EFFECTS OF MATERNAL NUTRITION DURING GESTATION ON VISCERA ENERGY USE IN RUMINANT DAMS AND OFFSPRING AND HYPOTHALAMIC NEUROHORMONE CONTENT IN THE OFFSPRING

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ABSTRACT

The extensive use of grazing systems for ruminant livestock and the high variation in forage quality throughout the year have important impacts on production. Changes in feed quality and availability cause alteration in the nutritional and physiological status of gestating animals. Modifications of the maternal nutritional environment throughout fetal development can have an impact on later performance of the offspring. Adjustments in maternal metabolism have been correlated with an increase in maternal energy use during pregnancy, and also further adjustments that occur in the dam's metabolism to provide adequate oxygen (O_2), nutrients, and energy for fetal growth and maternal maintenance systems. Moreover, energy utilized by fetal visceral tissues can be altered in response to changes in maternal feed intake. Prolonged changes in maternal feed intake during early pregnancy, the time which fetal brain development is taking place, can result in up- and/or down-regulation of neurohormones which play an important role in controlling long-term energy utilization and feed intake in the offspring. We designed three different studies with the main objective to investigate how maternal nutrient restriction throughout gestation or during different periods of gestation affects visceral organ metabolism in the dam and in the fetus. Furthermore, we aimed to understand the effects of fetal growth restriction on postnatal liver and small intestine mass, energy use, and content of hypothalamic neurohormones that control feed intake and energy metabolism. Our results indicate that, maternal hepatic and jejunal mass and energy use are impacted by nutrient restriction and strategic realimentation during different stages of gestation. Similar responses are also observed in fetal visceral development, metabolism and liver energy use in postnatal life. Moreover, arginine supplementation appears to be a nutritional strategy that diminishes the possible deleterious effects in maternal and fetal visceral metabolism in response to nutrient restriction.

Finally, maternal nutrient restriction throughout gestation decreased the number of cells expressing proopiomelanocortin (POMC) protein in the offspring hypothalamus, perhaps influencing energy metabolism in the offspring.

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LIST OF ABBREVIATIONS

ADF ..... acid detergent fiber

AgRP ..... agouti-related peptide

AI ..... artificial insemination

ARC ..... British Agricultural Research Council

ARH ..... arcuate nucleus

ATP ..... adenosine triphosphate

BCS ..... body condition score

BW ..... body weight

^{o}C ..... degrees Celsius

Ca ..... calcium

$CaCl_2$ ..... calcium chloride

cm ..... centimeters

Co ..... cobalt

CP ..... crude protein

CS ..... citrate synthase

Cu ..... coper

d ..... day

DM ..... dry matter

DMH ..... dorsomedial hypothalamus

DMI ..... dry matter intake

EBW ..... empty body weight

ETC ..... electron transport chain

Fe ..... iron

fx ..... fornix

g ..... gram

GIT ..... gastrointestinal tract

GH ..... growth hormone

h ..... hour

I ..... iodine

IU ..... international unit

IUGR ..... intrauterine growth restriction

K ..... potassium

KCl ..... potassium chloride

KH_2PO_4 ..... monopotassium phosphate

kg ..... kilogram

LH ..... lateral hypothalamus

Mg ..... magnesium

mg ..... milligram

min ..... minutes

mL ..... milliliters

$MgSO_4$ ..... magnesium sulfate

Mn ..... manganese

mM/µmol ..... micro molar

mol ..... molar

mmol ..... millimolar

mtDNA..... mitochondrial DNA

mTOR ..... mammalian target of rapamycin

N..... nitrogen

Na.....sodium

NaCl .....sodium chloride

$NaHCO_3$ ..... sodium bicarbonate

NDF..... neutral detergent fiber

NEm .....net energy maintenance

NO..... nitric oxide

NOS..... nitric oxide synthase

NPY.....neuropeptide Y

NRC ..... National Research Council

POMC .....proopiomelanocortin

ppm ..... parts per million

PVH/PVN ..... paraventricular nucleus

O_2 .....oxygen

SAS .....Statistical Analytical Software

Se..... selenium

SEM ..... standard error of the mean

TDN .....total digestible nutrients

T3 .....triiodothyronine

T4 ..... thyroxine

UCP2..... uncoupling protein 2

VMN .....ventral-medial hypothalamus

vs ..... versus

Zn .....zinc

3V.....third ventricle

µL.....microlitter

µm ..... micrometer

CHAPTER 1 - REVIEW OF LITERATURE

Introduction

Modifications of the maternal nutritional environment throughout fetal development can have an impact on later performance of the offspring. Prolonged changes in maternal feed intake during early pregnancy, the time which fetal brain development is taking place, can result in up- and/or down-regulation of neurohormones that play an important role in controlling how energy utilization and feed intake of these offspring will occur later in life. Moreover, maternal nutrition will also play a role in the amount of energy utilized by visceral organs of the dam and fetuses during gestation. Visceral organs, including liver and the small intestine, account for almost half of the body energy requirements for maintenance. Furthermore, the energy utilization of these organs is dependent on the amount and quality of the diet and physiological state of the animal.

Maintenance of energy balance and feed intake is regulated via a homeostatic system that includes central and peripheral organs. At the central level, the hypothalamic region of the brain is responsible for the synthesis and secretion of several neuropeptides that are involved in the control of energy homeostasis. During periods that are considered stable as far as food intake, peptides will work towards the maintenance between adequate intake and decreased energy expenditure or increased nutrient storage. These changes will also be controlled by circadian rhythms, physiological status and peripheral energy storage. Altogether, the signaling and the central responses to it are responsible for the control of how energy will be metabolized in the body.

The overall objective of this chapter is to review what is known in regards to how maternal nutrition throughout gestation affects energy utilization in the liver and small intestine of the dam and fetuses. Moreover, the impact of nutrient restriction during early gestation, a

period coinciding with early fetal brain development, and its influences on the expression of key hypothalamic neurohormones that control energy homeostasis and feeding behavior in the offspring, will be reviewed as well as some of the supplementation strategies that might be able to rescue animals from the deleterious effects that can be caused by nutrient restriction during different physiological states.

Energy metabolism in the ruminant

Energy can be measured only via its transformation between different forms (Kleiber, 1975). Chemical and heat are the two forms of energy utilized when measurements are related to animal nutrition. Thus, animal energy intake is in the chemical forms and via metabolism transforms it into heat that will be vital to maintain basic body function. Moreover, in order to support other physiological states such as growth, work, pregnancy, and lactation; energy is stored in chemical forms (Armsby, 1917). Chemical energy measurements are expressed as calories or joules (which can be interchangeably converted).

Committees commissioned by the British Agricultural Research Council (ARC), and the National Research Council (NRC) have formulated systems for the prediction of energy requirements and feed values for each species, based on review of experimental data on energy metabolism in various species (ARC, 1965 and 1980; NRC, 1976 and 1981). These systems take into consideration various aspects, including body weight, sex, activity, physiological state, environment, and amount and nutritive value of feed intake in order to predict energy requirement. Based on these systems, partitioning of energy in animals can be measured or predicted (NRC, 2000; Figure 1.1).

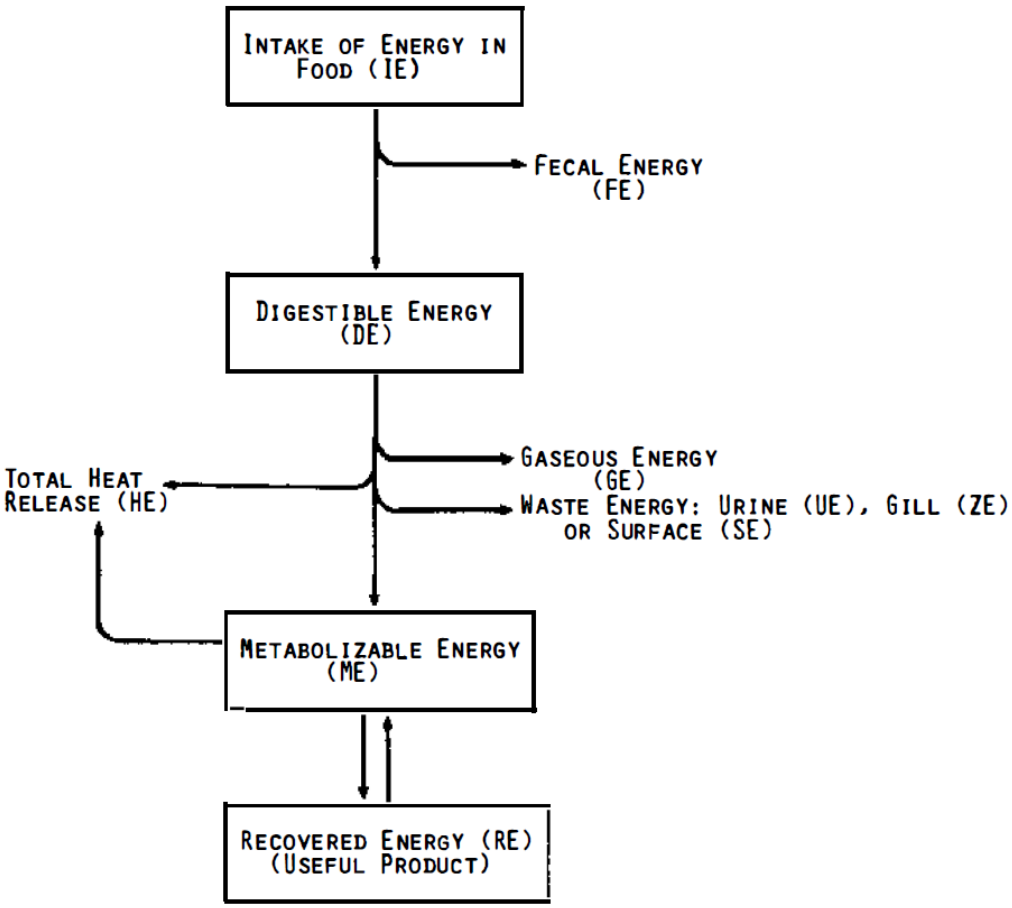

Figure 1.1. Partitioning of feed energy (NRC, 2000).

Baldwin and Bywater (1984) have previously discussed how the sources of variation in energy requirements influence the application of feeding systems when considering different physiologic and metabolic states. Furthermore, differences in relative organ weights are another source of variation when considering energy metabolism. Visceral organs have a high contribution (Baldwin et al., 1980) to whole animal energy expenditure when calculated relative to basal heat production. Changes in physiological state influence feed intake, which consequently affects visceral organ weight. Tissues in the body have the ability to increase or decrease in mass in response to their functional workload. Thus, young growing animals have higher energy expenditure per mass of these organs when compared to adults. When comparing organ energy expenditure related to metabolic body weight (MBW) between young and adult

animals, a difference of 50% in basal metabolism between both ages was observed when utilizing brain mass as a normalizing factor (Holliday et al., 1967). The same has been demonstrated when comparing adult animals in different physiological states (e.g. lactation, gestation; Smith and Baldwin, 1974), and for animals in the same state but on different planes of nutrition (Koong et al., 1982). Webster et al. (1975) suggested that the activities associated with metabolic output do not seem to be driven by the amount of energy input into the system, but generated by activity from different organs and tissues regenerating substrates that will ultimately produce ATP. Work, lactation, and growth have a great impact determining how much energy will be required from these organs.

Nutrient metabolism in the liver of the ruminant

The liver has many physiological functions, which include to determine the metabolic rate in the whole body, contribute to control of digestion, nutrient storage, and immunity (Tso and McGill, 2012). Studies have demonstrated a high proportion of energy expenditures in order to maintain Na^+, K^+-ATPase activity in the liver (McBride and Milligan, 1985). Furthermore, variation in energy expenditure in the liver differs depending on physiological states, and is more costly in growing animals when compared to adults. Variations in metabolic energy expenditure in the liver have also been associated with fluctuations in the concentrations of growth hormone (GH), and thyroid hormones (T3 and T4) in peripheral blood. A greater concentration of GH in young, growing, and lactating animals was associated with increased activity of Na^+, K^+ATPase (McBride et al., 1989; Gregg and Milligan, 1987). Hepatic protein synthesis also has a high energy cost, accounting for 25% of total hepatocyte utilization of ATP (McBride and Early, 1989). A tight association between protein accretion and ATP use suggests that regulation of protein synthesis is dependent on the energetic efficiency of the animal. Therefore, the time

course for protein synthesis completion, which is supported by protein degradation and secondary processes is relatively comparable to the level of Na+, K+-ATPase activity (McBride and Kelly, 1990). When accounting for total cellular energy consumption, protein turnover is one of the substrate cycles also to be considered. Along with metabolic fluxes, different substrates are involved in the allosteric regulation of precursors and end products in the cell. There is an energy cost to controlling metabolism in the cell, which varies depending on the physiological state of the animal.

Changes in energy use per unit of liver weight (kcal/g) in response to dietary energy intake are thought to be similar across different species (Johnson et al., 1990). Moreover, liver mass is thought to increase in response to overfeeding in gestating ewes (Wester et al., 1995; Fluharty and McClure, 1997; Swanson et al., 2000), as well as with advances in gestation (Caton et al., 2009). However, when proportional liver mass to BW were evaluated in response to different feed intake at different stages of gestation; it was observed that control-fed animals had a greater proportion than high-fed animals (Scheaffer et al., 2004b). Differences observed in the responses to intake were attributed to the higher BW gain in the high-fed group, which was mostly composed by body fat (Wallace et al., 1996).

Nutrient metabolism in the small intestine of the ruminant

Absorption of the hydrolyzed products of different nutrients such as carbohydrates, lipids, vitamins, electrolytes, water, and proteins occur in the small intestine. The luminal surface absorptive capacity is influenced by the presence of Kerkring's fold, villi, and microvilli. Cell proliferation, differentiation, and maturation are responsive to growth hormones and nutrient availability (Johnson et al., 2012). The absorptive mechanism occurs mainly via two different mechanisms (active or passive), which is dependent on the nutrient that is being absorbed. After

being absorbed from the intestinal lumen into the apical portion of the villi, nutrients are transported to the serosa via villi vascularity or central lacteal in the villa; where they enter either into the circulatory or lymphatic systems (Johnson et al., 2012). Maintenance of ionic homeostasis, nutrient uptake, and cell turnover are some of the functions in the small intestine that account for energy expenditure. Variations in the activity of peptide hormones, specific amino acid and sugar transporters, and mucosal and muscle gut mass occur in adaptation to changes in intake and requirements. The ability to make these changes has been demonstrated not only in the newborn (Widdowson, 1985), but also in the fetus (Reed et al., 2007; Carlson et al., 2009), and in the dam throughout gestation (Scheaffer et al., 2003; 2004b; Caton et al., 2009; Meyer et al., 2010b). Initial changes in small intestinal length, adaptation of mucosa, and mass are triggered postnatally in the pig within the first 24 h after suckling initiation (Dowling and Booth, 1967; Menge et al., 1983).

In the growing and adult animal, adaptation to changes in diet result in alterations in a number of proliferating jejunal mucosa cells, which can take place as early as 5 d after dietary intake is manipulated (Rompala and Hoagland, 1987). During gestation and lactation, vascularization of the small intestine and capillary area density also go through changes in order to adapt to the different requirements related to stages of gestation, as well as to changes in feed intake that might occur (Scheaffer et al., 2004a; Caton et al., 2009; Meyer et al., 2010b). Furthermore, protein turnover is also altered when feed intake is changed, as well as in response to changes in physiological state. Decreased small intestinal mass and epithelial cell turnover in the villi occur in response to low levels of intake (Lohrs et al., 1974; McNurlan et al., 1979). Perhaps, these responses can be related to an overall alteration in intracellular energy directed towards protein synthesis in the small intestine (McBride and Kelly, 1990). In a series of studies,

Scheaffer et al. (2001, 2003, 2004) demonstrated that alterations in tissue metabolic rate in pregnant animals are associated with changes in crypt cell proliferation in the intestinal mucosa rather than change in total mass suggesting that changes in metabolic rate can occur independent of changes in tissue mass.

O2 consumption as a measurement of energy use

Brody (1945) and Kleiber (1947) demonstrated the relationship between variations in adult animal metabolic rate in response to changes in body weight. Oxygen consumption has been previously determined on a whole body basis from animals during a fasting period; with effects of treatments being predicted for basal energy requirements (Webster et al., 1975; Rumsey et al., 1980). Moreover, when measuring O_2 uptake by the portal-drained viscera, it was observed that changes in response to fasting occur in the concentration of O_2 circulating in peripheral blood but not in the amount utilized by the viscera. Determining whole body O_2 consumption allows for further calculations on the ratio of tissue specific consumption in relation to the whole body. Webster (1983) observed that changes in heat production associated with digestion are one of the main components of whole-body O_2 consumption.

In vitro oxygen consumption has been utilized as a methodology to predict tissue specific metabolism (Krebs, 1950). Ferrell et al. (1976) were able to demonstrate via *in vitro* O_2 consumption that liver, heart, and kidney represent around 40% of the total body energy expenditures in pregnant and non-pregnant beef heifers. Therefore, it has been demonstrated that there is a disproportional amount of metabolism occurring in visceral tissues during pre and post-natal growth when we compare the organ mass ratio to the total body mass (Holliday et al., 1967; Bell et al., 1987).

Level of nutrition has been shown to directly affect the energy requirements for the maintenance of basic body functions at rest, which further correlates with visceral organ metabolism through changes in metabolizable energy intake, tissue specific and portal-drained viscera O_2 consumption, and blood flow to these organs (Webster et al., 1975; Huntington, 1984; Reynolds and Tyrell, 1987). Huntington et al. (1988) demonstrated that higher metabolizable energy intake results in increased energy flux across the portal-drained viscera in beef steers. Perhaps, this difference is promoted by changes in O_2 consumption and concentration of metabolites in the blood stream. Pregnancy also influences the level of metabolic rate in visceral organs. Freetly and Ferrell (1997) have shown an association between advancing pregnancy and a decrease in feed intake, which results in lower O_2 consumption by the liver. Moreover, this decrease in consumption has been shown to be associated with decreased in tissue mass (Burrin et al., 1990), thus being affected by changes in physiological demand and energy intake (Jenkins et al., 1986).

Citrate synthase activity as a measurement of mitochondrial function

Oxidative energy production takes place in mitochondria and has its level of activity controlled by mitochondrial metabolism. Mitochondrial function is controlled not only by nuclear encoded genes but also by cellular mechanisms and substrate concentration and activity (e.g. ATP phosphorylation, mitochondrial size, citrate synthase (CS) activity, etc.). Data on the effects of nutrient restriction on mitochondrial biogenesis in rodent models and humans have been inconsistent with some data reporting increases (Nisoli et al., 2005; Civitarese et al., 2007) and some data reporting no change (Hancock et al., 2011) in mitochondrial biogenesis.

Citrate synthase is one of the key regulatory enzymes in the citric acid cycle, controlling the condensation of oxaloacetate and acetyl CoA to form citrate. Moreover, CS activity has been

used as a marker for mitochondrial content and function (Spina et al., 1996; Benard et al., 2006). Changes in CS activity have been detected after O_2 concentration in venous blood has also been changed, and further related to observed dissociations in the mitochondria that led to changes in oxidative capacity (ACMS, 1995; Civitarese et al., 2007). Exercise training and muscle tissue activity have been widely utilized as models to access and understand how CS activity might be modulated in several species and at different ages (Spina et al., 1996; Starritt et al., 2000; Parco et al., 2003). Feed restriction has been demonstrated to decrease mitochondrial enzyme activity in liver tissue of rats (Dumas et al., 2004). The decreased activity of CS may occur due to a significant body weight and hepatic mass loss in response to caloric restriction. Liver mitochondria from rats that were restricted had less protons both under resting (state 4-oligomycin respiration) and phosphorylating (state 3 respiration) conditions (Dumas et al., 2004).

Maternal nutrition during pregnancy and changes in energy metabolism throughout the gestational period

Nutritional status is one of many factors that influences the maternal system through the programming of nutrient partitioning. Ultimately this system will dictate how fetal organ systems will develop and function (Wallace, 1948; Godfrey and Barker, 2000; Wu et al., 2006). Moreover, maternal undernutrition during the first two thirds of the gestational period may affect cardiovascular, endocrine and metabolic functionality, increasing the propensity to develop diseases associated with those systems (McMillen et al., 2001; Symonds et al., 2001).

On the maternal side, the adjustments in response to pregnancy are suggested to be homeostatic and homeorhetic (Bauman and Currie, 1980). In response to these adaptations that start to take place after conception, an increase in energy use by at least 50% has been previously observed to occur by the end of gestation in cattle (Reynolds and Redmer, 1993). Differences in

basal metabolic rate have been reported by Prentice et al. (1996) in pregnant women consuming adequate and inadequate amounts of energy, which demonstrates that there is an adaptation of the maternal system to the available nutrients in order to promote growth and delivery of healthy offspring. Therefore, in an attempt to promote adequate energy reserves for both fetal and maternal systems and also provide enough oxygen and nutrients to the fetus, the maternal system has to undergo metabolic adjustments throughout pregnancy (Stock and Metcalfe 1994).

Effects of nutrient restriction during gestation

Maternal nutrient restriction during pregnancy has been shown to have various effects on both the dam and fetus. The severity and consequences of these effects are dependent on the timing, duration, and rigorousness of the nutritional restriction. Overall, the changes in maternal energy intake and also body stores are going to reflect on the concentration of energy substrates available for maintenance of the dam as well as fetal growth, and of metabolic hormones. Reduced visceral organ mass in animals being nutritionally restricted during gestation has been described as an adaptation mechanism and ultimately decreases maintenance requirements (Burrin et al., 1990). Perhaps, these changes occur in order to allow the dam to maintain gestation and consequently support fetal growth during a period that is not favorable due to the decreased amount of nutrients available for absorption.

Nutrient restriction of ewes at d 50 of gestation resulted in decreased BW by d 135 (Faichney and White, 1987) and was associated with the level of restriction. One of the effects observed on the maternal side is on placental growth initiated at early gestation (Reynolds et al., 1990; Heasman et al., 1999; Vonnahme et al., 2007). Moreover, the gastrointestinal tract (GIT) along with the liver, represent approximately 10% of body mass but is responsible for approximately 50% of total energy expenditure (Koong et al., 1985; Reynolds et al., 1990).

Decreased mass of metabolically active tissues in response to nutrient restriction suggests that alterations in whole animal energy balance may occur. Nutrient restriction impacts the liver by decreasing organ mass and consequently decreasing metabolic activity (Scheaffer et al., 2004; Ferrell and Oltjen, 2008).

Small intestinal mass is affected by maternal nutrient restriction, having both mass and tissue composition being altered in order to adapt to the amount of nutrients available. Reductions in jejunal mass and its mucosal tissue have been observed in gestating ewes (Scheaffer et al., 2004b; Reed et al., 2007). The high energetic requirement for maintenance by the small intestine in ruminants is, perhaps, because it is one of the first organs to respond to decreased availability of nutrients (Ferrell and Jenkins, 1985; Bell, 1993). The mechanistic response of intestinal tissue to nutrient restriction involves alterations in villus height due to the decreased number of cells caused by a reduced rate of cell proliferation and migration, using mice as a model (Butzner et al., 1990; Syme and Husain 1981). Therefore, total mucosa area and absorptive area will be reduced. Moreover, jejunal capillary area and total mucosal vascularity also seem to be reduced with dietary restriction (Reed et al., 2007) which likely results in decreased blood flow into the jejunum.

Supplementation techniques to overcome nutrient restriction effects during the gestational period

Studies have focused on the interactions between feed supplementation and improvement on reproductive performance (Stalker et al., 2006) and also the possible effects on the performance of the offspring (Larson et al., 2009). An enhancement in postnatal growth and increased pregnancy rates in heifers in response to maternal protein supplementation during late

gestation has been observed (Martin et al., 2007). Maternal supplementation has promoted an increase in feed efficiency of heifers fed a low energy and high forage diet (Martin et al., 2007).

Supplementation has also been used to modify the possible effects that undernutrition can have on offspring development. When comparing birth weight of heifer calves born from dams that were supplemented during gestation with those that did not receive any supplementation, an enhancement of puberty onset was observed in those born heavier due to a higher amount of nutrients available to them during the fetal growth period (Southam et al., 1971). When energy and protein supplements were offered to cows being fed low quality forages, a reduced loss in BW and increase in birth weight of the offspring was observed (Clanton and Zimmerman, 1970). However, limited data are available showing effects of using other sources of supplements on maternal and fetal energy use as well as on fetal programing, in cattle.

Melatonin nutritional supplementation

Melatonin, is a hormone synthesized in pineal gland cells from the amino acid tryptophan in response to dark-light period. However, it has also been shown to be synthesized in the gut and to play a role in regulating gastrointestinal function. Konturek et al. (2007) reported that melatonin in the upper portion of the GIT has many different effects such as synchronization of circadian rhythm, control of free radical activity, and mucosal protection against different lesions in the GIT. Others have reported that melatonin supplementation increases TCA cycle substrate-induced respiration in rat liver (Reyes-Toso et al., 2006). It is also hypothesized that melatonin present in bile functions as a protection mechanism for the gastrointestinal mucosa against oxidative stress (Tan et al., 1999), as it acts as a potent endogenous free radical scavenger (Tan et al., 1993). When melatonin and Krebs cycle substrates were supplemented *in vitro* to a

mitochondrial preparation, an increase in respiratory rate was observed (Reyes-Toso et al., 2003).

Decreased rate of electron transport chain (ETC) activity in response to melatonin supplementation is hypothesized to occur in response to antioxidative effects. Perhaps, damage to protein complexes in the chain are diminished along with ubiquinone oxidation (Figure 1.2; Acuña-Castroviejo et al., 2001; 2003; 2007). Moreover, limitation in loss of intra-mitochondrial glutathione along with lower protein damage, might be capable of enhancement in the ETC as well as reduced mtDNA damage (Leon et al., 2005).

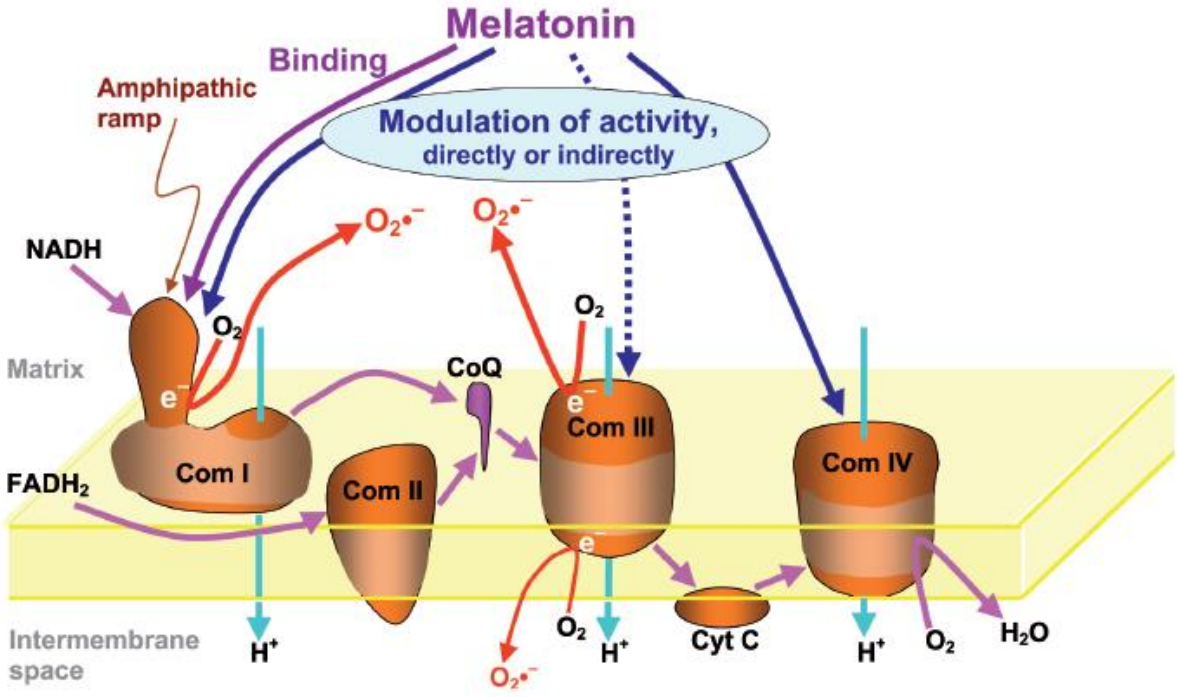

Figure 1.2. Overview of melatonin's actions at the electron transport chain (Hardeland et al., 2005).

Melatonin supplementation has also been shown to alter cardiovascular function (Paulis and Simko, 2007). Lemley et al. (2012) demonstrated increased umbilical blood flow in response to dietary melatonin supplemented throughout gestation.

Arginine nutritional supplementation

Arginine (Arg), a physiological substrate for nitric oxide (NO) and polyamine synthesis, has been suggested to function as a valuable supplement in the maternal diet to potentially help alleviate intra-uterine growth restriction (IUGR; Wu et al., 2006). Perhaps, these changes are stimulated by higher concentrations of NO and polyamines that are known to stimulate cell proliferation, migration, and remodeling, as well as angiogenesis (Wu et al., 2009). Moreover, NO and polyamines have been demonstrated to regulate fetal development, through contributing to nutrient delivery to the fetus as well as stimulating the use of the nutrients by the developing fetus despite maternal low level of nutrition (Wu et al., 2004). Increased blood flow in the utero-placenta is the mechanism that is suggested to act towards the rescue of the developing fetus (Wu et al., 2004). Since NO functions as an endothelium-derived relaxing factor, this increased blood flow might improve nutrients being transferred from maternal to fetal circulation (Reynolds et al., 2005). Altogether, in ovine models of IUGR, intravenous arginine administration has been shown to promote improvement of fetal growth (Wu et al., 2009).

Nitric oxide synthase (NOs) is involved in the regulation of energy metabolism in mammals (Joffin et al., 2012). The mechanism perhaps involves a potential dependence of leptin on NO in order to have an action on hepatic gluconeogenesis (Shiuchi et al., 2001). The diffusion of NO into cells is thought to act through cGMP-dependent pathways and via cross talk with AMP-activated protein kinase (AMPK), and other molecules by playing a role not only on blood pressure and neurotransmission, but also in whole-body homeostasis, and nutrient metabolism (Jobgen et al., 2006). Furthermore, physiological levels of arginine have been shown to activate the cellular signaling pathway mTOR (Bazer et al. 2010; Kong et al. 2012).

Effects of maternal nutrition on fetal growth and development

The maternal nutritional status maintained during pregnancy affects not only the dam as discussed above, but also has large effects on fetal development as thoroughly discussed previously (Hales and Barker, 2001; Wu et al., 2006). The level of nutrition received by the dam influences the maternal uterine environment resulting in decreased space for fetal growth and development, which results in intra-uterine growth restriction (IUGR; Wallace, 1948). Therefore, functional capacity of the placenta, transference of nutrients and oxygen via uteroplacental pathways, nutrient availability to the fetus, and metabolic pathways, are some of the several mechanisms potentially affected by the amount of nutrients received by the dam and that together affect fetal growth and development (Bell and Ehrhardt, 2002; Redmer et al., 2004; Fowden et al., 2005; Reynolds et al., 2005).

High rates of morbidity and mortality are associated with low birth weights (Mellor, 1983). Dysfunctions observed in the intestinal system resulting from IUGR is one of the factors that highly contributes to increased mortality rates in livestock (Trahair et al., 1997). In swine, small intestinal necrosis disorder is observed in piglets that underwent IUGR, which further impairs intestinal function and absorption of essential nutrients resulting in increased rates of mortality (Wu et al., 2004c and d). Thus, reduced efficiency in offspring can also be related to gastrointestinal dysfunction (Trahair et al., 1997), compromised skeletal muscle development (Hegarty and Allen, 1978), and smaller livers (Widdowson, 1971); which together are responsible for the coordination of nutrient metabolism in the whole animal.

Fetal liver and small intestine formation

Liver formation originates from the ventral foregut endoderm (Figure 1.3; Tremblay and Zaret, 2005), which is established during gastrulation of the endoderm germ layer that also forms

the midgut and the hindgut. Hepatic morphological formation is continued until after birth to complete the generation of all the characteristics of the hepatic tissue architecture.

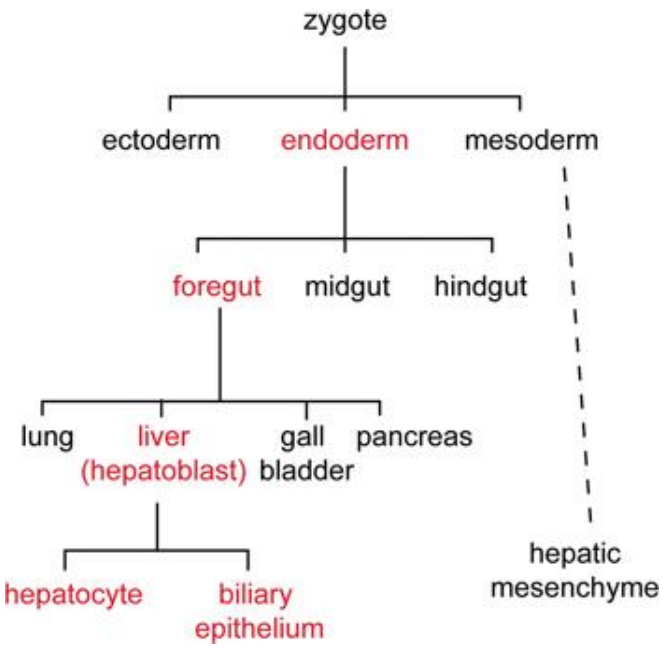

Figure 1.3. Liver cell lineage (Zorn, 2008).

Hepatocyte nuclear factor 4 (*Hnf4*) has been shown to be one of the main genes in the control of hepatic functionality. It appears that upregulation of this gene initially takes place during gastrulation and promotes the initiation of control of energy metabolism and other hepatic activities (Watt et al., 2003). Early investigations by Burch et al. (1963) demonstrated the presence of several key enzymes of glycolysis and gluconeogenesis in the embryonic liver. When using sheep as a model, it was observed that the liver had enzymatic activity for both glucose synthesis and degradation (Burch et al., 1963). In response to changes in maternal blood flow and nutritional status, the fetus has been shown capable of changing its blood glucose, fatty acid, and amino acid concentrations (Makowski et al., 1973; Gresham et al., 1972).

Intestinal formation has its origin from the endoderm and occurs around midgestation in response to the *Wnt* gene, which initiates the formation of villi from the stratified cuboidal epithelium (de Santa Barbara et al., 2003). Important functional alterations in intestinal morphology and function are observed during late gestation and early postnatal life to prepare the offspring for ingestion of less lipids and more carbohydrates (Drozdowski et al., 2010). In livestock species, timing and rate of fetal gastrointestinal tract maturation is dependent on enteral nutrient intake, which takes place both pre- and post-natally (Sangild, 2006). Towards midgestation, the fetus starts to receive nutrients through enteral nutrition by swallowing amniotic fluid (Sangild, 2006). Amniotic fluid contributes approximately 1% of the required protein and 10 to 20% of the required energy for the fetus (Mulvihill et al., 1985). Major developmental changes in the gastrointestinal tract appear to occur after swallowing is initiated, which stimulates prenatal mucosal differentiation of the gastrointestinal tract lumen (Trahair and Harding 1995; Trahair and Sangild, 1997).

Energy use by the fetal liver and small intestine during different stages of development and growth

The metabolic rate of the fetus, as measured utilizing an O_2 consumption technique, has been observed to be twice as high as in adults (Meschia et al., 1980). Increased rates of consumption are observed with the advance of gestation and are also attributed to increased fetal tissue mass. Glucose is considered the major metabolic substrate received by the fetus via uteroplacental transfer. Moreover, there is little need for gluconeogenic activity in the fetal liver, when compared to the level of activity observed in adults (Alexandre et al., 1966). Amino acids appear to be the main metabolic fuel for respiration in the sheep fetus (Gresham et al., 1972).

Lipid synthesis but not oxidation in the fetal liver is high during the majority of the gestational period (Jones, 1973).

Small intestinal development is divided into three different stages, starting with morphogenesis and proliferation of cells; followed by cell differentiation and maturation. Neville et al. (2010) demonstrated that maternal nutrient restriction affects fetal jejunal vascularization, although changes in angiogenic factors were not observed to support these changes. Decreased swallowing of amniotic fluid during gestation affects intestinal growth and differentiation (Tahair et al., 1995; 2002). Moreover, effects on concentration of plasma gastrin were shown to be altered depending on the level of intake of the fluid. However, brush border enzyme activity does not seem to be impacted by fetal amniotic fluid intake (Sangild et al., 1999).

Effects of changes in maternal nutrition on growth and metabolism of visceral organs of the fetus

In order to have a successful gestation and produce a healthy offspring, nutrient partitioning between the dam and the fetus is critical and has to be well maintained. In order for optimal fetal growth, it is important that the necessary adjustments of maternal metabolism in response to gestation occur in a manner that the necessary amounts of oxygen and nutrients are provided to the fetus. Changes in maternal feed intake during the gestational period have been shown to have different effects on growth rate and development of the offspring (Wu et al., 2006). These alterations are related to success rate of the gestation, which would be parturition of a healthy offspring. Furthermore, the rate of postnatal growth affects the incidence of cardiac predisposition and several metabolic disorders in the offspring (Barker, 1995).

Productivity and profitability are two of the main points of interest in livestock production. Thus, it is important that the dam is able to produce offspring with acceptable birth

weight and able to develop and grow in an efficient manner. Freetly et al. (2000) demonstrated that offering lower levels of nutrients to the dam during early and mid-gestation has a negative impact on calf birth weight. Moreover, decreased maternal intake during the last trimester will most likely impact fetal growth that is occurring at this stage and is highly dependent on nutrient flow to the gravid uterus (Ferrell and Koong, 1986).

Disturbances in maternal nutrient intake during pregnancy impact visceral organ growth and function (Hales and Barker, 1992). Different organs might respond differently to both lower and higher levels of nutrition. The responsiveness of intestinal tissues to IUGR can result in decreased small intestinal mass, altered intestinal villi morphology (Avila et al., 1989; Trahair et al., 1997; Wang et al., 2005), reduced number of cells in the jejunum (Reed et al., 2007), and altered vascularity (Neville et al., 2010) in the offspring. These changes in visceral organs during pregnancy may negatively impact nutrient absorption since the gastrointestinal tract serves as the main site of absorption and also as a major energy and nutrient sink due to its high metabolic activity and rapid turnover. Vonnahme et al. (2003) reported that reduced intake of energy during fetal organogenesis increased mass of the heart ventricles and liver of the offspring. Further, changes in fetal hepatic functionality in response to changes in maternal intake have been proposed (Vonnahme et al., 2003); however, no studies have examined metabolic activity and/or energy use by the fetal liver throughout gestation in ruminants.

Mechanisms of the hypothalamic control on energy balance and feed intake

Maintenance of energy balance and feed intake is regulated via a homeostatic system that includes central and peripheral organs (Morton et al., 2006). At the central nervous system level, the hypothalamic region of the brain is responsible for the synthesis and secretion of several neuropeptides that are involved in the control of energy homeostasis (Morton et al., 2006).

During periods of adequate feed availability, neuropeptides maintain the balance between feed intake, energy expenditure, and nutrient storage. These events are also controlled by circadian rhythms, physiological status, and peripheral energy storage (Kalsbeek et al., 2010). Altogether, signaling cascades and the central responses to it are responsible for the control of how energy will be metabolized in the body.

The hypothalamus plays an important role in the integration of central and peripheral signals that are essential for the regulation of energy homeostasis. The axis of communication between the gastrointestinal tract and adipose tissue with the brain are critical in the regulation of energy metabolism (Morton et al., 2006). The specific regions located throughout different hypothalamic regions such as the lateral hypothalamus (LH), arcuate nucleus (ARC), paraventricular hypothalamus (PVN), dorsomedial hypothalamus (DMH) and ventral-medial nucleus (VMN) play specific and key roles in this regulation (Morton et al., 2006). Within these specific hypothalamic nuclei, subsets of neurons with specific neurobiological phenotypes are responsive to glucose, fatty acids, amino acids and other fuel-related stimuli (Kalsbeek et al., 2010). In these nutrient-sensing neurons, nutrients act as signaling molecules to engage a complex set of neurochemical and neurophysiological responses, thereby regulating energy intake, the release of stored nutrients, and nutrient utilization in most tissues, thus compensating for decreased or increased energy availability.

Changes in neuropeptide expression in response to feed intake, physiological state, and energy metabolism

The brain has the capability to actively regulate signaling in response to environmental cues that perhaps regulate animal feeding behavior or physiological state. Initial work conducted by Hetherington and Ranson (1940) and by Anand and Brobeck (1951) demonstrated that

hyperphagia and obesity were induced when the VMN was lesioned, while aphagia and death by starvation were induced by lesions in the LH. As mentioned above, several hypothalamic nuclei are involved in the control of food intake and body weight regulation. Moreover, specific nuclei produce anorexigenic and orexigenic peptides, which orchestrate the regulation of energy homeostasis between the central nervous system and the peripheral organs. The orexigenic neurons that produce neuropeptide Y/agouti related peptide (NPY/AgRP) and anorexigenic proopiomelanocortin (POMC) are present in the ARC (Morton et al., 2006). Projections from these neurons modulate other hypothalamic nuclei, which will further promote responses to adapt metabolism.

Body composition and energy reserves are the primary signaling mechanism to send information to the brain via hormonal and nutrient related signals and will initiate responses from neurons; thus, regulating metabolism in adaptation to energy that is available (Schwartz et al., 2005). When rats were injected with a fatty acid synthase inhibitor, a decrease in food intake, body weight and NPY protein expression was observed (Loftus et al., 2000). Thus, fatty acid signaling has been demonstrated to play a role in the control of energy balance via hypothalamic regulation. Intracerebral infusion of lipid emulsion into the third ventricle region in the hypothalamus has been shown to alter insulin secretion in the pancreas and decrease gluconeogenesis in the liver (Clement et al., 2002; Cruciani-Guglielmacci et al., 2004). However, these effects can be altered by inhibiting the transport of long-chain fatty acids utilized for β-oxidation in the mitochondria.

Hypothalamic neurons have been identified as glucose sensors being responsive to plasma glucose levels through changing their firing rate and consequently modifying neuropeptide synthesis and secretion (Oomura et al., 1964). These neurons have been localized

in the VMH, ARC, and PVN areas of the hypothalamus (Kow and Pfaff, 1989; Funahashi et al., 1999; Song et al., 2001). Glucokinase has been shown to be present also in the brain, co-expressing in the ARC with NPY and POMC (Mulberg et al., 1998). These neurons make direct and indirect efferent contact; thus being capable of responding to metabolic cues from periphery organs.

Communication between the gastrointestinal tract and the hypothalamus

At the gastrointestinal tract level, immediately after feed intake a neural reflex is activated through direct contact with macronutrients, stimulating L-cells to release peptide YY (PYY; Pedersen-Bjergaard et al., 1996), which exhibits 70% homology with NPY. Peptide YY is released into the circulation and plateau is reached between 1 to 2 hours after a meal and remaining elevated for 6 hours, in mice and humans (Batterham et al., 2002). There are two forms of PYY observed in the circulation: PYY1–36 and PYY3–36. However, PYY3–36 is the major form in both the intestinal mucosal endocrine cells and the circulation (Batterham et al., 2002). Regarding PYY influencing NPY secretion, it is known that both peptides have affinity for the same Y2 receptor located in the ARC. It is also proposed that appetite is inhibited by PYY3–36 acting directly on the ARC via the Y2 receptor, a pre-synaptic inhibitory autoreceptor (Zimanyi et al., 1998). This increase in PYY secretion likely is correlated with a reduction in NPY secretion.

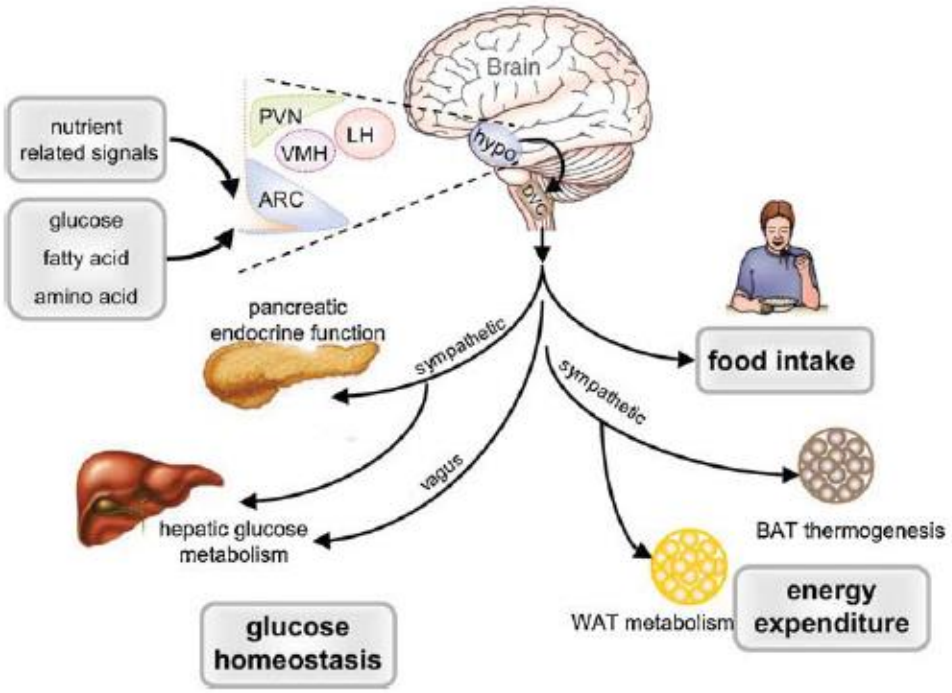

Figure 1.4. Pathway for effects of energy balance regulated by hypothalamic nutrient sensing on feed intake behavior and energy metabolism (Blouet and Schwartz, 2012).

Gastrointestinal hormones released upon feeding signal to the appetite centers in the brain and play a role in the regulation of satiety (Figure 1.4). The ARC behavior and metabolism related neurons express receptors for these hormones secreted from peripheral tissues (Korner and Leibel, 2003; Wynne et al., 2004). At the base of the third ventricle, those cross the semi permeable blood brain barrier and bind to receptors located within the ARC that will further regulate synthesis and secretion of NPY, AgRP and POMC (Figure 1.5; Abbot et al., 2005), depending on the signaling. In response, these neuropeptides will be activated or deactivated further affecting food intake (Wynne et al., 2004).

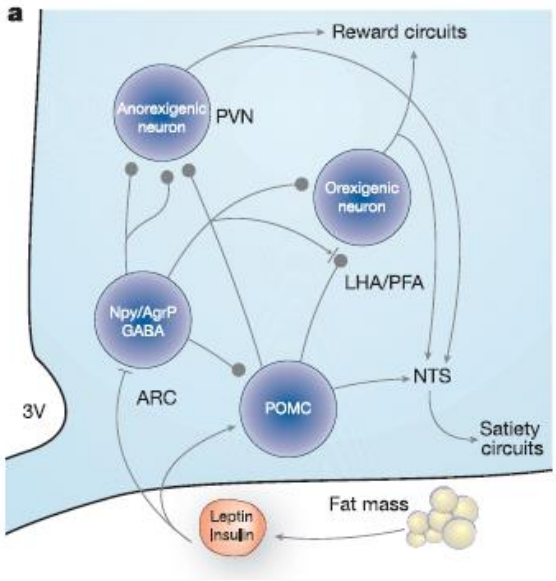

Figure 1.5. Hypothalamic nucleis and neuropeptides involved in the regulation of feeding behavior and energy metabolism (Morton et al, 2006).

How hypothalamic neurohormone secretion may affect food intake and energy metabolism

The hypothalamic region has a large impact on energy balance and feeding behavior through a circuit of neuropeptide expression that modulates intake (Morton et al, 2006). Alteration in feed intake and composition of feed may result in up- or down-regulation of these neuropeptides and also trigger modifications at the peripheral and central circulation of a series of hormones. Moreover, these effects can be modified during perinatal and/or neonatal stages through the manipulation of maternal nutrition, which will cause alterations in hormonal secretion that will signal the fetus through placental transport and consequently alter how the hypothalamic circuit will be initiated.

Numerous orexigenic and anorexigenic peptides have been previously described (Kalra et al., 1999; Schwartz et al., 2000; Hillebrand et al., 2002) as part of the neuropeptidergic circuit that plays a role in controlling energy homeostasis. Among these known peptides are two neuronal types localized in the ARC, NPY and POMC, which play critical opposite roles in regulating food and energy intake.

Besides the central and peripheral responses through hormonal secretion that positively or negatively controls NPY expression, there are also metabolic signals, such as glucose concentration that is able to initiate a counter-regulatory response by this neuropeptide. The presence of glucose sensing neurons in the same nuclei as NPY suggests that increased concentration of plasma glucose might stimulate these neurons and have an increased expression of POMC as a response (Ibrahim et al., 2003). Lipid concentrations in plasma also have a stimulatory effect on POMC indicating that energy reserves are high and intake can be decreased.

Increases in leptin signaling to the hypothalamus may rapidly inhibit food intake by altering the secretion of NPY (Flier, 2004). Increases in leptin secretion also increases POMC and decreases NPY and AgRP expression; however, in the case of leptin deficiency, a reduction in POMC and an increase in NPY and AgRP expression are observed.

NPY and AgRP mechanisms of action on the control of energy utilization and feed intake

Neuropeptide Y is the most abundant neuropeptide in the hypothalamus (Williams et al., 1991) and one of its physiological roles is as an orexigenic agent (Gehlert and Hipskind, 1997). At the central nervous system level, it increases the amount of feed intake without altering frequency, which implies that satiety perception might be altered (Paez and Myers, 1991). Activity is modulated by the hindbrain and limbic structures and dependent on energy availability being upregulated during periods of food restriction and returning to baseline after adequate intake is met (Beck, 2006). Moreover, it is suggested that the desire for carbohydrate intake is increased compared to fat and protein (Beck et al., 1992). However, overexpression of NPY in the DMH via manipulation of gene expression resulted in enhanced food intake, body weight, and exaggerated diet-induced obesity in normal weight rats (Yang et al., 2009).

Knockdown models for NPY in the DMH have been investigated and shown that there is an improvement in glucose homeostasis and insulin resistance that has been hypothesized to occur due to an indirect effect of NPY on pancreatic/liver function that can lead to increased energy expenditure (Bartness, 2011). The biological actions of this neuropeptide occur in different regions of the body and are mediated by six different receptors (Y1-Y6; Widdowson et al., 1997b; Burkhoff et al., 1998).

Agouti-related peptide is also an orexigenic peptide that plays a role in food intake along with NPY. It has been shown to be co-expressed with NPY (Morton and Schwartz, 2001) in the ARC. Together, AgRP and NPY neurons respond by upregulating expression in situations of negative energy balance (Mizuno et al., 2003) and sensing changes in secretion of gut related hormones (Nakazato et al., 2001). Ghrelin and PYY3-36 (NPY-2R) receptors have been localized on NPY and AgRP neurons (Pritchard et al., 2004). The mode of action of AgRP has been thought to occur via antagonization of anorexic effects promoted by POMC; therefore, regulating food intake (Yang et al., 1999).

POMC mechanisms of action on the control of energy utilization and feed intake

Proopiomelanocortin (POMC) is also located at the ARC of the hypothalamus and has an opposite effect compared to NPY. As an effect of increased NPY/AgRP co-expression, the endogenous antagonist POMC is upregulated through activation promoted by its central melanocortin receptor α-MSH. Increases in POMC expression results in a reduction in feed intake and consequently BW as its main effect. Previous research has demonstrated that POMC neurons are part of the formation of the melanocortin system (*POMC, PC1, MC4R* and *MC3R)*, which participates in the regulation of feeding behavior (Coll et al., 2004). Post-translational activity cleaves POMC into different melanocortins, which have different actions and are

synthesized and secreted in different nervous system and peripheral tissues. In addition to melanocortins, adrenocorticotrophin (ACTH) is found in the pituitary, MSHs (α-, β- and γ2-MSH) are found in the ARC nucleus, and in the nucleus tractus solitaries of the brainstem; and β-endorphin is found in peripheral tissues (Millington, 2007).

Proopiomelanocortin in the ARC nucleus has been shown to respond to levels of glucose by increasing neuronal excitability (Ibrahim et al., 2003). The mechanism involved is thought to include the closure of K_{ATP} channels located in the plasma membrane, induced by ATP (Ashford et al., 1990). The involvement of a mitochondrial protein, UCP2, in the mechanism of glucose sensing by POMC neurons has been previously demonstrated (Parton et al., 2007). UCP2 is expressed in many tissues, including pancreatic β-cells and in the ARC nucleus of the hypothalamus (Richard et al., 2001). In animals fed high-fat diets, an increase in UCP2 has been related to a loss in POMC neurons that contain receptor to sense the level of glucose (Parton et al., 2007). Therefore, these studies indicate that POMC neurons integrate the metabolic cues and synaptic input that ultimately help to control homeostasis in mammals.

Hypothalamic formation in fetus

Maternal nutrition has a large influence on the developmental programming of neurocircuits responsible for feeding behavior and energy homeostasis during the fetal perinatal stage (Bouret and and Simerly, 2006). Maternal undernutrition and overnutrition have been shown to cause permanent perturbations in the energy balance of the offspring (McMillen et al., 2005; Levin, 2006; Plagemann, 2006). The development of hypothalamic circuits (projection of nerves from ACR to the PVH, DMH, and LHA) occurs in the second week of life in mice, and during the final quarter of gestation in sheep (Bouret and Simerly, 2006). Adequate concentrations of leptin, produced by the fetal adipose tissue and transported via the placenta,

will determine via a trans-synapse mechanism how fetal NPY, AgRP and POMC are expressed (Armstrong and Montminy, 1993). A neonatal leptin surge is the signal needed by the hypothalamus to initiate increased expression of other neuropeptides.

Formation and distribution of neurons occur at a high rate during the embryonic period and later in life as a reparatory mechanism in the adult brain (Gotz and Huttner, 2005). Evidence supports that this mechanism is also responsive for organization and activation of hypothalamic neuronal circuits in response to changes in whole body or tissue specific metabolic rates (Kokoeva et al., 2005). Hypothalamic progenitor cells in the newborn are thought to be programed to regulate appetite via commitment and differentiation to neurons responsible for the synthesis and secretion of neuropeptides such as NPY/AgRP and POMC (Lee et al., 2012). Thus, it is suggested that the nutritional environment to which the embryo is exposed throughout gestation has an effect on the formation and distribution of neurogenic cells (Desai et al., 2011).

Statement of the problem and overall objectives

The impacts of nutrient restriction on the nutritional and physiological status of the dam and also the consequences on the development of the fetus have been reviewed in this chapter. Moreover, the deleterious effects of IUGR on fetal development and consequently on offspring performance have also been reviewed. Tissue specific energy use has been previously investigated. However, there is a limited amount of work that has been done in pregnant animals and fetuses. Furthermore, information on the use of nutritional techniques such as supplementation and realimentation and their impacts on visceral organ metabolism in fetus and dam are limited.

Most of the relevant work investigating the central nervous system mechanisms involved in the regulation of feed intake and energy utilization has been done in monogastric models. The

hypothalamus plays a critical role in energy balance and feeding behavior through a circuit of neurons expressing numerous neuropeptides. Alteration in feed intake and composition of feed may result in up- or down-regulation of these neuropeptides and trigger modifications in peripheral and central release of hormones. Moreover, these circuits can be modified during perinatal and/or neonatal stages through the manipulation of maternal nutrition. Alterations in maternal nutrition modify hormonal secretions that are transduced via the placenta to alter activation of the hypothalamic circuit in the fetus. Several studies have been conducted that together, paint a bigger picture illustrating how all these circuits are activated and maintained. A few of these, including the studies that demonstrate how manipulation of maternal nutrition affects offspring development, were done in farm animals. These reports also indicate that maternal diet during pregnancy has the potential to be a tool to produce more efficient offspring as a result of altered expression of neuropeptides that regulate feed intake and absorption of nutrients. Finally, research is needed to determine if proper nutrition during the neonatal period is able to rescue some of the systems that might be altered due to disturbed prenatal nutrition. Thus, the overall objectives of the series of studies presented in the following chapters are to better understand how nutrient restriction during pregnancy impacts visceral organ mass and energy utilization of the dam and the fetus. Moreover, we analyzed supplementation with melatonin and arginine, as well as realimentation as possible approaches that can be utilized to help sheep and cows and their fetuses to overcome the deleterious effects of nutrient restriction. We investigated how maternal nutrient restriction affects neurohormonal content in the ovine offspring and how arginine supplementation might impact NPY, AgRP, and POMC protein content.

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CHAPTER 2 - EFFECTS OF NUTRIENT RESTRICTION AND MELATONIN SUPPLEMENTATION ON MATERNAL AND FETAL HEPATIC AND SMALL INTESTINAL ENERGY UTILIZATION [11]

Abstract

To determine how nutrient restriction and melatonin supplementation influence ewe and fetal hepatic and small intestinal energy use, 32 primiparous ewes on d 50 of gestation were fed 60% (RES) or 100% (ADQ) of NRC recommendations with 0 (CON) or 5 mg/d (MEL) of dietary melatonin. On d 130 of gestation, small intestine and liver were weighed and collected. Data were analyzed as a completely randomized design with a 2 × 2 factorial arrangement of treatments. Liver weight (g/kg EBW) decreased ($P = 0.02$) in RES ewes. Jejunum weight (g/kg BW) increased (interaction $P = 0.04$) in ADQ-MEL ewes compared with all other treatments. Total *in vitro* O_2 consumption (mol/min/tissue) and total citrate synthase activity (mol/min/tissue and mol/min/kg EBW) in liver decreased ($P \leq 0.03$) in RES ewes. Oxygen consumption (mol/min/kg EBW) increased (interaction $P = 0.02$) in jejunum of ADQ-CON versus RES-MEL and ADQ-CON. Citrate synthase activity (mol/min/kg of EBW) increased (interaction $P = 0.03$) in jejunum of ADQ-MEL compared with RES-MEL and ADQ-CON. Fetal liver weight (g/kg BW) decreased ($P = 0.02$) in RES versus ADQ. Fetal small intestine weight (g/kg BW) decreased (interaction $P = 0.05$) in RES-MEL versus ADQ-MEL. Total O_2 consumption (mol/min/tissue) and total citrate synthase activity (mol/min/kg of BW) in fetal liver decreased ($P \leq 0.05$) in RES versus ADQ. Fetal small intestinal O_2 consumption (m/min/kg of BW) was greater (interaction $P = 0.03$) in RES-CON and ADQ-MEL than RES-MEL and ADQ-CON.

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Maternal nutrient restriction had a greater effect than melatonin supplementation on liver and jejunum mass and energy utilization in dams and fetuses. Because intestinal mass and energy utilization were more responsive to melatonin supplementation in ewes fed adequate nutrition compared to restricted ewes, melatonin may have limited use as a therapeutic supplement to help overcome potential negative effects of nutrient restriction.

Introduction

The extensive use of grazing systems for ruminant livestock and the high variation in forage quality throughout the year have important impacts on production. Changes in feed quality and availability alter the nutritional and physiological status of gestating animals (Wu et al., 2006; Caton and Hess, 2010). Maternal homeostatic and homeorhetic adjustments are necessary during pregnancy (Bauman and Currie, 1980) due to the importance that nutrient partitioning between maternal and fetal tissues has on species survival and fetal development. These adjustments have been correlated with an increase in maternal energy use during pregnancy (Ferrell and Jenkins, 1985), and also further adjustments that occur in the dam's metabolism to provide adequate oxygen (O_2), nutrients, and energy reserves for fetal growth and maternal maintenance systems. Alterations in small intestinal and hepatic mass and metabolism, two key tissues influencing total body energy expenditure (Koong et al., 1985; Reynolds et al., 1991), have been observed when different nutritional treatments have been implemented in growing cattle (Wang et al., 2009a, b, c).

Maternal nutrient restriction can result in fetal intra-uterine growth restriction (IUGR; McMillen et al., 2001; Vonnahme et al., 2003) potentially reducing growth of fetal organs (Hales and Barker, 1992). Moreover, the responsiveness of intestinal tissues to IUGR can result in decreased small intestinal mass, altered intestinal villi morphology (Avila et al., 1989; Trahair et

al., 1997; Wang et al., 2005), reduced number of cells in the jejunum (Reed et al., 2007), and vascularity (Neville et al., 2010) in the offspring. These changes in visceral organs during pregnancy may negatively impact nutrient absorption since the gastrointestinal tract (GIT) serves as the main site of absorption and also as a major energy and nutrient sink due to its high metabolic activity and rapid turnover.

However, it is not as well understood as morphological changes how nutrient restriction during pregnancy influences energy utilization and maintenance requirements of tissues (oxygen consumption and mitochondrial function) in maternal and fetal tissues. Restriction may affect tissue energy use decreasing the rate of activity of metabolic cycles that will produce ATP and consume O_2. Data on the effects of nutrient restriction on mitochondrial biogenesis in rodent models and humans have been inconsistent with some data reporting increases (Civitarese et al., 2007; Nisoli et al., 2005) and some data reporting no change (Hancock et al., 2011) in mitochondrial biogenesis.

Melatonin likely plays a role in regulating gastrointestinal function. Konturek et al. (2007) reported that melatonin in the upper portion of the GIT has many different effects such as synchronization of circadian rhythm, control of free radical activity, and mucosal protection against different lesions in the GIT. We hypothesize that dietary melatonin may be a useful therapeutic supplement to prevent GIT disturbances and improve efficiency of energy and nutrient utilization in ewes (and fetuses) undergoing IUGR.

The objectives of the present study were to examine the effects of maternal nutrient restriction with or without melatonin supplementation on maternal and fetal intestinal and hepatic mass and energy utilization as measured through analysis of *in vitro* oxygen consumption and citrate synthase activity (a marker of mitochondrial biogenesis).

Material and Methods

Animal care and use protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Animals and experimental design

Animal care and dietary treatments (Table 2.1) as previously described (Lemley et al. 2012). Briefly, a total of 32 nulliparous ewes carrying singleton fetuses were transported at d 28 of gestation to a temperature (14°C) and light-dark cycle (12:12-h; lights on at 07:00 and off at 19:00) controlled room. Animals were housed in individual pens (0.91 x 1.2 m) and fed to meet or exceed nutrient recommendations for early gestation (NRC, 2007). Animals had their breeding dates intentionally distributed from September 2010 to December 2010 (Lemley el al., 2012). Maternal BW was determined in a weekly basis and at the beginning of experimental period (d 50 of gestation) averaged 45.6 ± 0.5 kg (Lemley et al, 2012). Average daily gain was higher in the ADQ group (85 ± 7 g/d) when compared to RES group (-49 ± 7 g/d; Lemley et al., 2012).

Table 2.1. Treatment dietary composition fed to ewes.

Item	%
Ingredient, % of dry matter	
Beet pulp, dehydrated	29
Alfalfa meal, dehydrated	34
Ground corn	9
Soybean meal	4
Wheat middlings	24
Diet composition, % of dry matter	
Crude protein	15.8
Neutral detergent fiber	36.5
Acid detergent fiber	20.8
Calcium	1.11
Phosphorus	0.58
Ash	7.8
Metabolizable energy, Mcal/kg	2.66

Lemley et al, 2012.

On d 50 of gestation, dietary treatments were implemented and were continued until d 130 of gestation. Ewes were alloted to treatments in a 2 × 2 factorial (plane of nutrition by melatonin supplementation; n = 8 per treatment) arrangement of treatments and were fed at 100% (ADQ; adequate diet) or 60% (RES; restricted) of nutrient recommendations (NRC, 2007) and supplemented with 0 (CON) or 5 mg of melatonin rumen-fragile (MEL; Spectrum Chemical Mfg., Gardena, CA). Melatonin supplementation level was determined by a preliminary experiment that tested the effects of the supplementation on peripheral serum concentration of melatonin (Lemley et al, 2012). Adequate ewes were fed to receive adequate nutrients and energy for maintenance and fetal growth. One ewe had to be removed (ADQ-CON) from the study due to a perforated esophagus.

Feed was offered and adjusted as previously reported (Lemley et al., 2012), as well as the process to prepare the melatonin supplement and feeding. The maternal undernutrition model utilized during mid- to late-gestation has been previously reported to decrease fetal and birth weights in previous research (Lekatz et al., 2010; Meyer et al., 2010; Reed et al., 2007).

Visceral organ measurements, tissue collection, and analysis

Ewes were anesthetized at d 130 of gestation prior to tissue collection to collect uterine blood samples, as described by Lemley et al. (2012) and were then euthanized (sodium pentobarbital; Fort Dodge Animal Health, Fort Dodge, Iowa). Fetuses and viscera (including digesta) were removed and weighed. The liver (without the gall bladder) and the jejunum (30-cm section for maternal and 10-cm section for fetal) were removed for further analyses. The liver sample was collected from the left lobe. The landmarks utilized to define the correct portion of the jejunum to be collected began at a point adjacent to the third vascular branch caudal to the mesenteric-ileocecal vein junction. At this point, a 30-cm segment was collected for further

analyses. A second 30-cm segment was taken following the first section for measurement of stripped weight. Demarcations of the 3 sections of the small intestine were made using similar methods as previously reported (Soto-Navarro et al 2004; Caton et al., 2009; Yunusova et al., 2013). Similar approaches were used for sample collection of fetal small intestine. For fetal intestine, individual segments were not weighed separately. Therefore, fetal small intestinal oxygen consumption and citrate synthase activity are reported relative to total small intestinal weight for fetuses and relative to jejunum weight for ewes. After collection, the intestine was dissected, gently stripped of fat and digesta, and weighed.

A portion of each sample was snap-frozen in liquid N super-cooled with isopentene and stored at -80C until analyses. The remaining liver and jejunum/small intestine sample (200 mg) was collected and placed in Krebs-Ringer bicarbonate buffer (118.1 mM NaCl, 25.0 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$), and transported to the laboratory for *in vitro* O_2 consumption analysis (see below).

Hepatic and small intestinal in vitro O_2 consumption

As previously described by Scheaffer et al. (2003), tissues were sliced (0.5 mm thick) with a Stadie-Riggs microtome (Thomas, Philadelphia, PA). Tissues were placed into Petri dishes containing Krebs-Ringer buffer fortified with sodium pyruvate (5.0 m*M*), sodium glutamate (5.0 m*M*), sodium acetate (4.5 m*M*), glucose (25.0 m*M*), and malic acid (4.5 m*M*) buffer at 37°C. Subsamples (200 ± 10 mg; Reynolds et al., 1990) of the sliced tissues were placed into test chambers containing 3 mL of the same buffer and a Clarke polariographic electrode (model 5300, Yellow Springs Instruments, Yellow Springs, OH) to determine *in vitro* O_2 consumption for 5 min. Samples were analyzed in duplicate and the slope of the line created by the chart recorder was utilized to calculate O_2 consumption per test chamber. Oxygen

consumption (µmol/min) was then calculated per mg of tissue (µmol/min/mg tissue), per mg of protein (µmol/min/mg protein), per total organ weight (mol/min/tissue) and relative to empty BW (mol/min/kg).

Hepatic and small intestinal protein

Protein concentrations in maternal and fetal tissues were analyzed using the colorimetric bicinchoninic acid (BCA) method (Smith, et al. 1985; Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) using a micro plate reader (SPECTRAmax^{TM} 340, Molecular Devices Corporation, Sunnyvale, CA).

Citrate synthase activity

Citrate synthase activity was used as a marker for mitochondrial content (Rockl et al., 2007; Heilbronn et al., 2007) and was evaluated according to the methodology described by Srere (1969) using a commercially available kit (Sigma-Aldrich, St. Louis-USA). Samples (100 mg) of maternal and fetal liver and small intestine were homogenized in 2 mL of CelLyticTM MT Mammalian Tissue Lysis/Extraction Reagent (Sigma-Aldrich, St. Louis, MO). Lysed samples were centrifuged for 10 min at 16,000 × g to pellet the tissue debris and the supernatant extract was transferred to a chilled vial. Samples were pipetted (2 µL) to a 96 well plate (Thermo Scientific, Rockford, IL) and the protocol was followed from the citrate synthase activity kit (Sigma Aldrich).

Statistical analysis

Data were analyzed as a completely randomized design with a 2 × 2 factorial arrangement of treatments utilizing the maximum likelihood covariance test (PROC MIXED, SAS Inst., Cary, NC). The model included breeding date, melatonin treatment, nutritional treatment, and the interaction between melatonin treatment and nutritional treatment. Breeding

date was used as a covariate due to the range of breeding dates from early September (13.5 h of daylight) to late December (8 h of daylight). Significance was declared at $P \leq 0.05$ and a tendency was reported if $0.05 < P < 0.10$.

Results

Empty body weight of ewes decreased ($P < 0.001$) in RES ewes as compared with ADQ ewes (Table 2.2). Liver weight (g and g/kg EBW) decreased ($P \leq 0.02$) in RES ewes as compared with ADQ ewes. Protein concentration and content relative to BW (mg/g and g/kg EBW) in the maternal liver decreased ($P \leq 0.04$) and protein content (g/liver) tended to decrease ($P = 0.08$) in RES ewes compared with ADQ ewes. There was an interaction ($P = 0.04$) between plane of nutrition and melatonin supplementation for liver protein (mg/g), as protein concentration increased in ADQ-MEL versus ADQ-CON and RES-MEL ewes. Maternal jejunal weight (g) decreased ($P < 0.001$) in RES ewes as compared to ADQ ewes. There was an interaction ($P = 0.04$) between plane of nutrition and melatonin supplementation for jejunal weight relative to EBW (g/kg EBW), as jejunal weight increased in ADQ-MEL ewes versus all other treatment groups. Protein content (g/jejunum) in maternal jejunum decreased ($P < 0.001$) in RES ewes compared with ADQ ewes. There was an interaction ($P = 0.02$) between plane of nutrition and melatonin supplementation for protein content relative to EBW (g/kg of EBW), as protein content increased in ADQ-MEL ewes versus all other treatment groups.

Table 2.2. Influence of dietary melatonin supplementation and nutrient restriction on maternal empty body weight, liver and small intestinal mass, and protein concentration and content.

Item	Treatment							
	RES*		ADQ†			*P*-Value		
	CON‡	MEL§	CON	MEL	SEM	Mel	Nutr	M x N
EBW¶, kg	36.7	35.8	47.4	45.0	1.33	0.21	<0.001	0.57
Liver weight								
G	419	425	586	588	13.1	0.78	<0.001	0.87
g/kg EBW	11.5	11.9	12.5	13.1	0.431	0.23	0.02	0.80
Liver protein								
mg/g	180[ab]	173[a]	173[a]	194[b]	6.94	0.31	0.28	0.04
G	75.9	73.5	102	114	4.21	0.21	<0.001	0.08
g/kg EBW	2.09	2.07	2.18	2.56	0.137	0.20	0.04	0.15
Jejunum weight								
G	149	140	194	223	11.6	0.41	<0.001	0.11
g/kg EBW	4.09[a]	3.88[a]	4.09[a]	4.95[b]	0.249	0.20	0.04	0.04
Jejunum protein								
mg/g	108	109	108	111	3.46	0.43	0.80	0.77
G	16.0	15.0	20.9	24.8	1.26	0.26	<0.001	0.06
g/kg EBW	0.441[a]	0.418[a]	0.442[a]	0.550[b]	0.028	0.13	0.02	0.02

* RES = Fed at 60% of nutrient recommendations.
†ADQ = Fed at 100% of nutrient recommendations.
‡CON = no melatonin supplementation.
§MEL = 5 mg of melatonin supplementation.
¶EBW = Empty body weight; weight of body minus digesta.
[a,b] Means followed by different lower case letter (a, b) are different ($P \leq 0.05$).

Total *in vitro* O_2 consumption (mol/min/tissue) in maternal liver decreased ($P < 0.001$) in RES ewes as compared with ADQ ewes (Table 2.3). In maternal liver, total citrate synthase activity (mol/min/tissue and mol/min/kg EBW) decreased ($P \leq 0.03$) in RES ewes as compared with ADQ ewes. Jejunum oxygen consumption per mg tissue and per mg protein decreased ($P \leq 0.03$) in ADQ as compared with RES ewes. There was a plane of nutrition by melatonin supplementation interaction ($P = 0.02$) for total O_2 consumption (mol/min/tissue) in the jejunum, as consumption increased in ADQ-MEL compared with all other treatments. There was a plane of nutrition by melatonin supplementation interaction ($P = 0.01$) for total O_2 consumption relative to EBW (mol/min/kg), as consumption was greatest in ADQ-MEL, least in ADQ-CON and RES-MEL, with RES-CON intermediate. There was a significant ($P = 0.03$) plane of nutrition by melatonin supplementation interaction for total citrate synthase activity (mol/min/tissue) in the jejunum as activity increased in ADQ-MEL versus all other treatment groups. There was a significant ($P = 0.007$) plane of nutrition by melatonin supplementation interaction for total citrate synthase activity relative to EBW (mol/min/kg of EBW) in the jejunum as activity was greatest in ADQ-MEL, least in ADQ-CON and RES-MEL, with RES-CON intermediate.

Table 2.3. Influence of dietary melatonin supplementation and nutrient restriction on maternal *in vitro* oxygen consumption and citrate synthase activity in liver and small intestine.

Item	Treatment							
	RES*		ADQ†			*P*-Value		
	CON‡	MEL§	CON	MEL	SEM	Mel	Nutr	M x N
Liver oxygen consumption								
µmol/min/mg tissue	64.0	63.3	65.8	64.1	4.22	0.78	0.76	0.90
µmol/min/mg protein	363	373	396	332	31.7	0.41	0.91	0.26
mol/min/tissue	26.8	26.7	39.0	38.1	2.71	0.86	<0.001	0.87
mol/min/kg EBW	0.739	0.750	0.833	0.849	0.067	0.84	0.17	0.97
Liver citrate synthase								
mmol/min/mg tissue	2.10	2.27	2.37	2.41	0.140	0.46	0.15	0.63
mmol/min/mg protein	11.6	13.2	13.9	12.4	0.810	0.99	0.33	0.07
mol/min/tissue	880	966	1388	1416	81.1	0.48	<0.001	0.72
mol/min/kg EBW	24.4	27.0	29.7	31.8	2.24	0.30	0.03	0.91
Jejunum oxygen consumption								
µmol/min/mg tissue	91.1	80.1	65.2	74.2	6.07	0.87	0.02	0.11
µmol/min/mg protein	840	724	627	651	63.5	0.48	0.03	0.28
mol/min/tissue	14.0^{a}	11.5^{a}	11.7^{a}	17.1^{b}	1.58	0.37	0.32	0.02
mol/min/kg EBW	0.386^{a}	0.326^{ab}	0.242^{b}	0.382^{a}	0.037	0.30	0.25	0.01
Jejunum citrate synthase								
mmol/min/mg tissue	2.94	2.45	2.17	2.68	0.276	0.99	0.33	0.08
mmol/min/mg protein	27.8	22.2	20.0	23.9	2.40	0.71	0.21	0.06
mol/min/tissue	433^{a}	336^{a}	430^{a}	596^{b}	56.9	0.54	0.03	0.03
mol/min/kg EBW	11.8^{ab}	9.2^{a}	8.9^{a}	13.2^{b}	1.17	0.46	0.62	0.007

* RES = Fed at 60% of nutrient recommendations.
†ADQ = Fed at 100% of nutrient recommendations.
‡CON = no melatonin supplementation.
§MEL = 5 mg of melatonin supplementation.
[a,b] Means followed by different lower case letter (a, b) are different ($P \leq 0.05$).

Fetal body weight and fetal liver and small intestine weight were previously published (Lemley et al., 2012). Fetal BW decreased ($P < 0.001$) in the RES group as compared to the ADQ group (Table 2.4). Fetal liver weight (g and g/kg BW) decreased ($P \leq 0.02$) in the RES group as compared to the ADQ group. There was an interaction ($P = 0.02$) between plane of nutrition and melatonin supplementation in fetal small intestine weight (g) as small intestine weight was greatest in fetuses from RES-MEL ewes, lightest in ADQ-CON and ADQ-MEL, with RES-CON intermediate. There was an interaction ($P = 0.05$) between plane of nutrition and melatonin supplementation in fetal small intestine weight relative to BW (g/kg BW) as small intestine was greatest in fetuses from ADQ-MEL ewes, lightest in fetuses from RES-MEL ewes, with RES-CON and ADQ-CON intermediate. Protein concentration (mg/g) in fetal small intestine decreased ($P = 0.008$) in fetuses from RES ewes compared to fetuses from ADQ ewes. There was an interaction ($P \leq 0.003$) between plane of nutrition and melatonin supplementation for small intestine protein content (g and g/kg BW) as protein content was greatest in ADQ-MEL, least in RES-MEL, with RES-CON and ADQ-CON intermediate.

Table 2.4. Influence of maternal dietary melatonin supplementation and nutrient restriction on fetal empty body weight, liver and small intestinal mass, and protein concentration and content.

Item	Treatment							
	RES*		ADQ†			*P*-Value		
	CON‡	MEL§	CON	MEL	SEM	Mel	Nutr	M x N
BW, kg	3.17	3.07	3.41	3.69	0.107	0.40	<0.001	0.08
Liver weight								
G	77.0	75.1	96.4	97.0	3.82	0.87	<0.001	0.74
g/kg BW	24.4	24.8	28.6	26.3	1.05	0.35	0.02	0.21
Liver protein								
mg/g	141	130	128	141	9.35	0.88	0.89	0.19
G	10.7	9.44	12.48	14.13	1.17	0.88	0.02	0.22
g/kg BW	3.43	3.12	3.68	3.83	0.355	0.82	0.21	0.52
Small intestine weight								
G	40.9^{ab}	33.9^{a}	42.2^{bc}	48.8^{c}	2.75	0.93	0.01	0.02
g/kg BW	12.8^{ab}	11.1^{a}	12.4^{ab}	13.2^{b}	0.642	0.52	0.25	0.05
Small intestine protein								
mg/g	86.5	82.2	94.1	95.0	6.63	0.65	0.008	0.48
G	3.46^{a}	2.69^{b}	3.92^{a}	4.70^{c}	0.205	0.97	<0.001	<0.001
g/kg BW	1.09^{a}	0.89^{b}	1.15^{ac}	1.27^{c}	0.049	0.36	<0.001	0.003

* RES = Fed at 60% of nutrient recommendations.
†ADQ = Fed at 100% of nutrient recommendations.
‡CON = no melatonin supplementation.
§MEL = 5 mg of melatonin supplementation.
[a,b,c] Means followed by different lower case letter (a, b) are different ($P \leq 0.05$).

In fetal liver tissue, total O_2 consumption (mol/min/tissue) decreased ($P = 0.01$) in the RES group compared to the ADQ group (Table 2.5). Total citrate synthase activity (mol/min/tissue and mol/min/kg of BW) in fetal liver decreased ($P \leq 0.05$) in the RES group as compared to the ADQ group. There was an interaction ($P \leq 0.03$) between plane of nutrition and melatonin supplementation for fetal small intestinal O_2 consumption (μmol/min/mg and mol/min/kg), as RES-CON and ADQ-MEL had increased consumption compared to RES-MEL and ADQ-CON. Fetal small intestinal O_2 consumption per mg protein (μmol/min/mg of protein) increased ($P = 0.004$) in the RES group as compared to the ADQ group. There was an interaction ($P = 0.03$) between plane of nutrition and melatonin supplementation for fetal small intestinal total O_2 consumption (mol/min/tissue), as oxygen consumption was greatest in RES-CON, least in RES-MEL and ADQ-CON, with ADQ-MEL intermediate.

Table 2.5. Influence of maternal dietary melatonin supplementation and nutrient restriction on fetal *in vitro* oxygen consumption and citrate synthase activity in liver and small intestine.

Item	Treatment							
	RES*		ADQ†			*P*-Value		
	CON‡	MEL§	CON	MEL	SEM	Mel	Nutr	M x N
Liver oxygen consumption								
µmol/min/mg tissue	61.1	60.9	59.6	60.9	2.24	0.80	0.76	0.72
µmol/min/mg protein	485	534	827	402	193	0.33	0.62	0.22
mol/min/tissue	4.80	4.56	5.77	5.80	0.377	0.79	0.01	0.72
mol/min/kg BW	1.54	1.53	1.69	1.60	0.091	0.58	0.28	0.61
Liver citrate synthase								
mmol/min/mg tissue	2.00	1.87	1.99	2.11	0.128	0.96	0.40	0.33
mmol/min/mg protein	14.5	15.0	16.0	14.8	0.856	0.70	0.42	0.32
mol/min/tissue	154	136	191	204	12.6	0.87	<0.001	0.23
mol/min/kg BW	49.0	45.5	56.2	55.4	3.97	0.58	0.05	0.74
Small intestine oxygen consumption								
µmol/min/mg tissue	74.0[a]	60.2[b]	51.2[b]	56.4[b]	4.19	0.31	0.009	0.03
µmol/min/mg protein	850	718	567	605	57.2	0.41	0.004	0.14
mol/min/tissue	3.00[a]	1.98[b]	2.12[b]	2.66[ab]	0.267	0.37	0.74	0.006
mol/min/kg BW	0.949[a]	0.662[b]	0.626[b]	0.736[a]	0.073	0.23	0.13	0.01
Small intestine citrate synthase								
mmol/min/mg tissue	2.22	1.97	2.51	2.33	0.200	0.28	0.14	0.86
mmol/min/mg protein	26.6	24.2	26.4	23.8	2.10	0.23	0.90	0.96
mol/min/tissue	92.3	66.0	105	112	9.83	0.33	0.009	0.10
mol/min/kg BW	28.9	21.6	30.6	30.3	2.62	0.15	0.07	0.19

* RES = Fed at 60% of nutrient recommendations.
† ADQ = Fed at 100% of nutrient recommendations.
‡ CON = no melatonin supplementation.
§ MEL = 5 mg of melatonin supplementation.
[a,b] Means followed by different lower case letter (a, b) are different ($P \leq 0.05$).

Discussion

In the current study, maternal empty body weight, liver weight, jejunum weight, and protein content in liver and jejunum decreased in response to feed restriction. The decreased amount of nutrients available to support maintenance and pregnancy may result in a shift in the amount of nutrients being consumed from the gut to functions that will allow the dam to survive and to maintain gestation. Previous studies reported decreased organ masses resulting from nutrient restriction (Scheaffer et al., 2004), as well as an association with maintenance energy expenditure (Ferrell and Oltjen, 2008). When nutrient restriction occurred during gestation (Reed et al., 2007; Scheaffer et al., 2004), maternal and fetal gastrointestinal and liver tissue masses generally decreased (Bell, 1993; Osbergy et al., 2002; Vonnahme et al., 2003). Nutrient restriction of ewes at d 50 of gestation resulted in decreased BW by d 135 (Faichney and White, 1987) that was associated with the level of restriction. Moreover, the GIT along with the liver, represent approximately 10% of body mass but is responsible for approximately 50% of total energy expenditure (Koong et al., 1985; Reynolds et al., 1991). Decreased mass of metabolically active tissues in response to nutrient restriction suggests that alterations in whole animal energy balance may occur.

Limited information is available on the effects of supplementation of dietary melatonin on hepatic and small intestinal mass. The observed interaction between nutrient restriction and melatonin supplementation for jejunum weight with increases observed due to MEL supplementation in ADQ groups but not in RES groups may suggest that melatonin supplementation has a greater effect on jejunum mass and protein content in ADQ than RES ewes. Perhaps the limitation in the amount of available nutrients to the intestine results in the inability of these tissues to respond to melatonin supplementation in regards to tissue mass.

Variation in O_2 consumption and citrate synthase activity can be utilized as an indication of changes in energy use and mitochondrial activity (Civitarese et al., 2007). *In vitro* O_2 consumption and citrate synthase activity per mg of maternal liver was not influenced by treatment, suggesting that changes in energy use and mitochondrial mass per mg of tissue was not influenced by dietary treatment and that the observed decrease in total *in vitro* O_2 consumption and total citrate synthase activity in maternal liver in RES ewes was the result of changes in liver mass. Previous research has suggested that liver O_2 consumption increases during gestation as measured using catheterized ewes (Freetly and Ferrell, 1998). Others have reported that melatonin supplementation increases TCA cycle substrate-induced respiration in rat liver (Reyes-Toso et al., 2006). The observed increase in jejunum O_2 consumption and citrate synthase activity per g of jejunum in ADQ ewes suggests that nutrient restriction may increase energy use per unit of tissue. In agreement with the tissue mass interaction results described above, total O_2 consumption and citrate synthase activity were increased with MEL supplementation in ADQ ewes but not in RES ewes, suggesting that the jejunum may be responsive to MEL supplementation only in ADQ ewes. In steers, small intestinal O_2 consumption did not differ due to dietary treatment and different levels of feed intake (Kelly et al., 2001). However, others have reported that *in vitro* O_2 consumption of duodenal mucosa (per mg) in growing wethers increased with increased feed intake (McBride and Milligan, 1985). Changes in GIT *in vitro* O_2 consumption may reflect possible changes in intestinal nutrient supply (Kelly et al. 1993). Nutrient restriction also decreased fetal BW, liver and small intestinal weight (Lemley et al., 2012) and protein content in this study. When the interaction between plane of nutrition and melatonin supplementation was taken into consideration, an increase in jejunal weight and jejunal protein content was observed in the adequately fed ewes supplemented

with melatonin. It has been demonstrated that undernourished sheep have compromised placental vascularity which influences transport of nutrients and oxygen to the fetus which impacts fetal growth (Thomas and Kott, 1995). Our findings are in agreement with previous reports that suggested decreased fetal weight resulting from nutrient restriction (Clarke et al., 1998; Heasman et al., 1999; Osbergy et al., 2002; Reed et al., 2007). Vonnahme et al. (2003) also observed lighter GIT weight in fetuses from nutrient restricted ewes at d 135 of gestation when reported on an absolute basis (g) but GIT weights were greater when expressed as a percentage of fetal BW. Asymmetrical growth of fetuses' organs during development may impact the difference in results obtained in different studies. The increase in maternal and fetal organ mass with melatonin supplementation when dams were fed adequate nutrients may be related to the ability of melatonin in the GIT (Bubenik et al., 1992; Huether, 1994) to increase absorption of nutrients (Sotak et al., 2000; Song et al., 2005). In livestock species, timing and rate of fetal GIT maturation is dependent on enteral nutrient intake, which takes place both pre and postnatally (Sangild, 2006). Towards midgestation, the fetus starts to receive nutrients through enteral nutrition swallowing amniotic fluid (Sangild, 2006). Major developmental changes in the gut appear to occur after swallowing is initiated, which stimulates prenatal mucosal differentiation of the GIT lumen (Trahair and Harding 1995; Trahair and Sangild, 1997, 2002). Furthermore, it is hypothesized that melatonin present in bile functions as a protection mechanism for the gastrointestinal mucosa against oxidative stress (Tan et al., 1999), since it acts as a potent endogenous free radical scavenger (Tan et al., 1993) and as an antioxidant (Reiter, 1998)

The observed decrease in maternal hepatic O_2 consumption and citrate synthase activity in the RES group also occurred in fetal tissue indicating that there is decreased energy use in both maternal and fetal liver. When interactions between melatonin supplementation and plane

of nutrition were analyzed in fetuses, an increase in jejunal O_2 consumption was observed in the CON-RES compared to CON-ADQ; however, melatonin supplementation to nutrient restricted ewes (MEL-RES) decreased jejunal O_2 consumption to the level of CON-ADQ. This observation may be due to the effect of melatonin on down-regulating pro-oxidant enzymes which are thought to be regulated on a tissue-specific basis (Hardeland, 2005). However, citrate synthase activity does not follow the same pattern, perhaps because cellular maturation and enzymatic activity onset may not be completed at this point of fetal development (Drozdowski et al., 2010). It has been observed that mitochondrial biogenesis can be a result of cellular proliferation and differentiation, which happens simultaneously with increases in Na,K-ATPase enzymatic activity during the perinatal period in rats (Prieur et al., 1995). Moreover, perinatal mitochondrial maturation in the rat kidney and liver is controlled by adrenal hormones (Prieur et al., 1997).

This study examined the effects of two levels of nutrition with or without one level of melatonin supplementation in pregnant ewes from d 50 to 130 of gestation on maternal and foetal liver and small intestinal mass and O_2 consumption. Additional research could be developed to examine effects of different levels of restriction or different levels of melatonin supplementation during different stages of gestation and to further study the mechanisms by which nutrition and melatonin influence tissue energy metabolism. In conclusion, our findings suggest that maternal nutrient restriction had a greater effect than melatonin supplementation on liver and jejunum mass and energy utilization in dams and foetuses. However, the observed interactions between melatonin and plane of nutrition generally suggest that jejunum mass and energy utilization are more responsive to melatonin supplementation in ewes fed adequate nutrition compare with restricted ewes. Furthermore, energy use measured through O_2 consumption and citrate synthase activity was reduced with nutrient restriction in hepatic tissue

but increased in intestinal tissue, suggesting a possible compensatory effect in this organ. Our results suggest that melatonin supplementation may increase intestinal mass and energy use in adequately fed ewes but not in restricted ewes, and therefore, it may have limited use as a therapeutic supplement to help overcome potential negative effects of nutrient restriction on liver and intestinal function in ewes and offspring.

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CHAPTER 3 - EFFECTS OF NUTRIENT RESTRICTION FOLLOWED BY REALIMENTATION IN BEEF COWS DURING EARLY- AND MID-GESTATION ON MATERNAL AND FETAL SMALL INTESTINAL MASS AND IN VITRO OXYGEN CONSUMPTION

Abstract

This study examined how maternal and fetal BW, liver and jejunal mass and energy use change throughout gestation and in response to maternal nutrient restriction in beef cows during early- to mid-gestation followed by realimentation. At d 30 of gestation, cows (initial BW = 620 ± 11.3 kg and BCS = 5.1 ± 0.1) were randomly assigned to 1 of 3 treatment groups: 1) 100% NRC recommendations throughout the experiment (CCC; n = 18); 2) 60% NRC recommendations from d 30 until 85 of pregnancy, then realimented to 100% of recommendations (RCC; n = 16); or 3) 60% of NRC recommendations from d 30 until 140, then realimented to 100% of recommendations (RRC; n = 12). Cows were slaughtered at d 85 (n = 6 and 6 for R and C), 140 (n = 6, 5, and 6 for RR, RC and CC), and 254 (n = 6, 5, and 6 for RRC, RCC and CCC) of gestation. Maternal liver oxygen consumption linearly increased ($P \leq 0.04$) and jejunal weight linearly decreased ($P = 0.04$) as gestation advanced. For fetus, BW, and hepatic and small intestinal absolute mass, protein content and oxygen consumption linearly increased ($P \leq 0.04$) as pregnancy advanced. However, hepatic mass and O_2 consumption relative to BW linearly decreased ($P \leq 0.001$) in the liver and had a quadratic effect ($P \leq 0.002$) in the small intestine. At d 85, fetal jejunal oxygen consumption (mol/min/kg BW) was lower ($P = 0.02$) in the R group when compared with the C group. At d 140, maternal liver weight (g) decreased ($P = 0.02$) in RC and RR cows when compared with CC and fetal jejunual oxygen consumption (mmol/min/mg tissue and mmol/min/g protein) was greater ($P \leq 0.02$) in RC when

compared with RR. At d 254, maternal hepatic oxygen consumption (absolute and relative to BW) was lower ($P \leq 0.04$) in the RCC cows when compared with RRC. Fetal liver weight was lower ($P = 0.05$) in the CCC group when compared with RCC and RRC, and liver protein content (g/liver) was lower ($P = 0.009$) in the CCC group when compared with RCC and RRC and lower ($P = 0.03$) in RCC when compared with RRC. Changes in visceral organ mass and energy use in the dam being fed to the requirements related to each stage of gestation, demonstrate how the maternal adaptations might occur in response to advance in pregnancy. Moreover, the changes in response to nutrient restriction in both the dam and fetus may indicate an adaptation to lower amount of nutrients by altering mass and metabolism in an attempt to alter digestive capacity and tissue metabolism, potentially returning to similar values after longer periods of realimentation as observed in their non-restricted counterparts.

Introduction

Pregnancy results in an increase (that varies between 20 to 50%) in maternal metabolic rate (measured via O_2 consumption), in most mammals (Stock and Metcalfe, 1994). Around 50% of the metabolic increase is related to the gravid uterus, while the other portion is related to metabolic changes in the dam's tissues such as the heart, the muscles related to the respiratory system, and the kidney (Bauman and Currie, 1980). Moreover, the small intestine and liver along with other organs that form the gastrointestinal tract (GIT) represent around 10% of total body mass; however, a large proportion of the total energy expenditure occurs in these organs (Koong et al., 1985; Reynolds et al., 1991). Timing and rate of fetal GIT maturation is dependent on enteral nutrient intake, which takes place during pre- and post-natal life (Sangild, 2006). Towards midgestation, the fetus starts to receive nutrients through enteral nutrition by swallowing of amniotic fluid (Sangild, 2006). Major developmental changes in the gut appear to

occur after swallowing is initiated, which stimulates prenatal mucosal differentiation of the GIT lumen (Trahair and Harding, 1995; Trahair and Sangild, 1997; Trahair and Sangild, 2000).

Variation in gestational nutritional status, results in effects on the dam (Hess et al., 2005) and on the offspring (Godfrey and Barker, 2000; Wu et al., 2006; Caton and Hess, 2010). Nutrient restriction results in a decrease in visceral organ mass (Scheaffer et al., 2004b) and impacts maintenance energy expenditure (Ferrell and Oltjen, 2008). Researchers have suggested that when gestating animals are nutrient-restricted (Scheaffer et al., 2004b; Reed et al., 2007), fetal intestinal mass increases (Osgerby et al., 2002) and liver mass decreases (Bell 1993); however, one study has observed an increase in liver mass (Vonnahme et al., 2003). Energy balance may be altered in response to changes in mass of metabolically active tissues. The maternal system may respond to alterations in nutrient intake by programming nutrient partitioning and consequently changing growth and development rate, as well as function of the major fetal organs (Godfrey and Barker, 2000; Wu et al., 2006; Wallace, 1948; Wallace et al., 1999). On the fetal side, nutrient restriction during mid- to late-gestation reduces growth and alters differentiation of the intestine, and consequently reduces body growth (Trahair and Sangild, 2000; Buchmiller et al., 1993; Sangild et al., 2002).

Fetuses from dams that were realimented during midgestation were able to increase their weight to what was observed in control animals (Dandrea et al., 1999). Maternal glucose and fat metabolism, and fetal tissue weights were not affected by early- to mid-gestation nutrient restriction followed by re-feeding to a well-fed level of intake (Brameld et al., 2000). Moreover, realimentation during late gestation appears to promote GIT growth in IUGR fetuses (Buchmiller et al., 1992; Buchmiller et al., 1994). Because of the potential of maternal nutrient restriction to result in changes in small intestinal and liver mass and/or energy use in both dam and fetus, our

objectives were to determine how nutrient restriction and realimentation during different times during gestation influences maternal and fetal small intestinal mass and energy use. We hypothesized that maternal realimentation during early- or mid-gestation will increase small intestinal and liver mass and decrease O_2 consumption caused by nutrient restriction, in both dam and fetus.

Materials and methods

Animals, housing, and diet

All procedures involving animals were approved by the North Dakota State University (NDSU) Animal Care and Use Committee. Forty six cross-bred multiparous gestating and non-lactating crossbred beef cows were managed as previously described (Gonzales et al., 2013; Camacho et al., 2014). Prior to the start of the experimental period, cows were adapted to the Calan gate feeding system for individual feeding and were fed a common hay diet to meet or exceed NRC recommendations for net energy, metabolizable protein, minerals, and vitamins (NRC, 2000) until d 30 of gestation. Cows were assigned to 1 of 6 breeding groups (n = 4 to 11 cows per breeding group with all treatments begin represented in each breeding group) with breeding dates ranging from July 13 to October 24, 2011. Cows were synchronized, monitored for estrous behavior, and bred using semen from one Angus bull. Pregnancy was confirmed via transrectal ultrasonography on d 28 or 29 post-insemination. Pregnant cows (d 30 of gestation; initial BW = 620 ± 11.3 kg and BCS = 5.1 ± 0.1) were grouped by d of insemination (breeding group) and body weight, and randomly assigned to treatment groups: control (C; 100% NRC; n = 18) and nutrient restriction (R; 60% NRC; n = 30). On d 85, cows were slaughtered (Con, n = 6 and Res, n = 6), remained on control (CC; n = 12) and restricted (RR; n = 12) treatments, or were realimented to control (RC; n = 11). On d 140, cows were slaughtered (CC, n = 6; RC, n = 5;

RR, n = 6), remained on control (CCC, n = 6; RCC, n = 5), or were realimented to control (RRC, n = 6). On d 254, all remaining cows were slaughtered (CCC, n = 6; RCC, n = 5; RRC, n = 6). Two animals, one from the RC group and one from the RCC group were removed from the study due to early embryonic loss and due to a twin pregnancy, respectively.

Cows were fed chopped grass hay (86.1% dry matter [DM], 8.0% crude protein [CP], 57.9% total digestible nutrients [TDN], and 1.3% fat) at 60 or 100% of their dietary net energy requirements (NRC, 2000), and a mineral and vitamin supplement (12% Ca; 5% Mg; 5% K; 180 ppm Co; 5,100 mg/kg Cu; 375 mg/kg I; 1.2% Fe; 2.7% Mn; 132 mg/kg Se; 2.7% Zn; 570,000 IU/kg Vit A; 160,000 IU/kg Vit D-3; 2,700 IU/kg Vit E) was top-dressed to meet or exceed mineral and vitamin requirements (NRC, 2000).

Visceral organ measurements, tissue collection, and analysis

Cows were weighed and slaughtered at the NDSU meat laboratory. Fetuses and viscera (including digesta) were removed and weighed. The liver (without the gall bladder) and the jejunum (150-cm section for maternal and 15 to 20 cm for fetal) were removed for further analyses. The liver sample was collected from the lobe opposite the gall bladder. The landmarks utilized to define the correct portion of the jejunum to be collected began at a point adjacent to the third vascular branch caudal to the mesenteric-ileocecal vein junction. A second 150-cm segment was taken following the first section for measurement of stripped weight. Demarcations of the 3 sections of the small intestine were made using similar methods as previously reported (Soto-Navarro et al., 2004; Caton et al., 2009; Yunusova et al., 2013). The intestine was dissected, gently stripped of fat and digesta, and weighed. However, for fetuses at d 85 of gestation the whole small intestine was collected since it was not possible to collect just a portion of the jejunum due to the small size of the small intestine.

A portion of each sample was snap-frozen in liquid N super-cooled with isopentene and stored at -80°C until analyses. The remaining liver and jejunum samples were collected and placed in Krebs-Ringer bicarbonate buffer (118.1 mM NaCl, 25.0 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$), and transported to the laboratory for *in vitro* O_2 consumption analysis (see below).

Hepatic and jejunal in vitro O_2 consumption

As previously described by Scheaffer et al. (2003) and Prezotto et al. (2014), tissues were sliced (0.5 mm thick) with a Stadie-Riggs microtome (Thomas, Philadelphia, PA). Tissues were placed into Petri dishes containing Krebs-Ringer buffer fortified with sodium pyruvate (5.0 mM), sodium glutamate (5.0 mM), sodium acetate (4.5 mM), glucose (25.0 mM), and malic acid (4.5 mM) buffer at 37°C. Subsamples (200 ± 10 mg; Reynolds et al., 1990) of the sliced tissues were placed into test chambers containing 3 mL of the same buffer and a Clarke polariographic electrode (model 5300, Yellow Springs Instruments, Yellow Springs, OH) to analyze for *in vitro* O_2 consumption for 5 min. Samples were analyzed in duplicate and the slope of the line created by the chart recorder was utilized to calculate O_2 consumption per test chamber. Oxygen consumption (µmol/min) was then calculated per mg of tissue (µmol/min/mg), per total organ weight (mol/min/tissue) and relative to BW (mol/min/kg of BW).

Hepatic and jejunal protein

Protein concentrations in maternal and fetal tissues were analyzed using the colorimetric bicinchoninic acid (BCA) method (Smith et al., 1985; Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL) using a micro plate reader (SPECTRAmax^{TM} 340, Molecular Devices Corporation, Sunnyvale, CA).

Calculations and statistical analysis

Oxygen consumption (mmol/min/g) was calculated from the data obtained from the analyses of duplicate samples weighing approximately 200 mg of each tissue and then extrapolated per g of protein (mmol/min/g protein), per total tissue weight (mmol/min/tissue), and relative to BW (mmol/min/kg BW). Data were analyzed as a completely randomized design with treatment included in the model statement for the dams and treatment and sex for the fetuses. Differences between means were determined using contrast statements analyzing linear and quadratic effects in the control group over time to determine effects of advancing gestation. Contrast statement coefficients for linear and quadratic effects were determined using the IML procedure of SAS. Effects of treatments were determined using contrast statements within slaughter days (d 85, d 140, or d 254 of gestation) to determine the effect of nutrient restriction on d 85 (C vs R), nutrient restriction on d 140 (CC vs RR and RC), nutrient restriction vs realimention on d 140 (RC vs RR), nutrient realimentation on d 254 (CCC vs RCC and RRC), and the effect of length of realimentation (RCC vs RRC). Significance was declared at $P \leq 0.05$ and tendency was reported if $0.05 < P < 0.10$.

Results

Maternal hepatic oxygen consumption (mol/min/kg BW) linearly decreased ($P \leq 0.04$) with advancing gestation (Table 3.1). However, a quadratic effect was observed ($P = 0.01$) for total hepatic O_2 consumption (mol/min/liver); with consumption decreasing from d 85 until d 140 and then increasing from d 140 until d 254. Jejunal weight (g) tended to linearly decrease ($P = 0.10$) and decreased when analyzed relative to BW (g/kg BW; $P = 0.04$) as gestation advanced. Protein concentration (mg/g) and oxygen consumption (mol/min/kg BW) in the jejunum tended to linearly increase ($P = 0.08$) and to decrease ($P = 0.06$), respectively with advancing gestation.

When comparing the different treatment groups within the different gestational periods, at d 85, none of the measured variables for mass and O_2 consumption in the maternal tissue differed due to nutrient restriction (Table 3.1). At d 140 of gestation, maternal BW tended to be lower ($P = 0.10$) in cows that had been restricted compared to control (CC vs. RR and RC). Maternal hepatic mass (g) decreased ($P = 0.02$; Table 3.1) in RC and RR when compared with CC cows. Maternal hepatic (mol/min/liver) and jejunal (μmol/min/mg jejunum) oxygen consumption tended to be lower ($P \leq 0.09$; Table 3.1) in RC and RR when compared with CC cows. At d 254 of pregnancy, maternal hepatic oxygen consumption increased ($P \leq 0.04$) in cows exposed to a longer length of realimentation when compared to cows exposed to a shorter period of realementation (RCC vs. RRC). Jejunal protein content (g/jejunum) tended to decrease ($P = 0.08$) in RRC when compared with RCC cows (Table 3.1).

Table 3.1. Influence of stage of gestation and dietary restriction and realimentation on maternal hepatic and jejunal weights and oxygen consumption.

Item	Stage of Gestation									*P*-value						
	Early (Day 85)			Mid (Day 140)				Late (Day 254)								
	C	R	CC	RC	RR	CCC	RCC	RRC	SEM	L	Q	C vs R	CC vs others	RC vs RR	CCC vs others	RCC vs RRC
BW, kg	558	540	606	571	531	610	619	606	28.7	0.22	0.35	0.62	0.10	0.30	0.94	0.73
Hepatic weight																
g	4262	4035	4376	3958	3765	4314	4331	4537	190.7	0.90	0.66	0.36	0.02	0.46	0.58	0.43
g/kg BW	7.70	7.48	7.41	6.95	7.12	7.08	7.01	7.52	0.279	0.10	0.77	0.55	0.25	0.65	0.56	0.19
Hepatic protein																
mg/g	114	112	115	103	114	117	120	115	6.37	0.72	0.98	0.85	0.40	0.20	0.90	0.59
g/liver	486	393	504	409	429	504	520	520	43.6	0.78	0.82	0.11	0.10	0.73	0.76	0.99
g/kg BW	0.874	0.721	0.836	0.719	0.817	0.827	0.843	0.863	0.0729	0.65	0.79	0.11	0.42	0.33	0.76	0.84
Hepatic oxygen consumption																
µmol/min/mg liver	19.6	16.7	18.6	19.8	15.5	29.4	25.7	34.9	3.12	0.01	0.25	0.48	0.78	0.32	0.79	0.04
µmol/min/mg protein	174	159	161	196	131	255	214	303	29.4	0.02	0.25	0.71	0.94	0.11	0.92	0.03
mol/min/liver	84.1	66.7	61.6	42.0	28.2	126.3	110.3	158.5	12.07	0.002	0.01	0.27	0.06	0.40	0.56	0.006
mol/min/kg BW	0.151	0.125	0.136	0.137	0.112	0.208	0.179	0.264	0.0238	0.04	0.23	0.41	0.67	0.44	0.65	0.01
Jejunal weight																
g	1476	1161	1134	1275	1200	987	1326	926	207.6	0.10	0.44	0.25	0.66	0.79	0.56	0.16
g/kg BW	2.71	2.17	1.93	2.19	2.30	1.61	2.20	1.53	0.380	0.04	0.33	0.27	0.47	0.83	0.56	0.21
Jejunal protein																
mg/g	47.1	49.1	48.4	45.8	48.7	54.9	57.1	54.0	3.83	0.08	0.47	0.69	0.83	0.58	0.89	0.56
g/jejunum	71.2	57.4	48.4	57.1	55.5	52.9	75.9	50.7	10.5	0.28	0.19	0.31	0.53	0.91	0.39	0.08
g/kg BW	0.132	0.107	0.080	0.098	0.107	0.087	0.125	0.083	0.019	0.14	0.11	0.31	0.32	0.74	0.42	0.11
Jejunal oxygen consumption																
µmol/min/mg jejunum	10.68	8.78	12.53	10.73	7.62	10.60	9.72	12.87	1.682	0.82	0.34	0.37	0.09	0.18	0.72	0.18
µmol/min/mg protein	228	178	261	238	158	197	172	239	32.1	0.32	0.27	0.24	0.11	0.08	0.81	0.13
mol/min/jejunum	16.5	10.0	14.1	13.2	8.3	10.2	12.9	11.7	3.02	0.11	0.92	0.10	0.34	0.24	0.55	0.78
mol/min/kg BW	0.030	0.018	0.024	0.023	0.016	0.016	0.021	0.020	0.0054	0.06	0.73	0.10	0.51	0.35	0.52	0.83

C = control, fed at 100% of NRC (2000) recommendations, R = restricted, fed at 60% of NRC (1996) recommendations starting at d 30 and continuing until d 85 (R) or 140 (RR).

n = 6, 6 6, 5, 6, 6, 5, 6 for C, R, CC, RC, RR, CCC, RCC, and RRC treatments, respectively.

L = linear effects of stage of gestation with C, CC, and CCC treatments; Q = quadratic effects of stage of gestation with C, CC, and CCC treatments; C vs R; CC vs others; RC vs RR; CCC vs others; RCC vs RRC = contrast statements within slaughter days (d 85, d 140, or d 254 of gestation).

A quadratic effect was observed with advancing gestation in fetal BW, absolute hepatic mass and protein content, and small intestinal mass, protein content and oxygen consumption ($P \leq 0.006$; Table 3.2). The fetal variables listed above exponentially increased as gestation advanced. When the measurements were reported relative to BW a quadratic effect ($P \leq 0.001$) was observed with an exponential decrease observed with advancing gestation. Fetal hepatic oxygen consumption (mol/min/kg BW) linearly decreased ($P \leq 0.004$) during the gestational period. When comparing the different treatment groups within the different gestational periods, at d 85, small intestinal O_2 consumption was lower in the R group when compared with C ($P = 0.02$; Table 2). At d 140, fetal small intestinal oxygen consumption was lower in the group with longer length of restriction (RR) when compared with realimented (RC) group. At d 254, fetal hepatic weight (g) and protein content (g/liver) were higher ($P \leq 0.05$; Table 2) in longer length of realimentation (RCC) and shorter length of realimentation (RRC) groups when compared with CCC group. When comparing a shorter length of realimentation with longer length of realimentation groups, hepatic protein content was greater in the group exposed to a shorter length of realimentation ($P = 0.03$) and hepatic oxygen consumption tended to be greater in the same group ($P = 0.08$; Table 2).

Table 3.2. Influence of stage of gestation and dietary restriction and realimentation on fetal hepatic and small intestinal weights and oxygen consumption.

Item	Stage of Gestation															
	Early (Day 85)			Mid (Day 140)			Late (Day 254)			*P*-value						
	C	R	CC	RC	RR	CCC	RCC	RRC	SEM	L	Q	C vs R	CC vs others	RC vs RR	CCC vs others	RCC vs RRC
BW, kg	0.120	0.200	2.00	2.16	2.09	34.5	35.1	37.1	1.15	<.0001	<.0001	0.95	0.92	0.96	0.22	0.19
Hepatic weight																
g	4.3	5.3	69	76	74	585	622	667	26	<.0001	<.0001	0.98	0.83	0.94	0.05	0.20
g/kg BW	37	37	35	36	34	17	18	18	1.1	<.0001	0.001	0.82	0.81	0.47	0.41	0.88
Hepatic protein																
mg/g	71	73	78	79	76	84	87	92	4.4	0.04	0.66	0.74	0.98	0.61	0.28	0.38
g/liver	0.305	0.983	5.0	6.2	5.0	49	53	61	2.7	<.0001	0.001	0.85	0.85	0.74	0.009	0.03
g/kg BW	2.7	2.7	2.6	2.8	2.6	1.4	1.6	1.7	0.147	<.0001	0.02	0.74	0.59	0.43	0.25	0.62
Hepatic oxygen consumption																
µmol/min/mg liver	34	32	33	36	28	19	18	21	4.1	0.004	0.45	0.69	0.71	0.10	0.88	0.52
µmol/min/mg protein	485	437	419	453	326	236	207	236	57	0.001	0.81	0.51	0.64	0.11	0.82	0.71
mol/min/liver	0.146	0.063	2.3	2.7	2.0	11	11	14	1.2	<.0001	0.29	0.96	0.99	0.67	0.41	0.08
mol/min/kg BW	1.2	1.2	1.1	1.3	0.898	0.312	0.326	0.380	0.140	<.0001	0.30	0.84	0.87	0.06	0.82	0.77
Small Intestinal weight[4]																
g	2.2	2.0	11	11	12	289	292	301	12	<.0001	<.0001	0.99	1.00	0.95	0.58	0.55
g/kg BW	19	18	5.1	5.2	5.6	8.3	8.5	8.2	0.658	<.0001	<.0001	0.30	0.78	0.65	0.94	0.69
Small Intestinal protein[4]																
mg/g	39	40	45	42	42	45	46	51	3.8	0.29	0.41	0.96	0.49	0.99	0.42	0.34
g/small intestine	0.084	0.112	0.503	0.473	0.482	13	14	15	1.1	<.0001	0.003	0.99	0.98	1.00	0.33	0.22
g/kg BW	0.748	0.750	0.253	0.219	0.232	0.379	0.390	0.414	0.051	<.0001	<.0001	0.97	0.60	0.85	0.68	0.71
Small Intestinal oxygen consumption[4]																
µmol/min/mg small intestine	12	8.6	9.4	13	7.8	13	13	14	1.5	0.30	0.12	0.11	0.58	0.01	0.95	0.60
µmol/min/mg protein	291	232	212	311	193	291	282	270	38	0.71	0.08	0.28	0.32	0.02	0.71	0.82
mol/min/small intestine	0.027	-0.070	0.152	0.106	0.178	3.8	3.7	4.1	0.330	<.0001	0.006	0.82	0.98	0.87	0.74	0.34
mol/min/kg BW	0.220	0.162	0.054	0.063	0.040	0.110	0.105	0.111	0.019	0.002	<.0001	0.02	0.91	0.37	0.91	0.81

C = control, fed at 100% of NRC (2000) recommendations, R = restricted, fed at 60% of NRC (1996) recommendations starting at d 30 and continuing until d 85 (R) or 140 (RR).

n = 6, 6 6, 5, 6, 6, 5, 6 for C, R, CC, RC, RR, CCC, RCC, and RRC treatments, respectively.

L = linear effects of stage of gestation with C, CC, and CCC treatments; Q = quadratic effects of stage of gestation with C, CC, and CCC treatments; C vs R; CC vs others; RC vs RR; CCC vs others; RCC vs RRC = contrast statements within slaughter days (d 85, d 140, or d 254 of gestation).

Discussion

Tissue mass and metabolic activity influence energy use by tissues (Goetsch, 1998). Previous researchers (Scheaffer et al., 2003; Scheaffer et al., 2004b) have demonstrated that changes in the GIT occur with the advance of pregnancy, which can also be influenced by changes in nutrient intake (Carlson et al., 2009). We demonstrated in the current study the alterations in maternal hepatic energy use, and jejunal mass and energy use in response to different stages of gestation. Oxygen consumption (and thus energy expenditure) in visceral tissues accounts for a large proportion of whole body oxygen consumption and it has been observed to vary depending on level of intake (Ruckebusch and Thivend, 1980; Dumas et al., 2004), physiological status (Reynolds and Huntington, 1988), and animal age (Verstegen, 1989). In our study, maternal hepatic energy use was reduced as pregnancy advanced, while the tissue mass was maintained. Our results contradict the suggestions made by Freetly and Ferrell (1998) that liver O_2 consumption increases throughout gestation when measured using catheterized ewes. The difference in results may be explained by the fact that in our experiment tissue specific O_2 consumption was measured, rather than total consumption as measured using net-flux approaches. Moreover, jejunal mass relative to BW was also decreased during gestation, followed by a tendency for a reduction in tissue energy use. The decreased mass of the jejunum that we observed also contradicts previous research that demonstrated that mass either remained unchanged or increased in response to pregnancy (Scheaffer et al., 2003, 2004). Thus, it appears that mass was decreased as an adaptation mechanism in order for the dam to support fetal growth throughout gestation, since intestinal cell turnover and function is known to have a high energy requirement. The decreased energy use may also be an indicator of this adaptation as gestation advanced. Perhaps, the energy obtained from digestion is being directed more towards the

support for fetal development, rather than to support tissue maintenance. These results can be supported by the data published by Camacho et al. (2014) which suggests that maternal BW and BCS decreased regardless to the dietary treatment throughout gestation. However, an increase was observed when realimentation was offered.

Dietary changes appear to modulate how the maternal GIT adapts to both the luminal nutrient supply and physiological state (Meyer et al., 2012). Timing of nutrient restriction during pregnancy could impact how tissues respond in terms of mass and energy use. In our study, no difference was observed in maternal tissue mass during early gestation, indicating that nutrient restriction did not affect tissue mass maintenance. However, hepatic oxygen consumption was decreased in response to the exposure to nutrient restriction in the first third of gestation. This decrease in hepatic energy use is in agreement with previous research (Koong et al., 1982; Ferrell and Koong, 1985; Ferrell et al., 1986; Burrin et al., 1990). Changes in mass and O_2 consumption have been previously attributed to occur in response to decreased dry matter intake and consequently metabolizable energy by nutrient restricted animals. Perhaps, these changes occur to adjust to the restricted amount of available nutrients to maintain BW (Ferrell and Koong, 1985; Ferrell et al., 1986; Burrin et al., 1989, 1990).

The tendency in decreased maternal BW during mid-gestation (d 140) observed in the groups exposed to restricted nutrient intake (RC and RR) from either from d 30 to d 85 or d 30 to d 140 of gestation might occur in response to the increased nutrient demand in response to pregnancy; moreover, realimentation did not result in compensatory gain of RC cows, as previously reported. The effects of restriction on the dam were also observed on maternal liver weight (g), which decreased in the groups exposed to nutrient restriction. Perhaps, this is occurring as a response to decreased nutrient intake combined with the high tissue energy

requirement of this organ. The mass data is in agreement with a previous study showing that liver mass is decreased in response to nutrient restriction in pregnant ewes (Scheaffer et al., 2004b). Moreover, the tendency for maternal jejunal O_2 consumption to be lower in RR and RC cows suggests that the tissue was adapting to reduced nutrients available by decreasing energy use while maintaining tissue mass possibly in an attempt to improve energetic efficiency. At d 254, maternal hepatic O_2 consumption was decreased in RCC when compared with RRC, which may be occurring in response to increased feed intake after restriction during early gestation.

The increases in fetal BW and organ mass as pregnancy advanced were observed as expected; however, when related to BW a decrease in hepatic and small intestinal mass were observed. Furthermore, hepatic energy use decreased as mass increased; while small intestinal energy use responded quadratically decreasing until mid-gestation and increasing during the last period of gestation. The result observed in the intestinal tissue may be an indication that the energy requirement is increased in the last third of gestation when fetal body growth is occurring at a higher rate. Perhaps, it may also be linked to the fact that during mid-gestation enteral amniotic fluid starts to be ingested by the fetus and serves as a source that provides 10-20% of nutrients required by the fetus (Sangild, 2006). To our knowledge, limited information has been reported on changes of fetal tissue mass and tissue specific energy use in response to different stages of gestation in ruminants.

In response to changes in maternal nutritional intake, a decrease in fetal small intestinal O_2 consumption was observed in the restricted group at d 85. Moreover, this decreased energy utilization was not because of decreased tissue mass. It appears that an adaptation to the environment providing a restricted amount of nutrients is occurring since an increase in metabolic activity occurs during development (Dumas et al., 2004). Moreover, difference in fetal

small intestinal mass in response to changes in the amount of nutrients consumed by the dam was not observed during the entire gestational period. The lack of an effect on small intestinal mass does not agree with previous literature which reported that nutrient restriction during early to midgestation, in ovine and human caused alterations in fetal organ growth that were permanent and not influenced by supplementation later in gestation (Barker, 1995; Thomas and Kott, 1995; Godfrey and Robinson, 1998; Godfrey, 1999). The difference in results might be related to the level of restriction, length of restriction, and species. Lower absolute O_2 consumption in fetal small intestine persisted until mid-gestation in the restricted group. However, the increase observed in the group that was realimented at d 85 suggests that increased nutrients available promote increased metabolism in the small intestine; which perhaps, is utilized to support development that may have been delayed due to previous nutrient restriction.

Interestingly, at d 254 of gestation, the difference in fetal small intestinal O_2 consumption was not observed in the groups that had been exposed to restriction possibly because it occurred during early- or mid-gestation. The results suggest that changes in maternal small intestinal mass and O_2 consumption during gestation might have improved the efficiency of the small intestine of fetuses developing in a nutrient restricted environment. These metabolic changes during early- and mid-gestation could potentially contribute to adaptations of the fetus to become more efficient later in life in the utilization of nutrients as gestation advances (Camacho et al., 2014). Moreover, the higher fetal liver mass observed in the restricted groups is in agreement with previous studies (Vonnahme et al., 2003). Perhaps, hepatic mass is increased in response to the lower level of nutrients available to the fetus in order to support the high metabolic activity required by the developing fetus which is increasing body size at a fast rate at this time point.

This can be supported by our observation on energy use by the liver tending to be reduced in the group that was exposed to realimentation during mid-gestation.

In conclusion, maternal and fetal tissue mass and energy use are modulated by the progression of gestation. Moreover, nutrient restriction in pregnant cows during early and midgestation affects tissue mass and energy utilization within the small intestine and liver of the dam and the fetus. This may indicate that cows and fetuses have the ability to adapt to restriction by increasing or decreasing mass in an attempt to increase the digestion and utilization of feed or to alter maintenance requirements to maintain fetal growth and development. However, maternal realimentation has the potential to change the effects observed in cows and fetuses by late gestation. Dams may alter their metabolism to be more efficient during nutrient restriction to help maintain desirable fetal development of the liver and small intestine; although, more research is necessary to determine if this pattern of fetal development continues after birth.

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CHAPTER 4 - EFFECTS OF MATERNAL NUTRITION AND ARGININE SUPPLEMENTATION ON POSTNATAL LIVER AND JEJUNAL OXYGEN CONSUMPTION AND HYPOTHALAMIC NEUROPEPTIDE CONTENT IN OVINE OFFSPRING

Abstract

Maternal nutrient restriction during gestation exerts long-term effects on offspring health and performance. Energy utilized by fetal visceral tissues can be altered in response to changes in maternal feed intake. Prolonged nutritional changes during early pregnancy can impact hypothalamic neuropeptide mRNA and protein expression of proopiomelanocortin (POMC), agouti-related peptide (AgRP) and neuropeptide Y (NPY) in the offspring. Arginine supplementation has been shown to rescue some of the negative effects of intra-uterine growth restriction on the fetus. We tested the hypothesis that maternal arginine supplementation from d 54 of pregnancy until parturition would rescue the deleterious effects of nutrient restriction on hepatic and jejunal energy use and hypothalamic protein expression of POMC, NPY, and AgRP in female offspring (n = 18). Multiparous ewes (54 ± 4 d of gestation) were randomly assigned to dietary treatment; 100% of requirements (control, CON), 60% of control (restricted, RES), or RES plus rumen-protected arginine (180 mg/kg; RES-ARG). At parturition, offspring were immediately removed from their dam and placed on a common diet. At 54 ± 3 d of age, lambs were weighed and euthanized. The liver and jejunum were collected and weighed. *In vitro* O_2 consumption was conducted to estimate energy use in liver and jejunum samples (n = 6 per treatment). Liver O_2 consumption (mol/min/liver and mol/min/kg BW) was decreased ($P \leq 0.02$) in the RES and RES-ARG group when compared with CON. Immunohistochemistry assays were conducted to analyze NPY and AgRP protein content in the paraventricular nucleus (PVN) of the

hypothalamus, and POMC protein content in the arcuate nucleus (ARC; n = 3-4 per treatment). Intensity of staining for NPY tended to be decreased ($P = 0.10$) in RES and RES-ARG when compared with CON. Number of POMC cells in the ARC were decreased ($P \leq 0.03$) in the RES group when compared with RES-ARG. In conclusion, maternal nutrient restriction did not influence lamb BW, liver and jejunum mass, jejunum energy use, NPY or AgRP protein content in the PVN, and POMC protein content in the ARC. Further, supplementation of arginine to the gestating ewe failed to influence hepatic energy use in lambs from restricted ewes; however, number of POMC-containing cells was increased in the ARC, which could potentially influence feeding behavior and visceral energy metabolism.

Introduction

Modifications in the maternal nutritional environment throughout gestation impact offspring performance (Barker, 1995). Hales and Barker (2001) described the association between poor fetal and neonatal development with the increased risk in development of metabolic syndrome diseases as an adult. Intra-uterine growth restriction (IUGR) promoted development of cardiac and metabolic diseases (Barker, 1995), and also decreased small intestinal mass, altered intestinal villi morphology (Avila et al., 1989; Trahair et al., 1997; Wang et al., 2005), reduced number of jejunal cells (Reed et al., 2007), and gastrointestinal tract (GIT) vascularity (Neville et al., 2010) in the offspring. Maternal nutrient restriction may further affect offspring tissue energy use decreasing the rate of activity of metabolic cycles that will produce ATP and consume O_2. This is of particular importance as there is a disproportionate amount of metabolic activity occurring in visceral tissues during pre- and post-natal growth in the offspring (Holliday et al. 1967; Bell et al. 1987) and therefore, these tissues may be more sensitive to alterations in nutrient partitioning.

Within the central nervous system, maternal nutritional alterations impact the programming of the fetal hypothalamic-pituitary-adrenal axis (Bouret and Simerly, 2004; Buckley et al., 2005; Challis et al., 2005). Changes in fetal hypothalamic circuitry that control energy homeostasis have been demonstrated following alterations in maternal diet (Bouret and Simerly, 2006). Perhaps these alterations are compensated by changes in energy consumption and expenditure by the fetus (Bouret and Simerly, 2006). Changes in orexigenic (*NPY* and *AgRP*) and anorexigenic (*POMC*) neuropeptide expression in the arcuate nucleus of the hypothalamus (ARC) may regulate fetal energy consumption and utilization. NPY and AgRP are synthesized within the ARC, and then secreted by nerve terminals within the paraventricular nucleus (PVN; Leibowitz, 1991). Endogenous NPY has been proposed to regulate several hormones involved in the control of energy metabolism, such as corticosteroid, aldosterone, insulin, and vasopressin (Swanson et al., 1983). Injections of NPY into the PVN have been shown to increase food intake and respiratory quotient (McCarthy et al., 1991; Stanley et al., 1993). Broberger (1998) reported that NPY-containing fibers that project to the PVN coexpress the neuropeptide agouti-related peptide (AgRP). Gene expression and endogenous actions of NPY and AgRP are modulated similarly during dietary manipulations, circulating nutrients, metabolic action, and physiological state (Ziotopoulou, 2000). Proopiomelanocortin (POMC) neurons are also located in the ARC with fibers projecting to various hypothalamic nuclei (Millington, 2007). Posttranslational modifications of POMC produces α-MSH (Millan et al., 1985; Rubinstein et al., 1996), which has been shown to modulate metabolic homeostasis via synaptic inputs within the PVN (Cowley et al., 1999). Offspring born from ewes that were nutrient restricted during midgestation do not have altered *POMC* expression at birth; however, decreased *POMC* expression was observed in animals exposed to an obesity-promoting diet

postnatally (Montague et al. 1997; Strobel et al. 1998). Moreover, nutrient restriction of the dam during late gestation increased NPY expression and protein content in the ARC (Beck et al, 1990; Brady et al., 1990; O'Shea and Gundlach, 1991; Davies and Marks, 1994), thus demonstrating the influence of gestational diet on offspring performance.

Arginine supplementation has been proposed to alleviate IUGR when supplemented to the maternal diet (Wu et al., 2006), potentially through serving as a precursor to nitric oxide synthase (NOS). Nitric oxide synthase is involved in the regulation of energy metabolism in mammals (Joffin et al., 2012) and food intake (Hui and Chan, 1995). Moreover, NOS appears to contribute to the control of dietary intake through action as an orexigenic molecule in mice and rats (Stricker-Krongrad et al., 1996). Nitric oxide synthase may alter food intake through the inactivation of leptin receptors in the hypothalamus and consequently upregulating orexigenic neuropeptides (NPY and AgRP) and downregulating anorexigenic neuropeptides (POMC; White and Martin, 1997).

Methods to offset the detrimental effects of maternal nutrient restriction during pregnancy on offspring visceral mass and energy use has been studied previously; however, the central mechanisms regulating energy utilization in the offspring have not been fully elucidated in ruminants. Moreover, the ability of arginine supplementation to ameliorate deleterious effects on visceral energy use, in response to development in an IUGR environment, has not been demonstrated. Thus, the present study aimed to investigate the effects of arginine supplemented to nutrient restricted ewes throughout gestation on the hypothalamic protein expression of NPY, AgRP and POMC, liver and jejunal mass, and energy use in the offspring. We hypothesized that maternal nutrient restriction would reduce hepatic and jejunal mass and energy use of the

offspring, while increasing NPY and AgRP protein content in the PVN and decreasing POMC protein content in the ARC in the offspring.

Material and methods

Animal care and use protocols were approved by the North Dakota State University Institutional Animal Care and Use Committee.

Animals and experimental design

Animal care and dietary treatments (Table 4.1) were as previously described (Peine et al. 2013). Briefly, a total of 32 Rambouillet-cross ewes (67.7 ± 6.2 kg initial BW) were confirmed pregnant 41 ± 6.0 d after mating, via ultrasound and then housed individually (0.91 x 1.2 m pens) in a temperature-controlled room (14°C) and light dark cycles (12:12 hours; lights on 0700 and off at 1900). Ewes had free access to water and were fed daily at 0800 to meet or exceed nutrient recommendations for early gestation (NRC, 2007). Weekly BW measurements were taken to determine if dietary adjustments were needed and body condition scores were assessed every two weeks.

Table 4.1. Ingredient and nutrient composition of pelleted diet fed to ewes.

Item	%
Ingredient	
Alfalfa meal, dehydrated	34.0
Beet pulp, dehydrated	27.0
Wheat middlings	25.0
Ground corn	8.4
Soybean meal	5.0
Trace mineral premix[1]	0.6
Nutrient composition	
DM	91.9
CP	15.5
NDF	35.8
ADF	20.9

[1]Premix: 18 to 21% Ca, 9% P, 10 to 11% NaCl, 49.3 ppm Se, 700,000 IU/kg Vitamin A, 200,000 IU/kg Vitamin D, 400 IU/kg Vitamin E.

At d 54 ± 3.9 d of gestation animals were allocated in a completely randomized design to one of three treatments: 100% of dietary requirements (control, Con; based on NRC, 1985, 2007), 60% of control (restricted, Res), or Res with the addition of a rumen protected arginine supplement containing 180 mg arginine/kg BW (Res-Arg; based on initial BW). Arginine was mixed with 50 g of fine ground corn and fed once daily at 0800 before offering the pelleted diet. Both CON and RES ewes were also provided 50 g of fine ground corn daily, without the added rumen protected arginine. Treatments continued until parturition. Two Con and one Res ewe died (2 unknown causes and 1 pneumonia) before parturition.

Ewes were monitored continually during expected dates of parturition. At time of parturition, lambs were immediately removed from the dam (prior to suckling) and reared independently (Peine et al., 2013). Lambs were fed artificial colostrum (Lifeline Rescue Colostrum, APC, Ankeny, IA) at 19.1 mL/kg of lamb birth weight at 0 and 2 h post birth, and 25.5 mL/kg of lamb birth weight at 4, 8, 12, 16, and 20 h post birth to achieve 10.64 g IgG/kg lamb birth weight, as previously described by Meyer et al. (2010) and Neville et al. (2010).

Lambs were group-housed in a temperature controlled room and had free access to water. From 24 h post birth, lambs were bottle-fed milk replacer (Super Lamb Milk Replacer, Merrick's Inc., Middleton, WI; DM basis: 24% CP, 30% fat, 0.10% crude fiber, 0.5 to 1.0% Ca, 0.65% P, 0.3 ppm Se, 66,000 IU/kg vitamin A, 22,000 IU/kg vitamin D, and 330 IU/kg vitamin E) for *ad libitum* intake until a strong suckling response was observed. At that point, lambs were fed by a teat bucket system as previously described (Meyer et al., 2010; Neville et al., 2010). Creep feed (DM basis: 20% CP, 6% fat, 8% crude fiber, 1.4 to 1.9% Ca, 0.4% P, 0.5% to 1.5% NaCl, 0.3 ppm Se, 11,000 IU/kg vitamin A, 6,000 IU/kg vitamin D, and 100 IU/kg vitamin E) mixed with long stem mid-bloom alfalfa was also available for *ad libitum* intake.

Visceral organ measurements, tissue collection, and analysis

For the purposes of this experiment, female lambs (n = 18; 6 Con, 6 Res-Arg, and 6 Res) were euthanized via captive bolt and exsanguinated at 54 ± 3 d of age and tissues collected. Only females were used in order to evaluate sex-specific alterations in postnatal neuropeptide function. Viscera (including digesta) were removed and weighed. The liver (without the gall bladder) and the jejunum (15-cm section) were removed for further analyses. The liver sample was collected from the left lobe. The landmarks utilized to define the jejunum to be collected began at a point adjacent to the third vascular branch caudal to the mesenteric-ileocecal vein junction. At this point, a 15-cm segment was collected for further analyses. A second 15-cm segment was collected following the first section for measurement of stripped weight. Demarcations of the 3 sections of the small intestine were made using similar methods as previously reported (Soto-Navarro et al., 2004; Caton et al., 2009; Yunusova et al., 2013). After collection, the intestine was dissected, gently stripped of fat and digesta, and weighed.

Liver and jejunal samples (200 mg) were collected and placed in Krebs-Ringer bicarbonate buffer (118.1 mM NaCl, 25.0 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$) and transported to the laboratory for *in vitro* analysis of oxygen consumption (see below). After slaughter, a tissue block containing the hypothalamus was dissected, rapidly frozen in liquid nitrogen vapor, and stored at -80^o C.

Hepatic and small intestinal in vitro O_2 consumption

As previously described by Scheaffer et al. (2003) and Prezotto et al. (2014), tissues were sliced (0.5 mm thick) with a Stadie-Riggs microtome (Thomas, Philadelphia, PA). Tissues were placed into petri dishes containing Krebs-Ringer buffer fortified with sodium pyruvate (5.0 mM), sodium glutamate (5.0 mM), sodium acetate (4.5 mM), glucose (25.0 mM), and malic acid (4.5

mM) buffer at 37°C. Subsamples (200 ± 10 mg; Reynolds et al., 1990) of the sliced tissues were placed into test chambers containing 3 mL of the same buffer and a Clarke polariographic electrode (model 5300A Biological Oxygen Monitor, Yellow Springs Instruments, Yellow Springs, OH) to determine *in vitro* oxygen consumption for 5 min. Samples were analyzed in duplicate and utilizing the data generated by a RS-232 serial output for monitoring and displaying rate curves during sample runs. Oxygen consumption (μmol/min) was then calculated per gram of tissue (mol/min/g tissue), per total organ weight (mol/min/tissue), and relative to BW (mol/min/kg).

Hypothalamus processing and immunohistochemistry procedure

Coronal sections (20 μm) were cut from hypothalamic blocks from a subset of the female lambs (n = 11; 3 Con, 4 Res-Arg, and 4 Res) using a cryostat, mounted on SuperFrost Plus slides, and stored at -80°C until processed. A series of every 10^{th} section was used for detection of NPY, AgRP, and POMC protein expression by immunohistochemistry. Sections were thawed in an incubator at 37°C for 10 min and air-dried at room temperature for 10 min. Slides were incubated for 25 min in a 4% paraformaldehyde solution in phosphate buffered saline (Santa Cruz Biotechnology; Santa Cruz, CA) followed by two washes (5 min each) in 0.1% Triton X-100 in 1X tris-buffered saline solution, followed by a blocking solution containing 10% goat serum for 20 min. Sections were then incubated overnight with either rabbit anti-NPY (1:750, Sigma-Aldrich; St. Louis, MO), guinea pig anti-AgRP (1:750, Abcam; Cambridge, MA), or rabbit anti-POMC (1:250, GeneTex; Irvine, CA). Sections were washed twice (5 min each wash) in 0.1% Triton X-100 in 1X tris-buffered saline solution. For NPY and POMC staining, sections were incubated in goat anti-rabbit IgG H&L Alexa Fluor 633 (1:250, Life Technology; Grand Island, NY) for 1 hour. For AgRP staining, sections were incubated in goat anti-guinea pig IgG

H&L Alexa Fluor 555 (1:250, AbCam; Cambridge, MA) for 1 hour. Sections were washed two times (5 min each) in water and then mounted with VECTASHIELD with DAPI (Vector Laboratories; Burlingame, CA) and coverslip was added.

Imaging and analyses

Tissue sections were observed with a Zeiss Imager.M2 epifluorescence microscope. Every tenth section (200 μm apart) throughout the PVN (for determination of NPY and AgRP protein expression) and middle ARC (for determination of POMC protein expression) were analyzed for each animal. Photomicrographs were taken using a 10X objective and AxioCam HRm camera with a Zeiss piezo automated stage. The mosaic image of a large tissue area of approximately 100 pictures (10 × 10 pictures) on the slide was taken using the MosaiX module of Zeiss AxioVision software (Zeiss, Thornwood, NY). This method allowed creation of a single image covering the entire region of interest. The MosaiX images were then analyzed using the ImagePro Premier software (ImagePro Premier 9.0, Media Cybernetics, Silver Spring, MD) for intensity of staining, cell number, and intensity of staining per cell per section of tissue. The location and intensity of staining for NPY and AgRP were determined in three sections through the PVN. Number of cells and intensity of staining per cell for POMC were determined in three sections through the middle ARC. Sections were selected from each lamb to represent comparable sections through the regions described above. Anatomical identification of the middle ARC was selected as previously described by Lehman et al. (1993).

The intensity of staining in the PVN for NPY and AgRP was estimated by using three regions of interest (ROI) of 35 microns in diameter placed randomly over the PVN. The area containing detectable signal was determined by subtracting the background signal from the ROI within the PVN. Number of cells expressing POMC were automatically quantified and intensity

of staining per cell determined using a ROI placed over individual cells (ImagePro Premier Software).

Statistics

Data were analyzed as a completely randomized design with treatment included in the model statement (PROC GLM, SAS). Difference between means were determined using contrast statements (Con vs. Res and Res-Arg and Res vs. Res-Arg). Significance was declared at $P \leq 0.05$ and a tendency was reported if $0.05 < P < 0.10$.

Results

Body weight at d 54 ± 3 of age did not differ in female lambs born from dams exposed to different dietary treatment during gestation (Table 4.2). Liver and jejunal mass (g and g/kg BW) were also not influenced by treatment. Jejunum oxygen consumption (mmol/min/g, mol/min/jejunum, mol/min/kg of BW) was not different between treatment groups. Further, fetal liver oxygen consumption per gram of tissue (mmol/min/g) was not altered by dietary treatment. However, total liver oxygen consumption (mol/min/liver and mol/min/kg of BW) was lower in Res and Res-Arg treatments compared to Con ($P \leq 0.02$; Table 4.2), but not different ($P \geq 0.77$) between the Res and Res-Arg group.

Table 4.2. Influence of maternal dietary arginine supplementation and nutrient restriction on offspring body weight, liver and jejunum mass, and *in vitro* oxygen consumption in liver and small intestine.

	Treatment				Contrast *P*-value	
Item	Con	Res-Arg	Res	SEM	Con vs others	Res vs Res-Arg
BW, kg	22.9	23.5	21.0	1.20	0.64	0.16
Hepatic weight						
G	470	464	414	24.3	0.31	0.16
g/kg BW	20.7	19.7	19.8	0.716	0.30	0.99
Hepatic oxygen consumption						
mmol/min/mg liver	0.470	0.464	0.414	0.024	0.31	0.16
mmol/min/liver	181	155	145	10	0.04	0.53
mmol/min/kg BW	8.0	6.7	6.8	0.397	0.02	0.77
Jejunum weight						
G	163	172	186	27.2	0.63	0.71
g/kg BW	7.50	7.39	8.91	1.35	0.69	0.40
Jejunum oxygen consumption						
mmol/min/mg jejunum	0.163	0.172	0.186	0.027	0.63	0.71
mmol/min/jejunum	582	598	710	90	0.62	0.48
mmol/min/kg BW	2.4	2.5	3.2	0.528	0.50	0.35

Con = 100% of dietary requirements (NRC, 1985, 2007), Res = 60% of control, Res-Arg = Res with the addition of a rumen protected arginine supplement containing 180 mg arginine/kg BW (initial BW).
SEM = standard error of the mean.
Con vs. others, Res vs Res-Arg = difference between means determined using contrast statements.

Staining intensity of AgRP within the PVN did not differ between treatments (Figure 4.1). However, staining intensity of NPY in the PVN tended ($P \leq 0.10$) to be lower in the Res and Res-Arg groups when compared to the Con group (Figure 4.2), but did not differ ($P \geq 0.56$) when comparing the Res group with the Res-Arg group. Staining intensity of POMC within the ARC did not differ between treatments (Figure 4.3). However, total number of cells containing POMC in the ARC was greater in lambs in the Res-Arg group when compared to the Res group ($P \leq 0.03$; Figure 4.4), but did not differ when the Con group was compared to the other two treatment groups.

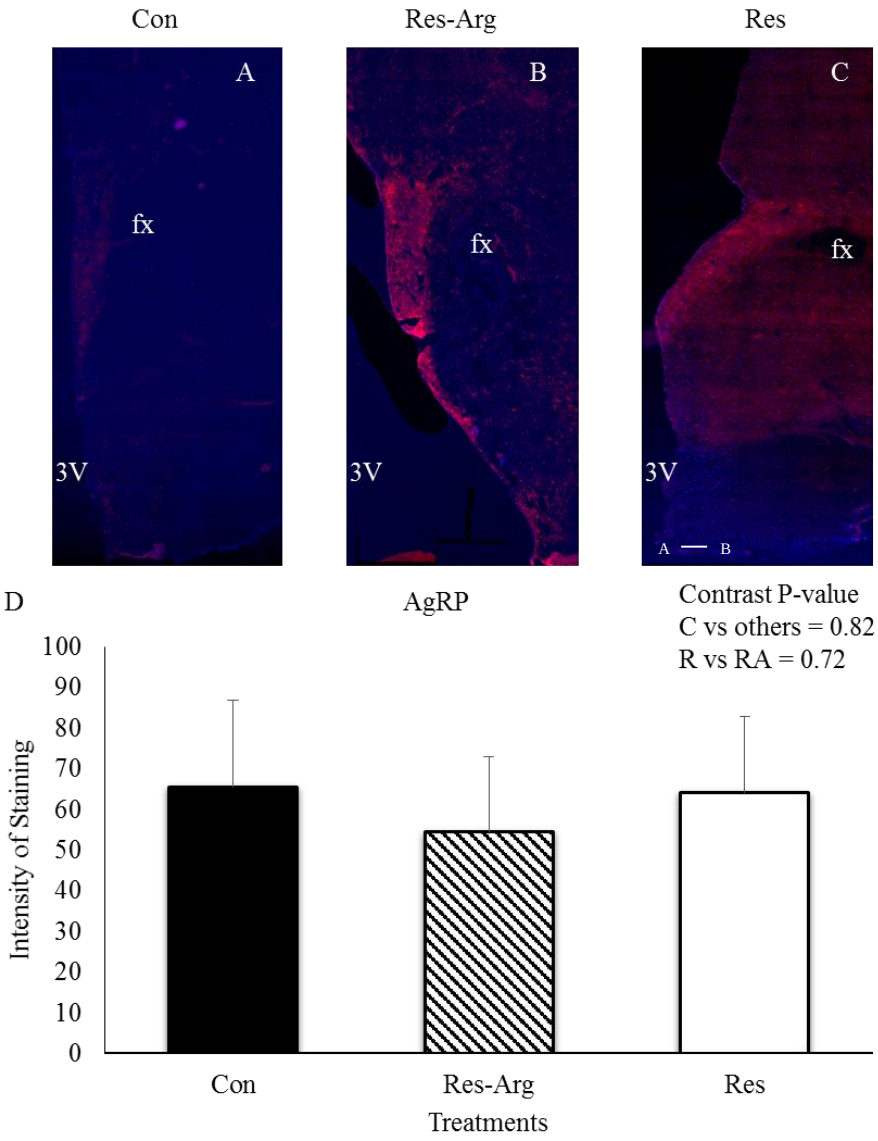

Figure 4.1. Protein content of AgRP in the paraventricular nucleus of the hypothalamus (PVN) of female lambs exposed to prenatal nutrition that was either: 100% of dietary requirements (control, Con; based on NRC, 1985, 2007), 60% of control (restricted, Res), or Res with the addition of a rumen protected arginine supplement containing 180 mg arginine/kg BW (Res-Arg; based on initial BW). Images of coronal sections processed for immuhistochemistry detection of AgRP protein concentration on the terminal nerve fibers in the PVN of representative lambs in the Con (A), Res-Arg (B), and Res (C) groups. Mozaic fluorescent image (A, B, C) at 10x magnification showing fibers stained (Alexafluor 633; far red) in sections at the level of the PVN. Mean (± SEM) intensity of staining of AgRP containing fibers (D) in the PVN was not different for Con contrasted with Res-Arg and Res (C vs others) animals, and Res contrasted with Res-Arg (R vs R-A) animals. fx, fornix; 3V, third ventricle. Scale bar: A-B = 500 µm.

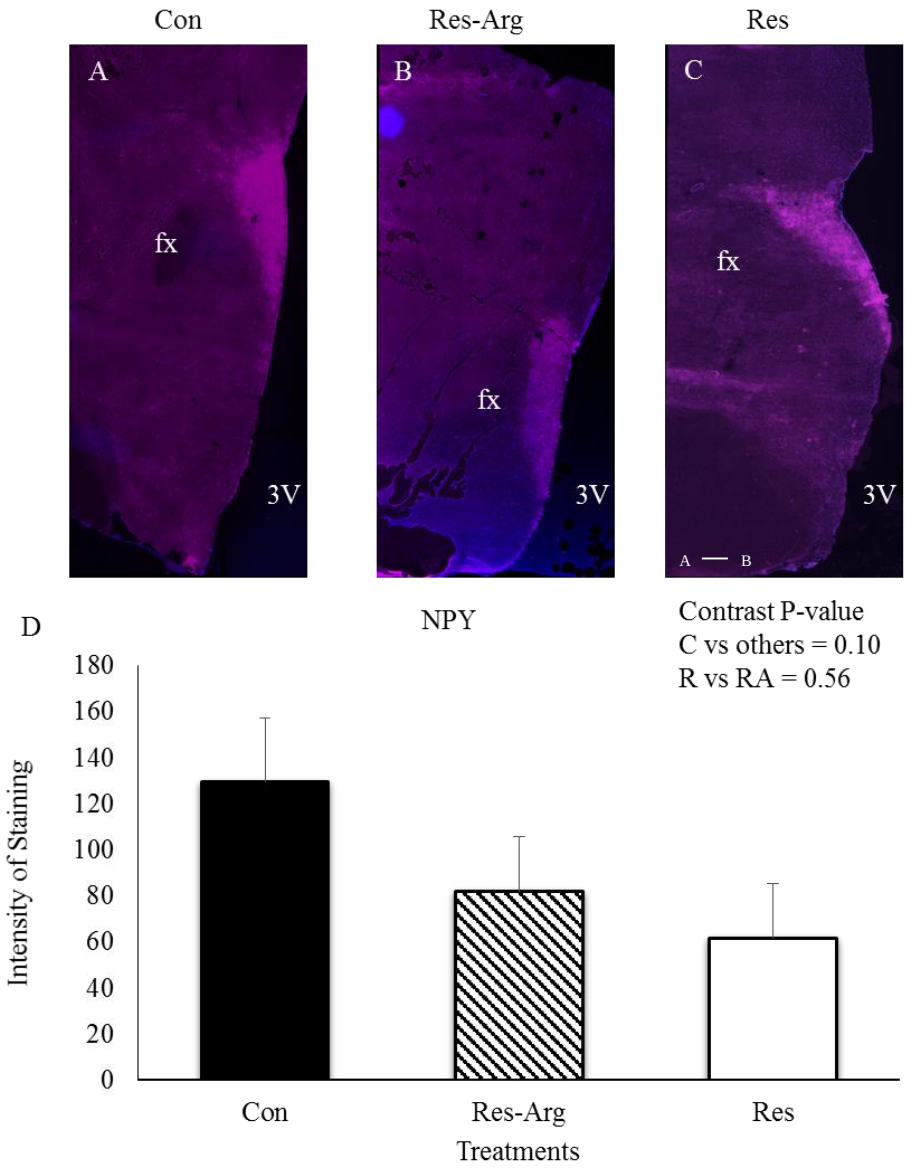

Figure 4.2. Protein content of NPY in the paraventricular nucleus of the hypothalamus (PVN) of female lambs exposed to prenatal nutrition that was either: 100% of dietary requirements (control, Con; based on NRC, 1985, 2007), 60% of control (restricted, Res), or Res with the addition of a rumen protected arginine supplement containing 180 mg arginine/kg BW (Res-Arg; based on initial BW). Images of coronal sections processed for immuhistochemistry detection of NPY protein concentration on the terminal nerve fibers in the PVN of representative lambs in the Con (A), Res-Arg (B), and Res (C) groups. Mozaic fluorescent image (A, B, C) at 10x magnification showing fibers stained (Alexafluor 633; far red) in sections at the level of the PVN. Mean (± SEM) intensity of staining of NPY containing fibers (D) in the PVN tended to be greater ($P \geq 0.10$) for Con contrasted with Res-Arg and Res (C vs others) animals. However, it was not different for Res contrasted with Res-Arg (R vs R-A) animals. fx, fornix; 3V, third ventricle. Scale bar: A-B = 500 µm.

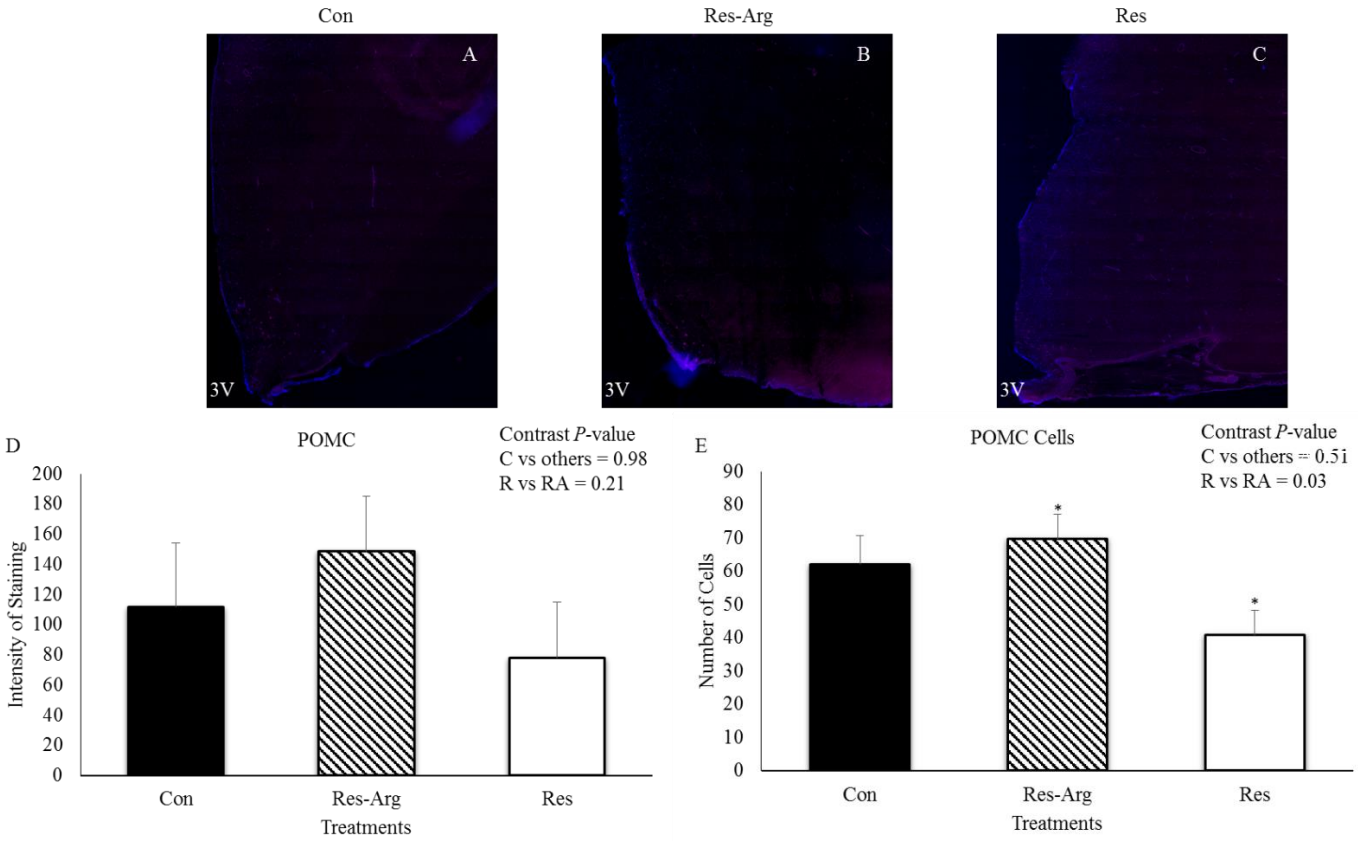

Figure 4.3. Protein content of POMC in the middle arcuate nucleus of the hypothalamus (mARC) of female lambs exposed to prenatal nutrition that was either: 100% of dietary requirements (control, Con; based on NRC, 1985, 2007), 60% of control (restricted, Res), or Res with the addition of a rumen protected arginine supplement containing 180 mg arginine/kg BW (Res-Arg; based on initial BW). Images of coronal sections processed for immuhistochemistry detection of POMC protein concentration in the cells in the mARC of representative lambs in the Con (A), Res-Arg (B), and Res (C) groups. Mozaic fluorescent image (A, B, C) at 10x magnification showing fibers stained (Alexafluor 555; red) in sections at the level of the mARC. Mean (± SEM) intensity of staining of POMC cells (D) in the mARC was not different for Con contrasted with Res-Arg and Res (C vs others) animals, and Res contrasted with Res-Arg (R vs R-A) animals. Number of cells containing POMC in the mARC (E) was not different for Con contrasted with Res-Arg and Res (C vs others) animals. However, it was greater for Res-Arg when contrasted with Res (*P ≤ 0.03; R vs R-A) animals 3V, third ventricle. Scale bar: A-B = 500 µm.

Discussion

Changes in maternal nutrition throughout gestation can influence fetal growth and postnatal offspring performance (Muhlhausler et al., 2006). Supplementation of arginine has been proposed to alleviate the deleterious consequences of IUGR (Wu et al., 2006). The impacts of IUGR on fetal liver and small intestinal oxygen consumption, as an indicator of energy use, have been previously described (Prezotto et al., 2014). Observations made in the present study indicate that BW, liver mass, and jejunal mass of female lambs do not suffer long term effects caused by maternal nutrient restriction during gestation when the offspring were offered a common diet for *ad libitum* intake after birth. Moreover, maternal nutrient restriction throughout gestation reduced hepatic oxygen consumption in the offspring. However, when analyzing lamb jejunal oxygen consumption, no differences were observed in response to prenatal maternal nutrient environment. Changes in gastrointestinal tract morphology and function in offspring influenced by alterations in maternal nutrition during gestation are not completely understood. Our laboratory has demonstrated that variations in oxygen consumption and mass in the fetal liver and small intestine occur in response to changes in maternal intake (Prezotto et al., 2013). However, to our knowledge no studies have been conducted to evaluate the long-term effects of prenatal nutritional environment on postnatal energy use within the GIT. Nutritionally programmed changes in the control of energy homeostasis have been described in different species (Vickers et al., 2001; Coupe et al., 2009; Sebert et al., 2009), which exert protracted effects in the regulation of energy balance. Thus, we propose that changes in visceral organ mass and functionality programmed during the prenatal period negatively impacts energetic efficiency of visceral organs in the female lamb.

The ARC-PVN NPYergic pathway is responsive to alterations in food intake (Sahu et al., 1988; Calza et al., 1989; Brady et al., 1990; Pages et al., 1993); with NPY content in the nerve terminals located within the PVN varying with energy status. The effects of nutrition on adult and fetal *NPY* and *AgRP* expression have been reported (Morton et al, 2006). The current study investigated the long term effects of maternal nutrition during gestation on hypothalamic orexigenic and anorexigenic protein expression in the offspring. Our results show that the staining intensity for NPY protein in the PVN tended to be lower in the Res and Res-Arg groups when compared with the Con group. Decreased neonatal *NPY* expression has been reported to occur in response to maternal nutrient restriction during early to mid-gestation (Sebert et al., 2009). Previous researchers have reported increased *NPY* expression in response to nutrient restriction (Leibowitz, 1991), or no change in expression in the fetus exposed to maternal nutrient restriction during gestation (Muhlhausler, 2006). Our study evaluated expression of NPY protein in the PVN, and our results are in contrast to the aforementioned report that evaluated NPY mRNA expression within the ARC of rodent pups of dams being either restricted to 50% (Sebert et al., 2009) or overfed to 160% (Muhlhausler, 2006) of maintenance requirements. Neuropeptide Y protein content within neuronal fibers located in the PVN of the female lambs tended to be lower for the animals born from nutrient restricted dams possibly because NPY secretion was stimulated to support feed intake and energy utilization that would result in the observed compensatory gain. Alternatively, the level of dietary restriction coupled with divergent placental-regulated nutrient partitioning across studies resulted in the different response in NPY expression. Furthermore, the absence of differences for AgRP were consistent with previous reports (Muhlhausler, 2006).

It appears that NPY and AgRP mRNA are more sensitive to variations in feed intake and energy status during postnatal life than nutrition during prenatal life (Muhlhausler et al., 2006). NPY and AgRP mRNA were not altered by maternal high feed intake during late gestation in sheep, nor in rats that were overfed neonatally (Plagemann et al., 1999; Muhlhausler et al., 2006). Both of these studies were able to demonstrate an inverse relationship between adiposity and NPY/AgRP mRNA concentrations. Therefore, this suggests that the sensitivity in the NPY/AgRP system to nutrition and energy status may be more responsive to fat stores, rather than to prenatal nutrition in the sheep or early postnatal nutrition in the rat. However, we do not know if the results observed in the current study would be different if data had been collected earlier in life as body weights and growth rates did differ at an earlier age (Peine et al., 2013).

Although the intensity for staining for POMC cells in the ARC was not different between the treatment groups, the number of POMC-expressing cells was greater in the Res group being supplemented with arginine when compared to the Res group. However, the number of cells was not different when those groups were compared with the Con group. Reduction in *POMC* expression has been shown to occur in animals exposed to a restricted diet (Millington, 2007). Reports were able to indicate increased dietary intake being related to the reduced *POMC* expression (Grill et al., 2002), or failed to demonstrate the association between POMC expression and changes in intake (Muhlhausler et al., 2006). Furthermore, increased hypothalamic expression of *POMC* was observed in response to increased intake by the ewe during late gestation (Muhlhausler et al., 2006). Although *POMC* expression was greater in this group of lambs, no changes in feed intake were observed during the first 30 d of postnatal life (Muhlhausler et al., 2006).

To our knowledge, this is the first report demonstrating an arginine-induced increase in POMC-expressing cells in a nutrient restricted model during gestation. The mechanism through which arginine is functioning to promote this increase in number of cells containing POMC may be via the serotonergic pathway. While we did not examine the effects of maternal arginine supplementation during gestation on the serotoninergic system, previous research demonstrates that central infusion of arginine may prevent the increase in feed intake (Iuras et al., 2013). Serotonin has been suggested to stimulate the expression of POMC while inhibiting the expression of AgRP present in the ARC (Heisler et al., 2006; Heisler and Lam, 2010). Therefore, exposure to increased arginine during the developmental period may promote greater number of cells containing POMC.

Our results on liver and on jejunal mass expand the current understanding of the effects of IUGR on offspring BW and visceral mass (Widdowson, 1971; Trahair et al., 1997; Freetly et al., 2000) and demonstrate that allowing lambs free access to feed during approximately the first 60 d of life may promote a compensatory BW gain and gastrointestinal mass gain. The suggestion of a compensatory gain is based on the data reported for this experiment by Peine et al. (2013) who recorded birth weight, and weekly BW in male and female lambs. The report by Peine et al. (2013) shows that during the first 30 d of life animals born from dams nutritionally restricted during gestation had lower BW when compared to the control group, with the lambs from arginine supplemented dams being intermediate. However, differences in body weight between treatments were lost by 40 d of age and remained similar between treatments throughout the remaining time of the experiment (Peine et al., 2013).

In conclusion, nutrient restriction from early gestation through parturition appears to result in a reduction of liver energy use and POMC-containing cell number within the ARC of

female lambs. Moreover, arginine supplementation was shown to rescue the reduction in POMC cell number which potentially could result in an increase in feed intake. However, maternal arginine supplementation to nutrient restricted ewes did not influence hepatic energy utilization in the offspring. More work is needed to determine the effects of maternal nutrient restriction and arginine supplementation on liver and intestinal energy use and hypothalamic POMC, NPY, and AgRP in offspring at different stages of development, including adulthood.

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CHAPTER 5 - GENERAL CONCLUSIONS

Based on previous research described in the review of literature, three experiments were conducted. The objectives were to expand the knowledge in regard to the effects of maternal nutrient restriction during gestation on the dam and fetus visceral organ metabolism, and on the protein content of neuropeptides in hypothalamic nuclei that play a role in feed intake and energy metabolism, of offspring exposed to IUGR. Moreover, we investigated how maternal supplementation with melatonin or arginine, and realimentation during different stages of gestation, would alleviate some of the effects of nutrient restriction on maternal and fetal liver and jejunum mass and energy use. We also studied whether maternal arginine supplementation would affect NPY, AgRP and/or POMC protein content in the hypothalamus of offspring that developed in an IUGR environment.

In the first experiment, we examined the effects of two levels of nutrition with or without melatonin supplementation in pregnant ewes from d 50 to 130 of gestation on maternal and fetal liver and small intestinal mass and O2 consumption. We concluded that maternal nutrient restriction had a greater effect than melatonin supplementation on liver and jejunum mass and energy utilization in dams and fetuses. However, the observed interactions between melatonin and plane of nutrition generally suggested that jejunum mass and energy utilization were more responsive to melatonin supplementation in ewes fed adequate nutrition compared with restricted ewes. Furthermore, energy use measured through O_2 consumption and citrate synthase activity was reduced with nutrient restriction in hepatic tissue but increased in intestinal tissue, suggesting a possible compensatory effect in this organ. Our results suggested that melatonin supplementation may increase intestinal mass and energy use in adequately fed ewes but not in restricted ewes, and therefore, it may have limited use as a therapeutic supplement to help

overcome potential negative effects of nutrient restriction on liver and intestinal function in ewes and offspring.

In the second experiment, our objects were to describe how maternal and fetal liver mass and oxygen consumption change as pregnancy of the cow progesses. Moreover, how duration of maternal nutrient restriction affect the dam and the fetus visceral organ metabolism during gestation, and also how realimentating the dam at different stages of pregnancy might affect mass and energy use of the liver and small intestine in the cow and in the fetus. In conclusion, we observed that maternal and fetal tissue mass and energy use are modulated by the progression of gestation. Moreover, nutrient restriction in pregnant cows during early and midgestation affects tissue mass and energy utilization within the small intestine and liver of the dam and the fetus. This may indicate that cows and fetuses have the ability to adapt to restriction by increasing or decreasing mass in an attempt to increase the digestion and utilization of feed or to alter maintenance requirements to support fetal growth and development. However, maternal realimentation has the potential to change the effects observed in cows and fetuses by late gestation. Dams may alter their metabolism to be more efficient during nutrient restriction to help maintain desirable fetal development of the liver and small intestine; although, more research is necessary to determine if this pattern of fetal development continues after birth.

In the third experiment, our objectives were to describe how maternal arginine supplementation to the gestating ewe affects hepatic and jejunal mass and energy use in the offspring. Moreover, we investigated whether arginine supplementation to the dam would affect protein concentration of NPY, AgRP and POMC in the female lamb hypothalamus. We concluded that nutrient restriction from early gestation through parturition appears to result in a reduction of liver energy use and POMC-containing cell number within the ARC of female

lambs. Moreover, arginine supplementation was shown to rescue the reduction in POMC cell number which potentially could result and an increase in feed intake. However, maternal arginine supplementation to nutrient restricted ewes did not influence hepatic energy utilization in the offspring. More work is needed to determine the effects of maternal nutrient restriction and arginine supplementation on liver and intestinal energy use and hypothalamic POMC, NPY, and AgRP in offspring at different stages of development, including adulthood.

In conclusion, this series of experiments have indicated that maternal nutrient restriction affects visceral mass and energy use in the dam and fetus. Maternal melatonin supplementation did not rescue the decreased mass and increased oxygen consumption observed in response to nutrient restriction, as we hypothesized. However, in the second experiment the deleterious effects were diminished when the dam was realimented during midgestation, suggesting that the dam is able to physiologically respond to the initial restriction in order to keep fetal development at an appropriate rate. Once nutrient consumption is increased, a compensatory mechanism promotes a rescue in liver and jejunal mass and O_2 consumption that are comparable with what is observed in the control group. Finally, we were able to demonstrate that maternal nutrient restriction throughout gestation has an effect on the offspring hepatic oxygen consumption, and also on number of cells in the ARC that synthesize POMC. Moreover, maternal arginine supplementation did not alleviate the effects of restriction on hepatic energy utilization as we hypothesized. However, the number of cell in the hypothalamus containing POMC protein where increased in response to arginine supplementation. Therefore, future studies need to be conducted to evaluate whether changes in number of POMC protein containing cells as a result of maternal arginine supplementation drives alterations in offspring feed intake and energy metabolism in the viscera.