg of total protein. Citrate synthase activity was calculated utilizing manufacturer methods and reported as units CS (mml/min/mg tissue).

Mitochondrial DNA Copy Numbers

Mitochondrial DNA was extracted from approximately 25 mg of maternal and fetal jejunum, liver, and skeletal muscle tissue utilizing the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Prior to lysis buffer addition, 4 µl of RNase A was added, mixed, and incubated for 2 minutes at room temperature to digest RNA present in the sample. DNA concentration was measured using a Qubit 3.0 fluorometer (Invitrogen, Life Technologies). If DNA concentration was high, samples were diluted to 40 ng/uL. Primers were designed for two mitochondrial genes: *ND2* (NADH dehydrogenase subunit 2) and *COX3* (cytochrome oxidase subunit III). The *ACTB* (actin B) gene was utilized as the nuclear control gene (Integrated DNA Technologies, Coralville, Iowa). For the analysis of mtDNA copy number, quantitate real-time PCR (qPCR) was utilized to amplify genomic DNA on a digital PCR system (QuantStudio 3.0, ThermoFischer Scientific). The reactions for each given gene were performed with approximately 40 ng genomic DNA in triplicates in a final volume of 10 uL PCR reaction mix using 500 nmol/L of the specified primers and the 2 x SYBR Green PCR mix (Biorad, Hercules, CA, USA). The PCR program was run as follows: 5min at 95°C; 40 cycles of 3sec at 95°C followed by 30s at 59°C; with a melting curve analysis of 0.5C increment every 5sec from 65°C to 95°C. Relative mtDNA copy numbers were calculated as: $\text{MtDNA copy number} = 2^{1+(Ct_{n\_gene}-Ct_{mt\_gene})}$, Ct indicating the reported average cycle threshold.

Calculation and Evaluation of Adjustment Factors for Respirometry Data

The following parameters were calculated in order to evaluate different normalization strategies and explore high-resolution respirometry reporting methods.

Oxygen Flux: Protein Concentration

High-resolution respirometry data was expressed as oxygen flux per tissue mass (pmol O_2 · s^{-1} · mg^{-1} tissue). Protein concentration was reported as (mg protein/g tissue); the following equation was utilized to normalize oxygen consumption to protein concentration, a similar function is available in the DatLab 4.0 Analysis Software (Oroboros Instruments):

$$(pmol\ O_2 \cdot s^{-1} \cdot mg^{-1}\ protein) = [(pmol\ O_2 \cdot s^{-1} \cdot mg^{-1}\ tissue) \times (mg\ protein/g\ tissue)]/1000$$

Oxygen Flux: Citrate Synthase

High-resolution respirometry data was expressed as oxygen flux per tissue mass (pmol O_2 · s^{-1} · mg^{-1} tissue). Citrate synthase activity was reported per mass of tissue sample (mmol/min/mg tissue), which allows for intrinsic reporting of this parameter. The following equation was originally reported by (Eigentler et al., 2012):

$$(pmol\ O_2 \cdot s^{-1} \cdot U\ CS) = (pmol\ O_2 \cdot s^{-1} \cdot mg^{-1}\ tissue)/(mmol/min/mg\ tissue)$$

Oxygen Flux: MtDNA

Mitochondrial DNA copy number was first converted to be reported by mg tissue given the specified amount and concentrations extracted from the original DNA sample:

$$MtDNA\ copy\ number/mg\ tissue = \frac{MtDNA\ copy\ number \times dsDNA\ concentration \times 100\ uL\ extracted\ DNA}{Dilution\ factor \times mass\ of\ tissue\ sample}$$

Once the mtDNA was reported per mg tissue, the following equation was used to report oxygen consumption per mtDNA copy number:

$$(pmol\ O_2 \cdot s^{-1} \cdot mtDNA\ copy\ number) = (pmol\ O_2 \cdot s^{-1} \cdot mg^{-1}\ tissue)/(mtDNA\ copy/mg\ tissue)$$

Statistical Analysis

Oxygen consumption data were analyzed as repeated measures using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). Fixed effects included treatment, trimester, and their interactions, and heifer was considered a random effect. Additionally, protein concentration, CS, and mtDNA copy number data were analyzed using the GLM procedure of SAS, with a fixed effect of treatment. The CORR procedure in SAS was used to analyze the correlations between maternal and fetal tissue oxygen consumption, protein concentration, CS, and mtDNA. In addition to the correlations between varying mitochondrial parameters amongst tissue types including: oxygen consumption, protein concentration, CS, and mtDNA. For all analyses, heifer was considered the experimental unit, and *P*-values ≤ 0.05 were considered significant.

Results

Tissue Oxygen Consumption

There was no treatment × trimester interaction for hepatic oxygen consumption rate ($P \geq 0.13$; Table 2.2). Liver mitochondrial function and oxygen consumption in F1 heifers was not influenced by gestational VTM supplementation ($P \geq 0.68$) or trimester of pregnancy ($P \geq 0.27$). Skeletal muscle oxygen consumption exhibited no treatment × trimester interaction ($P \geq 0.32$). However, LEAK respiration (L) in muscle was greater ($P = 0.05$) in CON heifers compared with VTM heifers. Additionally, L was greater ($P = 0.02$) in the third trimester compared to the second trimester (Table 2.2).

Table 2.2. Oxygen consumption (O2 Flux (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) measured in liver and muscle tissue during the 2nd and 3rd trimester of pregnancy in F1 heifers with gestationally differing backgrounds (CON or VTM).

	Treatment[2]							
	CON		VTM			*P*-values[3]		
Item[1]	2nd trimester	3rd trimester	2nd trimester	3rd trimester	SEM	TRT	Trimester	TRT x trimester
Liver								
L	2.64	2.30	2.38	2.55	0.28	0.99	0.83	0.53
P	8.91	10.75	9.71	9.05	0.81	0.70	0.61	0.29
PI	9.96	13.32	11.42	10.83	0.88	0.68	0.27	0.13
E	12.83	16.46	14.76	13.70	1.09	0.79	0.41	0.14
Muscle								
L	1.71	3.83	0.72	2.07	0.47	0.05	0.02	0.57
P	22.45	24.69	19.64	20.60	2.26	0.30	0.63	0.84
PI	22.84	29.09	23.61	22.51	2.52	0.43	0.48	0.32
E	26.69	33.27	28.47	31.91	3.01	0.96	0.26	0.72

[1] The items evaluated via high-resolution respirometry include: LEAK respiration (L), oxidative phosphorylation (OXPHOS) capacity (P), NADH-linked OXPHOS respiration (PI), and electron transfer capacity (E).
[2] Treatments were: CON - Control F1 heifers from dams that received a basal diet comprised of 60% grass hay, 30% corn silage, and 10% DDGS premix without vitamin and trace mineral supplement or VTM – Heifers from dams that received the basal diet above with addition of top-dressed vitamin and trace mineral supplement (113 $g \bullet heifer^{-1} \bullet d^{-1}$, Purina Wind & Rain Storm All-Season 7.5 Complete, Land O'Lakes, Inc., Arden Hills, MN)
[3] Significance considered at $P \leq 0.05$.

There was no treatment effect ($P \geq 0.37$) on jejunum oxygen consumption rates of the F1 offspring, or on hepatic, skeletal muscle, or jejunal oxygen consumption rates of F2 fetal offspring ($P \geq 0.17$). Jejunum mitochondrial function and oxygen consumption in F1 heifers (Table 2.3), as well as, fetal liver, skeletal muscle, and jejunum oxygen consumption of the F2 heifers were not influenced by F0 gestational VTM supplementation (Table 2.4 to 2.6).

There were no significant correlations between fetal and maternal liver oxygen consumption rates ($P \geq 0.28$), nor were there any correlations between F1 and F2 jejunum oxygen consumption ($P \geq 0.31$). However, within the skeletal muscle, F1 and F2 skeletal muscle

oxygen consumption of the OXPHOS and NADH-linked OXPHOS capacities were moderately correlated ($P \leq 0.02$).

Table 2.3. Oxygen consumption (O2 Flux (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) measured in jejunum tissue of F1 heifers with gestationally differing backgrounds (CON or VTM) at d 252 ± 3 of gestation.

	Treatment[2]		SEM	
Item[1]	CON	VTM		*P*-value[3]
Jejunum				
L	1.15	0.74	0.54	0.53
P	1.29	1.36	0.44	0.89
PI	4.76	6.37	1.33	0.39
E	5.36	6.52	1.33	0.55

[1] The items evaluated via high-resolution respirometry include: LEAK respiration (L), oxidative phosphorylation (OXPHOS) capacity (P), NADH-linked OXPHOS respiration (PI), and electron transfer capacity (E).
[2] Treatments were: CON - Control F1 heifers from dams that received a basal diet comprised of 60% grass hay, 30% corn silage, and 10% DDGS premix without vitamin and trace mineral supplement or VTM – Heifers from dams that received the basal diet above with addition of top-dressed vitamin and trace mineral supplement (113 g•heifer^{-1}•d^{-1}, Purina Wind & Rain Storm All-Season 7.5 Complete, Land O'Lakes, Inc., Arden Hills, MN)
[3] Significance considered at $P \leq 0.05$.

Table 2.4. Oxygen consumption (O2 Flux (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) measured in jejunum tissue of F2 heifers with grand-maternal gestationally differing backgrounds (CON or VTM).

	Treatment[2]			
	CON	VTM	SEM	*P*-value[3]
Item[1]	Fetal	Fetal		TRT
Jejunum				
L	2.74	2.06	0.40	0.17
P	7.67	7.58	1.07	0.95
PI	10.93	10.30	1.59	0.76
E	13.50	12.37	1.69	0.61

[1] The items evaluated via high-resolution respirometry include: LEAK respiration (L), oxidative phosphorylation (OXPHOS) capacity (P), NADH-linked OXPHOS respiration (PI), and electron transfer capacity (E).
[2] Treatments were: CON - Control F2 offspring from F1 heifers whose F0 dam received a basal diet comprised of 60% grass hay, 30% corn silage, and 10% DDGS premix without vitamin and trace mineral supplement or VTM – F2 offspring from F1 heifers whose F0 dam received the basal diet above with addition of top-dressed vitamin and trace mineral supplement (113 g•heifer^{-1}•d^{-1}, Purina Wind & Rain Storm All-Season 7.5 Complete, Land O'Lakes, Inc., Arden Hills, MN)
[3] Significance considered at $P \leq 0.05$.

Table 2.5. Oxygen consumption (O2 Flux (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) measured in liver tissue of F2 heifers with grand-maternal gestationally differing backgrounds (CON or VTM).

	Treatment[2]		SEM	*P*-value[3]
	CON	VTM		
Item[1]	Fetal	Fetal		TRT
Liver				
L	3.31	3.73	0.30	0.35
P	10.44	10.90	1.05	0.81
PI	12.27	12.95	1.34	0.79
E	17.00	17.36	1.18	0.83

[1] The items evaluated via high-resolution respirometry include: LEAK respiration (L), oxidative phosphorylation (OXPHOS) capacity (P), NADH-linked OXPHOS respiration (PI), and electron transfer capacity (E).
[2] Treatments were: CON - Control F2 offspring from F1 heifers whose F0 dam received a basal diet comprised of 60% grass hay, 30% corn silage, and 10% DDGS premix without vitamin and trace mineral supplement or VTM – F2 offspring from F1 heifers whose F0 dam received the basal diet above with addition of top-dressed vitamin and trace mineral supplement (113 $g \bullet heifer^{-1} \bullet d^{-1}$, Purina Wind & Rain Storm All-Season 7.5 Complete, Land O'Lakes, Inc., Arden Hills, MN)
[3] Significance considered at $P \leq 0.05$.

Table 2.6. Oxygen consumption (O2 Flux (pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) measured in muscle tissue of F2 heifers with grand-maternal gestationally differing backgrounds (CON or VTM).

	Treatment[2]		SEM	*P*-value[3]
	CON	VTM		
Item[1]	Fetal	Fetal		TRT
Muscle				
L	1.39	0.53	0.71	0.32
P	30.92	28.67	3.18	0.62
PI	35.72	34.02	4.59	0.87
E	37.54	37.46	4.37	0.74

[1] The items evaluated via high-resolution respirometry include: LEAK respiration (L), oxidative phosphorylation (OXPHOS) capacity (P), NADH-linked OXPHOS respiration (PI), and electron transfer capacity (E).
[2] Treatments were: CON - Control F2 offspring from F1 heifers whose F0 dam received a basal diet comprised of 60% grass hay, 30% corn silage, and 10% DDGS premix without vitamin and trace mineral supplement or VTM – F2 offspring from F1 heifers whose F0 dam received the basal diet above with addition of top-dressed vitamin and trace mineral supplement (113 $g \bullet heifer^{-1} \bullet d^{-1}$, Purina Wind & Rain Storm All-Season 7.5 Complete, Land O'Lakes, Inc., Arden Hills, MN)
[3] Significance considered at $P \leq 0.05$.

Evaluation of Oxygen Consumption Normalization Strategies

Protein concentration of all tissue types collected from both F1 heifers and F2 fetuses, were not influenced by F0 VTM supplementation ($P \geq 0.39$; Figure 1). Additionally, CS activity ($P \geq 0.13$; Figure 2) and mitochondrial DNA (mtDNA) copy number of ND2 ($P \geq 0.18$; Figure 3) and COX3 ($P \geq 0.24$; Figure 3) were not influenced by VTM supplementation.

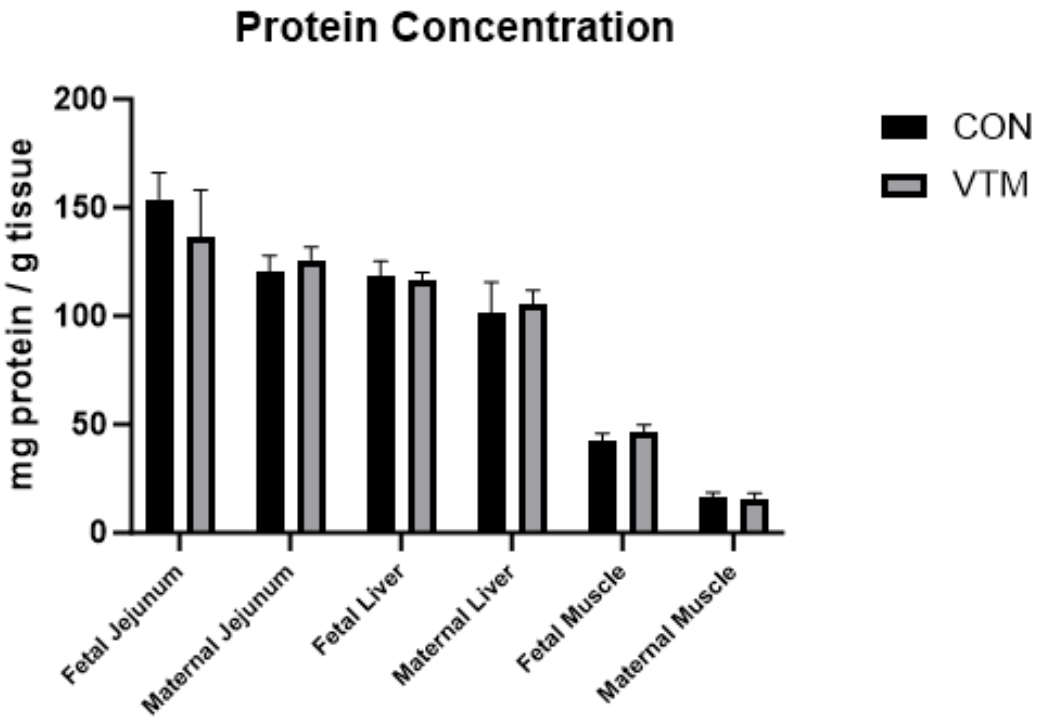

Figure 2.1. Protein concentration in jejunum, liver, and muscle of F1 heifers (maternal) and F2 fetuses (fetal) in response to F0 vitamin and mineral supplementation throughout gestation (CON, not supplemented vs. VTM, supplemented). Values are least square means with error bars depicting standard error. No significant difference in protein concentration was noted across tissue types between CON and VTM treatments ($P \geq 0.39$).

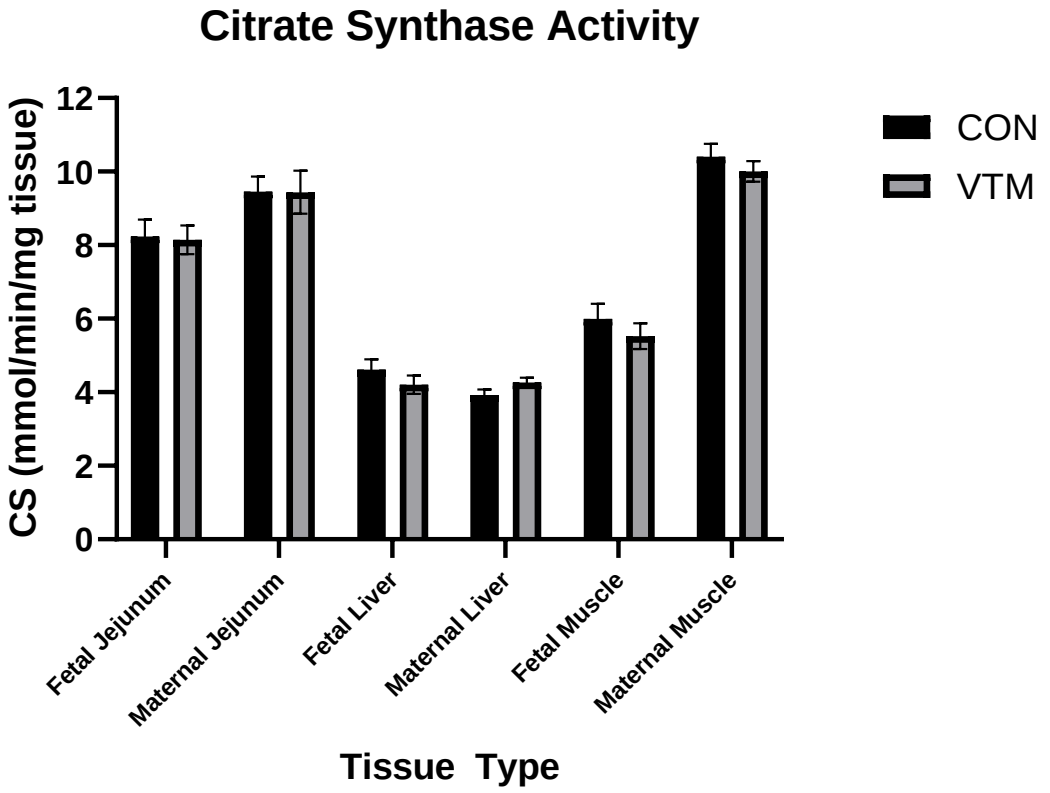

Figure 2.2. Citrate synthase activity in jejunum, liver, and muscle of F1 heifers (maternal) and F2 fetuses (fetal) in response to F0 vitamin and mineral supplementation throughout gestation (CON, not supplemented vs. VTM, supplemented). Values are least square means with error bars depicting standard error. No significant difference in CS activity was noted across tissue types between CON and VTM treatments ($P \geq 0.13$).

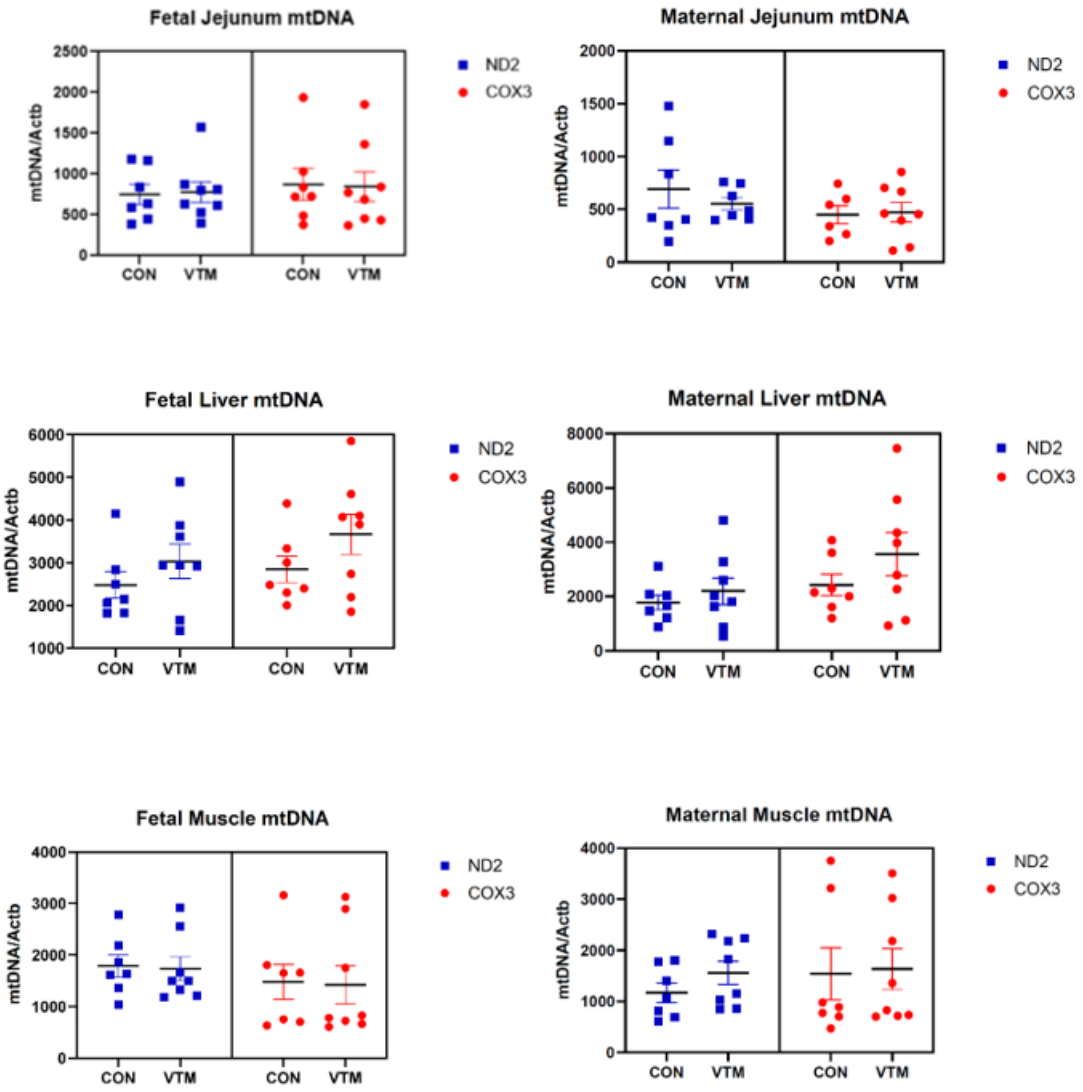

Figure 2.3. Mitochondrial DNA (mtDNA) copy number of ND2 and COX3 genes in jejunum, liver, and muscle of F1 heifers (maternal) and F2 fetuses (fetal) in response to F0 vitamin and mineral supplementation throughout gestation (CON, not supplemented vs. VTM, supplemented). Values are least square means with error bars depicting standard error. No significant difference in mtDNA was noted across tissue types between CON and VTM treatments ($P \geq 0.18$).

Citrate synthase activity and mtDNA results were utilized to intrinsically report respiratory capacities, in order to evaluate mitochondrial density's potential impact on oxygen consumption. Mitochondrial capacities intrinsically reported as (pmol/sec/U CS), showed that there was no significant difference in treatments across tissue type ($P \geq 0.17$), and was similarly non-significant when reported as pmol/sec/U COX3 ($P \geq 0.15$). However, when respiratory capacities were reported intrinsically, in relation to ND2 copy number, maternal muscle L in CON heifers trended similarly (P = 0.06; Figure 2.5) to its reported significantly increased integrative value ($P = 0.05$). Furthermore, maternal muscle NADH-linked OXPHOS respiration (PI), tended to be greater in CON heifers ($P = 0.085$). All other respiratory values reported as pmol/sec/U ND2, across tissue types, were not significantly different ($P \geq 0.12$).

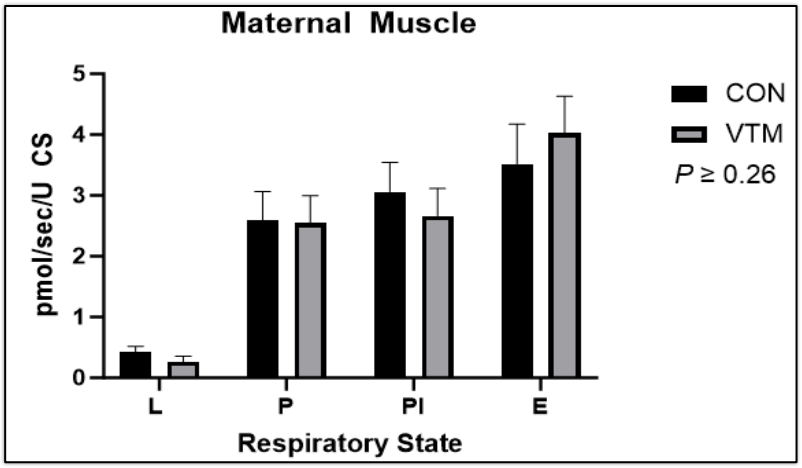

Figure 2.4. Oxygen consumption per unit, citrate synthase, in muscle of F1 heifers in response to F0 vitamin and mineral supplementation throughout gestation (CON, not supplemented vs. VTM, supplemented). Values are least square means with error bars depicting standard error. No significant difference in intrinsically reported oxygen consumption was noted across tissue types between CON and VTM treatments (P ≥ 0.06).

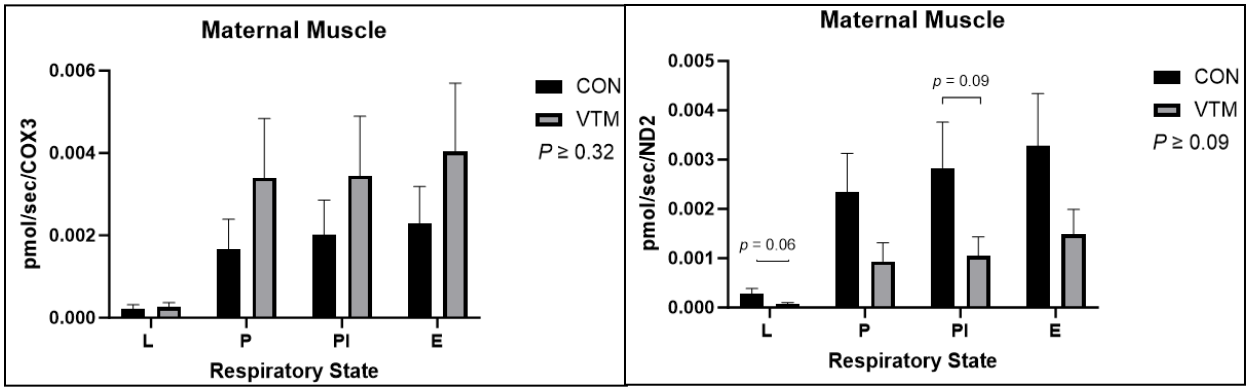

Figure 2.5. Oxygen consumption per unit ND2, and COX3; in muscle of F1 heifers in response to F0 vitamin and mineral supplementation throughout gestation (CON, not supplemented vs. VTM, supplemented). Values are least square means with error bars depicting standard error. No significant difference in intrinsically reported oxygen consumption noted across tissue types between CON and VTM treatments (P ≥ 0.06).

A strong correlation between ND2 and COX3 copy number was observed in the fetal liver (r = 0.936; *P* = 0.0001) and fetal muscle (r = 0.839; *P* = 0.0001). However, this trend in other tissue types including maternal jejunum, liver, muscle, and fetal jejunum were not strongly correlated (*P* ≥ 0.11). In the fetal jejunum, protein concentration normalized to mg tissue was strongly correlated with ND2 when reported as mg tissue (r = 0.73; *P* = 0.002), and tended to be moderately correlated with COX3 when reported as mg tissue (r = 0.45; *P* = 0.09). No other correlations were noted across the other density parameters in the additional tissue types. When evaluating oxygen consumption across the normalization strategies it was observed that oxygen consumption reported as pmol/sec/U ND2 and pmol/sec/U COX3, were highly correlated in fetal jejunum, liver, and muscle, as well as, maternal jejunum (*P* = < 0.0001). Additionally, pmol/sec/U ND2 and pmol/sec/ U CS were moderately correlated in fetal jejunum, muscle, and maternal jejunum (*P* ≤ 0.04). No correlations between normalization strategies were observed in the maternal liver (*P* ≤ 0.12). Maternal muscle was moderately correlated between pmol/sec/U ND2 and pmol/sec/ U CS, at the OXPHOS and NADH- linked OXPHOS respiratory states (r = 0.57; *P* ≤ 0.03); this trend was also seen between pmol/sec/U COX3 and pmol/sec/ UCS (r =

0.59; $P \leq 0.02$). No other strong or moderate correlations were observed across tissue type normalization strategies.

Discussion

This study assessed liver, skeletal muscle, and jejunum mitochondrial function and efficiency to evaluate the impacts of maternal VTM supplementation on the energy metabolism of F1 offspring during their first pregnancy and their F2 offspring at 252 ±3 days. Results showed that mitochondrial function and capacity in the liver and jejunum of F1 heifers were not influenced by F0 gestational VTM supplementation. However, skeletal muscle L was greater in F1 CON compared with F1 VTM heifers. Additionally, L was greater in the third trimester compared to the second trimester. Currently, there is not a large amount of research regarding skeletal muscle mitochondrial respiration modulations over the course of gestation. However, by observing an increase in L respiration, we can consider the exponential metabolic activity required in the third trimester of pregnancy (Bauman and Bruce Currie, 1980) and the increased presence of oxidative stress (Sciorsci et al., 2020), it is possible that increased reactive oxygen species induce proton leak, as they are mutually regulated (Nanayakkara et al., 2019). Evaluating mitochondrial density parameters of the first and second trimester would provide beneficial insight on additional modulations of mitochondria during gestation. Furthermore, continued evaluation of whole animal energetics and heat production during gestation, will benefit the presented data to deepen understanding of beef cattle gestational nutrient requirements. The increase in oxygen consumption we observed in the third trimester is in agreement with Prezotto et al. (2016), who observed that liver and jejunum oxygen consumption increased linearly throughout gestation.

The difference in respiration between CON and VTM heifers is most notable, as it provides a possible mode of action as to why VTM heifers exhibited a gain advantage compared to CON heifers (Hurlbert et al., 2024). Vitamin and mineral supplementation is an easily adoptable practice in the cattle industry and could allow producers to economically benefit through calf weight advantages. Hurlbert et al. (2024) reported weaning weights and yearling weights of the F1 VTM heifers involved with this study and observed that VTM heifers were, on average, 36 kg and 19 kg heavier than CON heifers, respectively. The observation that CON heifers tended to weigh less than VTM heifers may be related to differences in muscle metabolism, as LEAK respiration was greater in CON heifers. Greater oxygen consumption during LEAK respiration indicates increased proton leak and potentially decreased efficiency of mitochondrial respiration (Gnaiger E., 2020). Protons that leak or escape complex-1 are not utilized to produce ATP and, as a result, produce heat as a byproduct, resulting in a potentially less efficient animal. Research in aged mice hepatocytes supports the idea of less efficient energy utilization as it has been shown that incidences of increased proton leak results in a decrease in ATP turnover (Harper et al., 1998). Additionally, a compensatory effect was noted as oxygen consumption in hepatocytes increased in order to maintain the proton motor force of the electron transport chain (Harper et al., 1998).

Low-feed efficient animals may have compromised mitochondrial activity in complexes I and II of the electron transport chain. In a previous report evaluating feed efficiency of steers, greater oxygen consumption during basal respiration was observed in low-RFI steers than high-RFI steers (Casal et al., 2018). Casal's study largely focused on Complex II; therefore, it is important to explore potential differences in Complex I, which include proton leak and uncoupling proteins. By evaluating Complex-I, we have identified greater LEAK respiration and

potentially mitochondrial inefficiency in CON heifers. This is in agreement with a previous report (Bottje et al., 2002) in inefficient broiler chickens. Bottje et. al (2002) reported that inefficient broilers may have increased proton leak of Complex-I as less tightly-coupled ETC was observed in breast and leg muscle mitochondria.

As pregnancy progresses, maternal liver oxygen consumption linearly increases as well as jejunal oxygen consumption (Prezotto et al., 2016); this was observed alongside a decrease in jejunal mass – a potential compensatory mechanism during pregnancy. Energy conservation in liver and jejunum was previously observed by Scheaffer et al. (2003), noting although jejunal oxygen consumption increased linearly throughout gestation, oxygen consumption was less than non-pregnant heifers. Additionally, jejunal cell proliferation decreased from d 120 to d 200 (Scheaffer et al., 2003). In heifers fed 100% of their nutritional requirements, maternal oxygen consumption increased, jejunal mass decreased, and fetal hepatic and jejunal mass increased (Prezotto et al., 2016), whereas heifers that were nutritionally restricted had lower maternal hepatic mass and oxygen consumption, and greater maternal jejunal oxygen consumption. Fetal hepatic weight was greater compared to fetuses from dams provided 100% of their nutrient requirement and fetal jejunal oxygen consumption was lower in fetuses from restricted dams (Prezotto et al., 2016). These studies show the variety of modulations that take place to compensate for pregnancy and available nutrients throughout gestation, with further investigations required to evaluate micronutrient supply.

Knowing that the liver is a highly adaptable organ, it is important to consider that the F1 heifers were fed a common TMR which included vitamin and mineral supplement. The potential for the supplement to compensate for fetal programmed effects, as well as organ compensation, may exist (Prezotto et al., 2014). Based on the results of this experiment, it is likely that liver

function was not different between the gestationally different cohorts as they received the same diet post-weaning and throughout the experiment, which included vitamin and mineral supplement. Menezes et al. (2022) previously reported no differences in liver mitochondrial function and oxygen consumption at 30 hr post-birth in neonatal calves, between similar VTM and CON treatments as used in the current experiment (Menezes et al., 2022).

Signals and microbiome alterations of the GIT impact liver and muscle metabolism, functionality, and biogenesis (Carvalho et al., 2020). Menezes et al. (2022) observed a greater capacity for ATP production in neonatal calves from dams that received VTM supplementation during gestation as indicated by greater oxygen consumption during several stages of mitochondrial respiration (Menezes et al., 2022). Differences that may have been initially present at birth may have absolved as heifers grew, developed, and were placed on a similar diet. The high rate of protein and cellular turnover in the GIT could be a possible explanation for similar mitochondrial adaptions (Lobley, 2003). The GIT's ability to turnover its epithelial layer every 3 - 5 days is not only a protective mechanism but also supports the concept that of its ability to adapt to dietary changes, stressors, or microbiome alterations (Rath et al., 2018). Alternatively, programmed effects can remain latent over periods of time and may be displayed only when appropriate metabolic challenges or conditions are present (Pankey et al., 2017; Baumgaertner et al., 2024).

Mitochondrial function and capacity in the liver, skeletal muscle, and jejunum of F2 fetuses were not influenced by F0 gestational VTM supplementation at 0.9 gestation. Similarly, Aiken et al. (2015) reported that "grand-maternal" or F0 diet restriction had no effect on F2 offspring body weight, organ weight, or mitochondrial quantity three days post-parturition. However, as F2 rat offspring exposed to a gestationally restricted diet had increases in intra-

abdominal fat through post-natal development (Aiken et al., 2015). Further transgenerational studies are required to better understand the "grand-maternal" diet effect on the F1 ovarian follicle epigenetic characteristics and subsequent influence on the F2 generation of offspring. It is well established that maternal diet impact fetal development and programming. The F1 generation included in this study received the same nutritionally complete diet, therefore the F1 maternal diet may have had greater effect on the F2 generation than the indirect effect of F0 grandmaternal diet (Prizak et al., 2014). Future research is need to increase our understanding of the complex relationship among maternal and grandmaternal effects.

Evaluation of Adjustment Factors for Respirometry Data

The current study examined protein concentration, CS activity, and mtDNA quantity to evaluate treatment differences, as well as, compare oxygen consumption to these commonly used markers to aid in the standardization of Oxygraph-O2k data. By evaluating mitochondrial density, we can determine if oxygen consumption values observed differ due to modulations in mitochondrial function or if mitochondria density is increased or decreased. Evaluation of dietary selenium supplementation relative to integrative and intrinsic capacities were evaluated shown to be different depending on the data were reported (Owen et al., 2022). Oxygen consumption per mg tissue (integrative) was improved fo complex 1, however further evaluation of intrinsic CS and CCO activity indicated that this improvement was likely due to increased mitochondrial density (Owen et al., 2022). Thus, to better understand the mechanisms regulating differences in mitochondrial function, it is important to also report measures of mitochondrial density.

The observed lack of differences in protein concentration, citrate synthase activity, and mtDNA copy number in comparison to treatment suggest that the measured mitochondrial

respiratory capacities are not influenced by mitochondrial density. Overall, F0 maternal gestational VTM supplementation had no significant impacts on mitochondrial respiration or mitochondrial density in the maternal jejunum, maternal liver, fetal jejunum, fetal liver, and fetal muscle. Maternal muscle had increased LEAK respiration in CON F1 heifers, when reported integratively (per mg tissue) but measures of mitochondrial density were unaffected suggesting that CON heifers potentially had inefficiencies within complex-1 of the electron transport chain.

The ND2 mitochondrial gene codes for the production of NADH dehydrogenase 2, and is a part of mitochondria complex-1 within the electron transport chain (Tian et al., 2021). The COX3 mitochondrial gene codes for cytochrome c oxidase, which aids in driving oxidative phosphorylation and is a component of complex IV (Faitg et al., 2020). Reporting O2 flux in relation to MT-ND2, LEAK respiration tended increase ($P = 0.06$) in CON F1 heifers, although no significant difference in ND2 copy number between treatment groups was noted. This could represent an intrinsic indicator comparative to integrative mitochondrial capacities, whereas reporting data intrinsically through CS activity and COX3 copy number absolves any significant difference previously noted. Measuring mitochondrial density or even isolating mitochondria to evaluate intrinsic respiratory values may be warranted when changes in mitogenesis are speculated to be expected (Divakaruni and Jastroch, 2022).

Standardization of high-resolution respirometry and mitochondrial quantification of various tissue types could prove to be beneficial in future diagnostic practices. However, additional research data across tissue treatment types are needed to achieve a diagnostic standard. The use of biopsy techniques in livestock has the potential to indicate metabolic patterns which could be utilized to sort animals based on efficiency or indicate differences in animal health status. Continuing to study cellular and whole-animal energy metabolism could result in the

development of nutritional or animal selection management strategies to improve economic gain and provide more beef for consumers.

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CHAPTER 3: THE EFFECTS OF LIMIT-FEEDING A HIGH-CONCENTRATE OR HIGH-FORAGE DIET ON REPLACEMENT HEIFER AND FETAL OFFSPRING LIVER AND JEJUNUM TISSUE OXYGEN CONSUMPTION AND MITOCHONDRIAL FUNCTION[2]

Abstract

The study aimed to assess the impact of limit-feeding pregnant replacement heifers high-concentrate (HC) or high-forage (HF) diets on the energy utilization in liver and jejunum tissues of dam and offspring. We hypothesized that altering the diet would modulate tissue oxygen consumption and mitochondrial function. Once received at the NDSU Beef Cattle Research Complex, replacement heifers (n=20; initial body weight [BW] = 339.7 ± 33 kg) were blocked by initial body weight and randomly assigned to either a high-concentrate (HC; n = 10) or high-forage (HF; n = 10) diet targeting BW gains of 0.45 kg•heifer-1•d-1. After an adjustment period, heifers were on their allocated treatment diet for approximately 85 days prior to conception to male sexed semen, and remained on their respective dietary treatments until time of fetal tissue collection. Heifers were euthanized on d 180 ± 3 of gestation (n = 20; final body weight [BW] = 481.7 ± 33 kg), at which time maternal liver, jejunum, and fetal jejunum were collected, and tissue oxygen consumption and mitochondrial function were assessed via high-resolution respirometry (Oroboros Instruments, Innsbruck, Austria). The following respiratory states were evaluated: LEAK respiration (L), OXPHOS capacity (P), NADH-linked OXPHOS (PI), and

[2] The material in this chapter was co-authored by K. R. M. Slavick, J. L. Hurlbert, B. Kuzel, J. D. Kirsch, K. C. Swanson, and C. R. Dahlen. J. L. Hurlbert had primary responsibility for collecting liver and muscle biopsies and K. R. M. Slavick was the primary developer of the conclusions presented here. K. R. M. Slavick also drafted and revised all versions of this chapter, and C. R. Dahlen and K. C. Swanson assisted in revisions, statistical analysis, and other manuscript preparations.

electron transfer capacity (E). Data were analyzed using the GLM procedure of SAS with a fixed effect of treatment. Maternal and fetal jejunum were largely not influenced by maternal dietary treatment. However, high-forage heifers tended to have increased LEAK respiration of the maternal jejunum ($P \geq 0.095$) and maternal hepatic oxygen consumption was decreased in PI ($P = 0.037$) and E respiration ($P = 0.0367$), in HC heifers compared to HF heifers. The observed differences between HC and HF heifer mitochondrial respiration indicate an increased mitochondrial efficiency and improved ATP synthesis functionality in HF heifers.

Introduction

The majority of the cattle industry utilizes *ad libitum* feeding methods to provide nutrition to their cattle, whether in cow-calf operations, backgrounding beef production, or finishing feedlots. Research studying the effects of feeding diets with differing concentrate:forage ratio has been studied for many years (Blaxter and Wainman, 1964), and restricting feed intake has been shown to decrease cow energy requirements for maintenance (Koong et al., 1985). Limit-feeding high-energy diets to cattle has been previously introduced as a method to improve diet utilization and lower the energy requirements of the animal (Trubenbach et al., 2019).

High-concentrate diets, comprised of 70% concentrate feeds or more, are typically fed to support rapidly growing cattle on finishing feedlot diets (Terry et al., 2021) and are used to improve feedlot steer performance and consumer desirable carcass characteristics. However utilization of HCD with replacement heifers, designed to be retained for calf-rearing needs, warrants further research to develop an understanding of how it may impact function and energy utilization of key metabolic organs and on potential impacts on fetal programming.

Studies have shown the importance of meeting nutritional needs during the periods of puberty attainment, breeding, and gestation, both for the dam and future offspring (Caton et al., 2018; George et al., 2012). Previous observations show that *ad libitum* feeding of high-concentrate diets reduces first heifer lactation and mammary development (Swanson, 1960). However, when introducing limit-feeding strategies to reduce average daily gain (ADG) to a similar level as when forage-based diets are fed, there is no influence on heifer lactation and mammary development (Carson et al., 2000). Dairy cows in early lactation in pasture-based systems, compared to those on a well-balanced TMR, have shown impaired mitochondrial function, highlighting the importance of meeting the nutritional needs (García-Roche et al., 2022). Previous results have indicated greater efficiency of ATP synthesis in the jejunum of neonatal calves from vitamin and trace mineral supplemented dams (Menezes et al., 2022), which may indicate a greater capacity of nutrient utilization (Casal et al., 2018). Other maternal dietary modulations, such as nutrient restriction, result in decreased fetal jejunum oxygen consumption at d 85 of gestation (Prezotto et al., 2016). Research is lacking on the effects of changes in forage:concentrate on tissue mitochondrial function and energetic efficiency.

The scope of the study was to evaluate the influence of high-concentrate and high-forage diets, in a limit-fed setting, on liver and jejunum tissue oxygen consumption and mitochondrial function. Knowing that with high-concentrate diets, the proportion of propionate relative to acetate is increased (Chen et al., 2021), we hypothesized that the resulting differences in available substrates would influence liver and jejunum mitochondrial function of replacement heifers.

Materials and Methods

All animal care, management practices, and experimental procedures were approved by the North Dakota State University Institutional Animal Care and Use Committee (Protocol #20210043).

Animal, Housing, and Diet

One-hundred and nineteen crossbred Angus heifers, sourced from North Dakota State University's Central Grasslands Research Extension Center, were received at the NDSU Beef Research Complex in Fargo, ND, at approximately thirteen months of age. Replacement heifers were blocked by initial body weight (n=119; initial body weight [BW] = 339.7 ± 33 kg) and randomly assigned to either a high-concentrate (HC; n = 59) or high-forage (HF; n = 60) diet targeting BW gains of 0.45 kg•heifer-1•d-1. Heifers were ranked by body weight and sorted into one of 6 pens and fed individually via an electronic feed-bunk (Instentec Roughage Intake Control System, Hokofarm B.V., Marknesse, The Netherlands). All heifers were given a period of adaptation to adjust to the feeding system. High-concentrate heifers were given adequate time to adjust and step-up to their final treatment diet consisting of: 55% corn grain, 20% corn silage, 15% oat hay, 7.2% dried distillers grain, and 2.8% pre-mix. High-forage treatment diet consisted of 65% oat hay, 20% corn silage, 7.9% dried distillers' grain, 5% corn grain, and 2.1% pre-mix (Table 3.1). The adjustment period ended, and final diets were implemented 15 days prior to the first breeding. Heifers were weighed bi-weekly, and feed allotments were adjusted based on performance to achieve a target gain of 0.45 kg/day.

Table 3.1. Feed ingredients of diets delivered to gestating replacement heifers limit-fed either a high-concentrate (HC) or high-forage (HF) diet[1]

Ingredient, % DM	HC	HF
Corn Silage	20.0	20.0
DDGS	7.18	7.88
Corn Grain	55.0	5.0
Winter Wheat/Blended Hay	15.0	65.0
Limestone	0.90	0.50
Salt	0.30	0.20
Urea	0.85	
Monensin	0.02	
Trace Mineral Mix	0.05	
Vitamin A	0.20	
Vitamin D	0.20	
Vitamin E	0.10	
Total	100.0	

Heifers were subjected to a 7-d Select Synch + CIDR estrus synchronization protocol (Lamb et al., 2010) and bred via artificial insemination using male-sexed semen from a single sire. Pregnancy diagnosis was confirmed via transrectal ultrasonography on d 35 following AI, with fetal sex confirmation at d 65 following AI. Heifers (n = 46) of the first breed group continued on their respective treatment diet through gestation and were used for a different experiment. Heifers that were not pregnant at the first ultrasound (n = 32 HC and 29 HF, respectively) were resynchronized and bred to sexed male semen from a single sire 85 d after beginning to receive their respective treatment diets. The remaining heifers were subjected to a 7-d Select Synch + CIDR estrus synchronization protocol (Lamb et al., 2010) and bred via artificial insemination using male-sexed semen from a single sire. Pregnancy diagnosis was confirmed via transrectal ultrasonography on d 35 following AI, with fetal sex confirmation at d 65 following AI. Twenty heifers pregnant to the second insemination with male fetuses were used as experimental units for the current experiment (n = 10 HC, and n = 10 HF, respectively).

Heifers continued to be weighed bi-weekly, and feed allotments were adjusted based on performance to achieve a target gain of 0.45 kg/day for all heifers.

Tissue Collection

Heifers were slaughtered at 180 ± 3 days of gestation (final body weight [BW] = 481.7 ± 33 kg), via captive bolt and exsanguination. Internal organs, including the gravid uterus, were removed, weighed, and sampled. Fetuses were removed and weighed, and fetal jejunum (*20 mg)* was procured. Maternal liver (*20 mg)* was collected from the right anterior section of the right lobe, to mimic the site at which biopsies are commonly taken. Jejunum located by identifying the portal vein and mesenteric vein to demarcate the sections of the small intestine (Caton et al., 2009; Scheaffer et al., 2004). The fetal jejunum was transversely cut from the small intestine and then cut longitudinally to expose the inner mucosa. Due to the fragility of the fetal jejunum at d 180 of gestation, a section of the whole jejunum was used rather than a mucosal scrape. A one-meter section of maternal jejunum was transversely cut from the middle of the remaining segment of small intestine. The jejunum section was then cut longitudinally to expose the inner mucosa and rinsed with distilled water to remove digesta contents. Maternal mucosa was then scraped utilizing a glass microscope slide, similar to Trotta et al. (2020), and 0.5 g of scrapped epithelial mucosa was placed in a microtube containing ice-cold preservation media (BIOPS; 2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2$-$6H_2O$, 20 mM taurine, 15 mM Na_2phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, and 50 mM MES; pH 7.1; (Veksler et al., 1987). Additionally, the maternal liver and fetal jejunum were placed in BIOPS. Tissue samples were transported to the laboratory for high-resolution respirometry analysis.

High-Resolution Respirometry

Jejunum samples were removed from the microtube, blotted dry, and measured to a wet weight of 20 – 25 mg for maternal jejunum and 10 – 15 mg for fetal jejunum. For permeabilization, liver samples were placed in a microtube containing a saponin solution (0.1 ug/mL BIOPS) and incubated for 20 min at 4ºC. After the permeabilization period, liver samples were placed in a microtube containing respiration media (MIR05; 0.5 mM EGTA, 3 mM $MgCl_2$-$6H_2O$, 60 mM lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM d-sucrose, and 1 g/L BSA essentially fatty acid free, pH 7.1) and incubated for 10 min at 4 ºC. Samples were then blotted dry and measured to a wet weight of 4 – 6 mg. Samples were then placed in chambers of the Oroboros O2k Fluorespirometer (Oroboros Instruments, Innsbruck, Austria) to assess tissue oxygen consumption and mitochondrial function utilizing a substrate-inhibitor-uncoupler protocol.

One respirometer was utilized throughout the study. Each respirometer contains two 2.4-mL chambers (Chambers A and B). The instrument was calibrated at air saturation, before (background calibration) and on the day of collection (air calibration) with MiR05 buffer at 39 ºC. Both calibrations followed manufacturer's recommendations according to the type of tissue that was processed. Oxygen concentrations within the MiR05 buffer were recorded every 2 to 4-s intervals from which O_2 fluxes were calculated and corrected for calibration background oxygen flux (DatLab 4 analysis software; Oroboros Instruments).

A substrate-inhibitor-uncoupler protocol (SUIT) was utilized to assess oxygen consumption focused on Complex-1 of the electron transport chain, which is responsible for ATP production. Specifically, SUIT – 012 (Oroboros Instruments) was utilized to assess coupling control utilized NADH-linked substrates (Gnaiger E., 2020). Assay substrates were administered

to chambers as follows: 1) pyruvate (5 mM) immediately followed by malate (2 mM) to exhibit the resting oxygen flux compensating for proton leak (LEAK) during dissipative respiration (L); 2) ADP (2.5 mM) to determine oxidative phosphorylation (OXPHOS) capacity (P) 3) addition of cytochrome C (10 uM) utilized for quality control and indication of mitochondrial outer membrane integrity; 4) glutamate (10 mM) to exhibit NADH-linked oxidative phosphorylation respiration and capacity (PI); 5) uncoupler (UCCP) titration, administering 1uL amount until maximum uncoupled respiration was achieved to exhibit electron transfer capacity (E); 6) Antimycin A (2.5 uM) to inhibit complex III and examine baseline residual oxygen consumption of mitochondrial respiration. LEAK respiration describes the oxygen consumption utilized to compensate for proton leak, greater LEAK respiration indicates greater proton leak and heat production. Oxidative phosphorylation capacity indicates the respiratory capacity of mitochondria to produce ATP when abundant substrates are available. NADH-linked OXPHOS respiration evaluates the NADH electron transfer-pathway and respiration through glutamate dehydrogenase. Electron transfer capacity measures oxygen consumption in a noncoupled state.

Statistical Analysis

Data were analyzed using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC), with a fixed effect of treatment; results are reported as least square means (LSMEANS) in addition to their standard errors. The CORR procedure in SAS was used to analyze the correlations between maternal and fetal jejunum oxygen consumption parameters. For all analyses, heifer was considered the experimental unit and P-values ≤ 0.05 were considered significant, and tendencies were considered at $0.05 < P < 0.10$.

Results

Tissue Oxygen Consumption

Maternal and fetal jejunum were largely not influenced by maternal dietary treatment. However, high-forage heifers tended to have increased LEAK respiration ($P \geq 0.095$). No correlation between maternal and fetal jejunum oxygen consumption at any of the evaluated respiratory states was noted ($P \geq 0.43$). However, maternal hepatic LEAK respiration (L) and OXPHOS (P) in the liver tended to be greater ($P = 0.09$) in HF heifers compared to HC heifers. Additionally, NADH-linked OXPHOS (PI) and electron-transfer capacity (E) were greater ($P = 0.04$, $P = 0.04$) in the HF heifers compared to HC heifers.

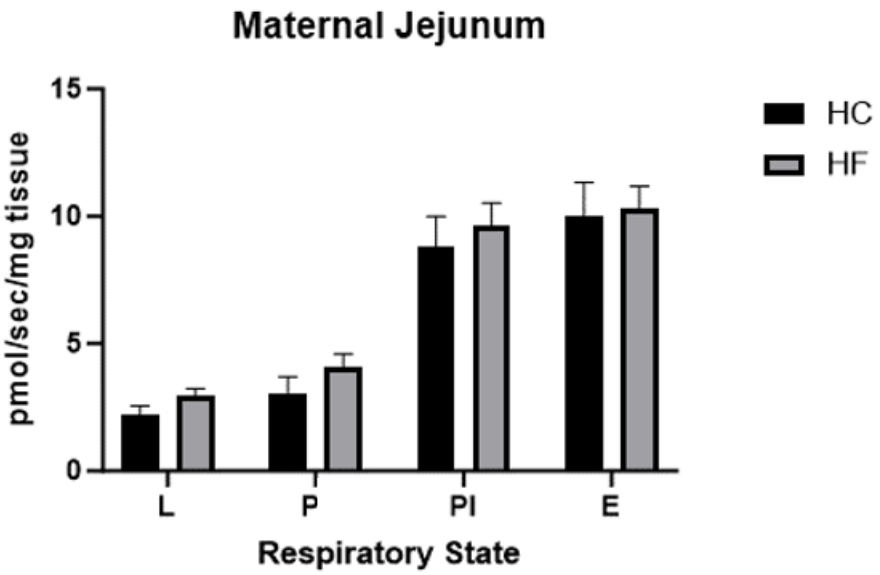

Figure 3.1. Oxygen consumption in the maternal jejunum of replacement heifers in response to consuming high-concentrate (HC) and high-forage (HF) diets during the first 2 trimesters of pregnancy. Values are least square means with error bars depicting standard error. No differences in oxygen consumption were observed in the P, PI, and E respiratory states; HF high-forage heifers tended to have increased LEAK respiration ($P \geq 0.095$).

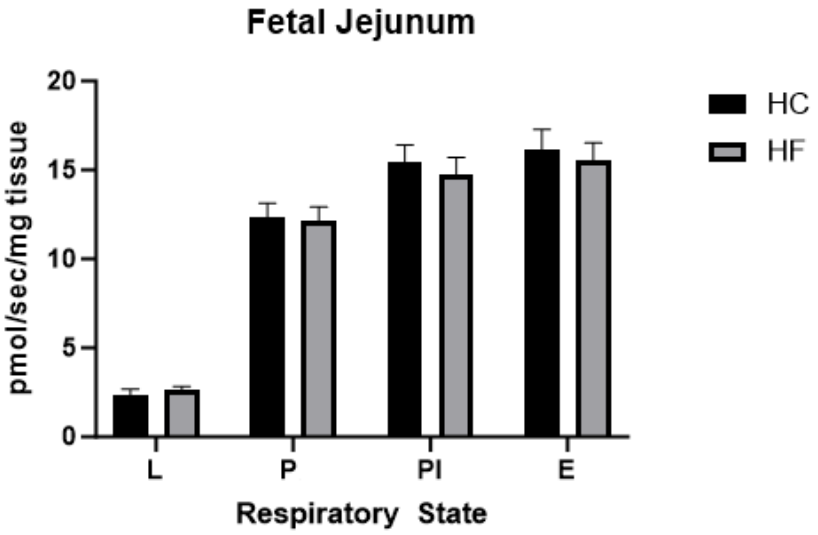

Figure 3.2. Oxygen consumption in the fetal jejunum from replacement heifers fed high-concentrate (HC) and high-forage (HF) dietary treatments. Values are least square means with error bars depicting standard error. No significant difference in oxygen consumption was noted across evaluated respiratory states between HC and HF treatments ($P \geq 0.51$).

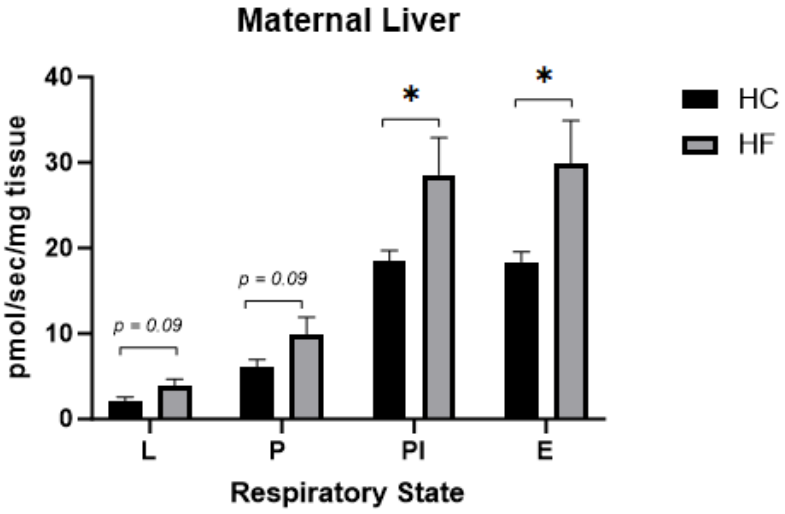

Figure 3.3. Oxygen consumption in the maternal liver of replacement heifers in response to high-concentrate (HC) and high-forage (HF) dietary treatments. Values are least square means with error bars depicting standard error. Dietary treatment impacted hepatic mitochondrial function. LEAK respiration (L) and OXPHOS (P) in the liver tended to be greater ($P = 0.09$) in HF heifers compared to HC heifers. Additionally, NADH-linked OXPHOS (PI) and electron-transfer capacity (E) were greater ($P = 0.04$, $P = 0.04$) in the HF heifers compared to HC heifers.

Discussion

The study assessed liver and jejunum mitochondrial function and efficiency to evaluate the impacts of limit-feeding HC and HF diet on the cellular energy metabolism of replacement heifers and their offspring. Results showed that maternal jejunum from HF heifers tended to have greater oxygen consumption at the LEAK respiratory state and that the fetal jejunum was not influenced by the dietary treatments. However, increased oxygen consumption in liver mitochondrial function were noted at every respiratory state evaluated in this study. These modulations may be attributed to the volatile fatty acids made available through the breakdown and metabolization of the diet treatments in the current study.

As dietary forage inclusion increases, concentrations of acetate increase and propionate decrease, resulting in an increased acetate:propionate ratio (Wang et al., 2020). The respective volatile fatty acids are metabolized differently in the body of the ruminant. Acetate accounts for 90-100% of the total VFA found in arterial blood, with peripheral tissues utilizing about 80% of acetate; acetate is utilized for lipogenesis or oxidized to generate ATP (Bergman, 1990), whereas, propionate is utilized by the liver for gluconeogenesis or oxidized to generate ATP (Lin et al., 2012).

In the current literature, no other study has evaluated the impacts of forage:concentrate inclusion in beef replacement heifers utilizing high-resolution respirometry. However, it has been observed that increased inclusion of calcium propionate modulates the rate of gluconeogenesis in the liver, and that diet can be modified to support gluconeogenesis (Zhang et al., 2015), whereas acetate is utilized in other tissues (Moffett et al., 2020). Due to the great energetic expense of gluconeogenesis, there is a potential for overall reduced ATP synthesis from the liver (Armentano, 1992). The high digestibility and fermentability of high-concentrate

diets are known to increase the fat content of cattle, even when limit-fed, and may pose a risk for an increase in visceral fat accumulation (Robelin, 1986). In human studies regarding fatty liver disease, reduced mitochondrial respiration and OXPHOS activity are observed (Moore et al., 2022). Further evaluations of protein concentration or fat content in liver should be evaluated to determine if our dietary treatments influenced liver fat concentrations potentially contributing to mitochondrial impairments in the liver. Independent of the liver, it has been observed that feeding HC diets can result in mitochondrial dysfunction in the mammary gland of dairy cows (Moffett et al., 2020). The observation of increased efficiency for ATP synthesis and electron transfer capacity in HF heifers could the result of the liver adapting to the lack of gluconeogenesis precursors and energy substrate availability, as acetate is metabolized in peripheral tissues.

Interestingly, no major differences between HC and HF heifer maternal or fetal jejunum oxygen were observed. However, research has suggested that increased concentrate inclusion increases rumen papilla surface area (Dieho et al., 2016), duodenal villi height, and jejunal villi height (Zitnan et al., 2003). The GIT experiences a great rate of intestinal epithelial cell turnover and is home to an expansive population of bacteria. Major impairments of jejunum mitochondrial function are often seen in instances of inflammation and disease (Mittal et al., 2011; Goudie et al., 2022). Dairy cattle consuming HC diets can result in increased incidence of subacute ruminal acidosis (SARA), microbiome perturbations, and inflammation (Sanz-Fernandez et al., 2020). The heifers in the current study did not show any signs of experiencing SARA likely because of they were limit-fed so that growth rates were similar to those of the forage-fed group.

This study found that HF-fed heifers had an increased hepatic efficiency for ATP synthesis and electron transfer capacity compared to that of HC-fed heifers. The diets evaluated

are known to be metabolized differently in the body of ruminants. Therefore, the modulations in mitochondrial function could reflect this differential metabolism and liver function. This research could potentially serve as a reference for future studies studying metabolic disorders by use of high-resolution respirometry.

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CHAPTER 4: SUMMARY AND CONCLUSIONS

Beef cows and replacement heifers account for 1/3 of the United States cattle inventory (Averill and Hollis, 2023). Successful retention within the cow herd is typically attributed to the dam's ability to gestate and rear a calf, with the average cow serving approximately five productive years in the herd (Schons et al., 1985) and some holding the longevity to successfully rear a calf upwards of 10 to 12 years of age (Roberts et al., 2015). Being that the beef cow's lifespan is substantially longer while consuming a predominately forage-based (Galyean and Goetsch, 2015), that is associated with a greater proportion of dietary intake excreted into the environment and increased methane production (Benchaar et al., 2001), poses a research avenue to evaluate dietary feeding strategies that improve cattle efficiency and reduce negative environmental impacts. Though we know that vitamin and trace mineral supplementation improves cattle growth, reproduction, and fetal outcomes and high-concentrate diet feeding is associated with lower methane output (Sakamoto et al., 2020), it is necessary to evaluate dietary feeding strategies and their impacts on mitochondrial respiration at a tissue level to better understand whole-animal energetics and nutrient utilization.

Though mitochondrial respiration differences were not observed in F2 offspring as a result of F0 vitamin and trace mineral supplementation, it is important to note the fetal programming impact observed in F1 offspring. This includes the increased muscle mitochondrial inefficiency at the LEAK respiratory state of F1 heifers from dams that did not receive VTM supplementation. Maternal VTM supplementation during gestation resulted in F1 heifers exhibiting less oxygen consumption at the LEAK respiratory state in skeletal muscle, a potential indicator of efficiency and reduction in cellular heat loss. The outcomes of project 1 leads into continued research of the F1 offsprings' whole-animal energy utilization and heat production

throughout pregnancy. Additionally, the observation of increased hepatic efficiency for ATP synthesis and electron transfer capacity in high-forage fed heifers and decreased hepatic oxygen consumption in high-concentrate fed heifers indicate differences in liver mitochondrial respiration and metabolism. These findings allow for the later evaluation of whole-animal energy expenditure needs and the environmental costs associated with feeding replacement heifers, high-forage, or high-concentrate diets.

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