DISSERTATION

INFLUENCE OF SHORT AND PROLONGED MATERNAL UNDERNUTRITION ON THE TWIN OVINE FETUS AND POSTNATAL LAMB

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2012

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ABSTRACT

INFLUENCE OF SHORT AND PROLONGED MATERNAL UNDERNUTRITION ON THE OVINE FETUS AND POSTNATAL LAMB

Introduction: Fetal, maternal and environmental factors are able to influence fetal and lamb growth and development. Fetal organ development and growth trajectory are highly vulnerable to maternal nutritional status during the early stages of gestation (Reynolds and Redmer 1995; Ford *et al*. 2007). Fetal intrauterine growth restriction (IUGR) can be induced by maternal undernutrition (UN) during gestation and result in smaller lambs at birth (Anthony *et al*. 2003). In the case of sub-optimal developmental conditions in-utero fetal programming occurs to increase the prospect of fetal survival (Lucas 1991). Further, a "thrifty phenotype" hypothesis indicates that maternal UN results in modified glucose utilization, insulin secretion and responsiveness during development (Hales *et al*. 1991). Although fetal programming may be crucial for continued growth of the IUGR fetus, it has potentially deleterious effects on offspring and is associated with the onset of metabolic diseases in adult humans (Stein *et al*. 1996; Barker 1998). The compensatory adaptations instigated by maternal UN, which could result from fluctuations in forage availability and quality, may have negative impacts on offspring growth, metabolic function, and carcass quality in sheep (Black 1983; Bell 1992; Anderson 1993; Kelley *et al*. 1996; Del Curto *et al*. 2000).

In sheep, twin pregnancy induces IUGR even when adequate nutrients are available to the ewe (Cleal *et al*. 2007; Ford *et al*. 2007; Quigley *et al*. 2008). In addition, prolonged nutrient restriction from early to late gestation can cause significant reductions in fetal and placental weights of mixed pregnancies (Mellor

1983). Although metabolic adaptations that result from maternal UN may contribute to increased neonatal mortality, some adaptations, such as insulin resistance and diabetes, may not become apparent until the offspring reaches adulthood (Barker *et al*. 1993).

Information is limited regarding the impact of maternal diet restriction, during early gestation and prolonged restriction, on twin pregnancies. We hypothesized that early gestational maternal UN followed by realimentation during late gestation would result in compensatory growth of twin fetuses, whereas continuous UN would result in fetal growth restriction, with control fed twin pregnancies presenting an intermediate phenotype.

Further, the postnatal impact of maternal UN from early- to mid-gestation or throughout gestation in the case of twin pregnancies is of interest since twinning is commonplace in sheep production. Therefore, the objective of this experiment was to determine the impact of maternal UN from 28 to 78 days of gestational age (dGA) and from 28 dGA to parturition on the growth trajectory, biometric measurements at birth and slaughter, and organ weights at slaughter of the lambs. We hypothesized that early gestational maternal undernutrition followed by realimentation during late gestation would enhance lamb growth, whereas, prolonged UN would suppress lamb growth.

Methods: Twelve ewes were fed 50% nutrient requirements (NR; NRC 2006) beginning at 28 dGA to induce IUGR. Five of those ewes were realimented to 100% NR at 79 dGA (LC). The other ewes receiving 50% NR were maintained on 50% NR through the end of the study (LL). Seven other ewes were fed 100% NR throughout the entire study (C). The experimental diet consisted of pelleted beet pulp (77.8% total digestible nutrients [TDN], 90.1% dry matter [DM], and 9.9% crude protein

[CP]) and a vitamin-mineral mixture to meet additional requirements. At 135 ± 1 dGA ewes were anesthetized by i.v. administration of sodium pentobarbital (27 mg/kg), the ewes were intubated and maintained on 1-3% isoflurane, and a midventral laparotomy was performed to remove products of conception for tissue harvest. Umbilical artery and vein blood was collected from each fetus; a uterine artery blood sample was collected from a subsample of ewes (n = 11) prior to delivery of the fetuses. Blood was collected into tubes containing 30 mg Sodium Fluoride and centrifuged for 10 minutes at 1,500 × *g* at 4°C to harvest plasma. Plasma was removed from the pellet and stored at -20°C for later analysis. Fetal body and organ weights and blood metabolite and hormone concentrations were measured. Blood gasses from umbilical and uterine blood were measured using an iSTAT blood analyzer.

In a separate study twenty-seven ewes were placed on the same feeding allotments as described above (C, n = 8; LC, n = 10; LL, n = 9) and the diets were maintained until parturition occurred. The experimental diet consisted of pelleted beet pulp (77.8% TDN, 90.1% DM, and 9.9% CP), which included a vitamin-mineral mixture to meet additional requirements. Lambs were allowed to suckle their dams freely or supplemented with a commercial milk replacer if no milk was available (Merrick's, Inc., Middleton, WI). Beginning at 2 wk postnatal (PN), lambs had free choice access lamb creep pellets and alfalfa hay (All*American Show Lamb Creep, Ranch-Way Feeds, Fort Collins, CO). Lambs were weaned from their mothers at 10 wk PN and gradually adjusted to a complete feed that was offered at 150% of the recommended feeding rate (All*American Show Lamb Complete, Ranch-Way Feeds, Fort Collins, CO). Ewes and lambs were weighed weekly and the ration was adjusted accordingly. Maternal jugular vein blood was collected during gestation and maternal and lamb blood samples were collected following parturition through 10 and 16 wk,

respectively. At birth and at 18 wk PN body weight, crown-rump length, abdominal circumference, thoracic circumference, hip girth (only at 18 wk PN), front and rear leg length, and gender were recorded. Ewes and lambs were weighed weekly following parturition. At 18 wk PN the lambs were killed at a local USDA inspected abattoir. Immediately following exsanguination the adrenals, brain, heart, intestine, kidneys, liver, pancreas and spleen were weighed individually. Blood glucose concentrations were measured with an YSI model 2700 Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Blood hormones including insulin, IGF-I, GH, and ghrelin were measured by radioimmunoassay.

All data are presented as LSmeans ± S.E.M. Means were separated at a significant level of $P < 0.05$ and considered as a trend at a level of $P < 0.10$. Each ewe was treated as one experimental unit and twin fetuses within the ewe were treated as repeated measurements. We hypothesized that restriction followed by realimentation would induce faster fetal growth, therefore, fetal and lamb blood data were analyzed by preplanned orthogonal contrasts: C vs. LC & LL and LC vs. LL. Any interaction by fetal sex observed is reported. Repeated measurements of ewe data were analyzed using the PROC MIXED model of SAS (SAS Inst. Carry, NC). Only ewes or lambs that survived through the entire study were included in analysis.

Results: Fetal body weight of the LC fetuses (4.64 **±** 0.15 kg) was greater than the LL fetuses (4.09 ± 0.13 kg; $P = 0.0132$). Brain (LC, 62.34 ± 1.84 g; LL, 54.62 ± 1.55 g), liver (LC, 123.03 ± 5.77 g; LL, 101.51 ± 4.89 g), and intestine (LC, 181.36 ± 6.57 g; LL, 160.41 ± 5.63 g) weights differed significantly between LC and LL fetuses at 135 dGA ($P \leq 0.05$). Uterine artery glucose concentrations did not differ ($P > 0.10$), but were numerically greater in LC dams than in C and LL dams. In the umbilical vein, LC fetuses (1.91 ± 0.20 mmol/L) tended to have greater ($P = 0.1041$) glucose

concentrations than LL (1.43 ± 0.18 mmol/L). The umbilical vein:umbilical artery glucose gradient was greater ($P = 0.0172$) in LC (0.39 ± 0.40) than LL fetuses (0.26 ± 0.03). Umbilical vein:umbilical artery O_2 content differences tended to be greater ($P = 0.0852$) in C (5.02 ± 0.43) than LC (4.43 ± 0.47) and LL (3.41 ± 0.47) fetuses. Uterine artery IGF-I, GH and INS were not impacted by treatment. Umbilical vein IGF-I was greater ($P = 0.0068$) in LC (74.3 ± 6.71 ng/mL) than LL (46.7 ± 5.62 ng/mL). Umbilical artery IGF-I (LC, 91.4 ± 8.97 ng/mL; LL, 66.6 ± 7.51 ng/mL) and INS (LC, 0.70 ± 0.15 ng/mL; LL, 0.24 ± 0.13 ng/mL) were greater ($P \leq 0.05$) in LC than in LL. These data suggest that maternal undernutrition followed by realimentation beginning at 79 dGA induces twin fetuses to undergo compensatory growth during late gestation, while continuous restriction induces a slower fetal metabolic rate and fetal growth.

In the second study lambs born to LC (5.41 ± 0.28 kg) ewes were heavier ($P = 0.0168$) than LL (4.34 ± 0.29 kg) lambs. Crown-rump length (CRL) of LC (17.90 ± 0.54 cm) lambs tended ($P = 0.0933$) to be greater than LL (16.38 ± 0.68 cm). Body weight of LC lambs was greater ($P < 0.05$) than LL lambs at 2 and 3 wk PP and the same trend ($P = 0.0654$) was observed at 4 wk PN. At 4, 16 and 17 wk PN body weight tended ($P < 0.10$) to be greater in C than LC & LL lambs. At 18 wk PN rear leg length of LC (63.92 ± 1.30 cm) lambs was greater ($P = 0.0287$) than LL (61.54 ± 1.26 cm). At 18 wk PN CRL of LC (114.46 ± 2.82 cm) lambs tended ($P = 0.0882$) to be greater than LL (107.30 ± 2.72 cm) lambs. At 18 wk PN brain weight was greater ($P = 0.0331$) in LC (107.98 ± 2.61 g) than LL (99.74 ± 2.47 g) lambs. Glucose (GLU) concentrations at 49 and 77 dGA were greater ($P < 0.05$) in C vs. LC & LL ewes; the same trend ($P < 0.10$) occurred at 63 dGA. At 105, 112, 126, and 147 dGA GLU was greater ($P < 0.05$) in LC vs. LL ewes. At 2 wk PN ewe GLU was greater ($P = 0.0327$)

in LC & LL than Control. Lamb GLU was reduced ($P = 0.0173$) in LC vs. LL at 14 wk PP. Insulin was greater ($P \leq 0.05$) in C vs. LC & LL ewes at 63, 77, 98, 105, 112, 119, 126, 133, and 140 dGA, while at 112 and 147 maternal INS was greater ($P < 0.05$) in LC vs. LL. Maternal IGF-I was greater ($P < 0.05$) at 112, 119, 126, 133, 140, and 147 dGA in C vs. LC & LL. Maternal IGF-I was greater ($P = 0.01$) at 147 dGA in LC vs. LL, with a similar trend ($P = 0.08$) at 2 wk PP. Maternal GH was greater ($P < 0.05$) at 49 and 140 dGA in LC & LL vs. C, with a similar trend ($P < 0.10$) at 119 dGA. At 35 dGA GH was greater ($P = 0.03$) in LC vs. LL, whereas at 49 ($P = 0.01$) and 112 dGA ($P = 0.08$) GH was greater in LL vs. LL. Ghrelin was greater ($P = 0.02$) in LC & LL vs. C ewes at 140 dGA, with a similar trend ($P < 0.10$) at 4 wk PP. Lamb INS was greater ($P = 0.04$) in C vs. LC & LL and greater ($P = 0.02$) in LC than LL at 2 wk PN. Lamb IGF-I tended to be greater ($P = 0.08$) in C vs. LC & LL at 4 wk PN. At Lamb GRL was greater ($P = 0.06$) in LC & LL vs. C at 2 wk PN. Elevated body weight and CRL of LC lambs at birth may indicate a shift in fetal metabolism to compensate for nutrient restriction during early gestation. Minimal differences between Control and nutrient restricted treatments indicate naturally occurring restriction in twin pregnancies. Elevated post-weaning GLU in LL lambs may indicate increased insulin resistance in these lambs. Umbilical IGF-I and insulin in LC indicate a shift in IGF-I and accelerated fetal growth as a result of realimentation following maternal UN. Prolonged and early- to mid-gestation maternal UN results in diminished INS and elevated GRL in early post-natal life. Further, elevated maternal GRL in the postpartum period may indicate a carryover impact of UN during gestation.

ACKNOWLEDGEMENTS

I would like to thank: Dr. Hyungchul Han for giving me the opportunity to pursue my PhD at Colorado State University; Dr. Russ Anthony for contributing his expertise and insight that helped to make many aspects of my research successful; Dr. Thomas Hansen for contributing his knowledge to my doctoral committee, as well as his encouragement and support to complete my degree; and Dr. Terry Engle for his insightful discussions and for his support and guidance which were vital to my success. I would also like to thank Dr. Lynne Kesel, Dr. Stacie Byers, and Dr. Tim Holt for their veterinary services. For the use of their facilities to during my research projects I thank the staff of the Agricultural Research, Development, and Education Center at Colorado State University and the staff of Innovative Foods, LLC.

I must also thank my fellow students, without whom conducting my research projects and collecting all of the samples would have been impossible. I particularly acknowledge the following individuals for their extraordinary contribution of their time and skills: Mikaela Vetters, Cornelia Flöercke, Elizabeth Domby, Jennifer Schutz, Kelley Neuhold, Emmanuel Caldera, Crystal Warner, Paul Repenning, Bo Bigler, Marco Dib, Katlin Hornig, Catie Cramer, Sophia Kerdoon, Natalie Blackmer, and Ryan Urie.

Finally, I will forever and ever be grateful to my family and friends that have supported me throughout this process with their love, generosity, prayers, and friendship. For their friendship, sharing in the highs and lows of graduate school, I am grateful to Cornelia Flöercke, Jennifer Schutz, Mikaela Vetters, and Elizabeth Domby. To my dad, Fred, I am grateful for your wisdom, guidance, love, and inspiration to pursue my interests and passions. To my mom, Jacki, without your

selfless love and care I would be in shambles, thank you for your unconditional love in all times and especially through my difficulties. To my sister, Melissa, thank you for inspiring me to work hard and to never settle for less than achieving my goals. I would also like to acknowledge my CO-family Ben, Kathryn, Kevin, and Megan Bright, for their love, support, and encouragement.

My dissertation is dedicated to my grandma, Virginia Sommer whose words, prayers and faith in the Lord have so often lifted me up.

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KEYWORDS

Maternal undernutrition, sheep, fetal growth

Chapter I. Introduction

Intrauterine growth restriction (IUGR) can be induced upon a growing fetus by restricting dietary intake of the gestating ewe (Anthony *et al*. 2003). Maternal nutrition is particularly critical during early gestation when organ development and the fetal growth trajectory are vulnerable to the nutritional environment (Reynolds and Redmer 1995; Ford *et al*. 2007). The developing fetus can adapt metabolically and physiologically to compensate for the sub-optimal environment, this is referred to as fetal programming (Lucas 1991). Particularly, the "thrifty phenotype" hypothesis has described that glucose utilization and insulin secretion and responsiveness may be altered by maternal undernutrition (Hales *et al*. 1991).

In the Western United States the availability and quality of forages varies depending on the season (Anderson 1993; Krysl and Hess 1993; Del Curto *et al*. 2000; Paterson *et al*. 2002). Thus, a fetus may experience IUGR when the pregnant ewe is exposed to undernutrition as a result of fluctuations in forage (Thomas and Kott 1995). Additionally twin pregnancy, which is common in sheep, may predispose the developing fetus to IUGR (Cleal *et al*. 2007; Ford *et al*. 2007; Quigley *et al*. 2008). Depending on the extent and duration of maternal undernutrition, as well as the number of fetuses being carried, the impact of maternal undernutrition varies. In singleton pregnancies, undernutrition during early gestation followed by adequate nutrition in late gestation does not impact fetal body weight near term or at birth (Heasman *et al*. 1998; Brameld *et al*. 2000). Similar results were reported regarding a mixture of singleton and twin lambs born to ewes that were undernourished in early gestation and then realimented (Ford *et al*. 2007).

Human epidemiological studies have provided significant evidence that indicate maternal undernutrition during gestation has potential repercussions on the adult offspring (Barker and Clark 1997; Edwards and McMillen 2001). Particularly, humans born to mothers undernourished during gestation may be predisposed to cardiovascular disease, as well as obesity and diabetes (Barker 1994; Godfrey and Barker 2000). In addition, low birth weight in humans is linked to the onset of cardiovascular and metabolic diseases in adulthood (Martyn and Barker 1994; Stein *et al*. 1996).

In sheep maternal undernutrition during early gestation results in smaller fetuses at mid-gestation, as well as reduced blood glucose concentration and left ventricular hypertrophy (Han *et al*. 2004; Ford *et al*. 2007). Further, maternal undernutrition is associated with reduced renal growth hormone (GH), insulin-like growth factor (IGF), and glucocorticoid receptors, increased arterial blood pressure, and alteration of cardiovascular development (Edwards and McMillen 2001; Edwards and McMillen 2002a; Brennan *et al*. 2005). Undernutrition during gestation in sheep also results in elevated fetal blood GH concentrations (Koritnik *et al*. 1981; Bauer *et al*. 1995). Furthermore, fetal blood IGF-I concentrations are reduced as a result of maternal undernutrition (Bassett *et al*. 1990a).

Variable maternal undernutrition, may result in alterations in the activity of the GH/IGF axis, which could impact postnatal growth trajectories. Thus this research aims to directly compare the effect of early- to mid-gestation and prolonged nutrient restriction on the fetal and postnatal growth response, as well as the circulating concentrations of the important regulators of growth and metabolism. As well, these studies aim to determine the effect of maternal undernutrition on twin pregnancies since information specifically detailing the circumstance of twin pregnancy is limited.

Chapter II. Literature Review

Introduction

The impact of maternal undernutrition on fetal and offspring growth and development is of considerable interest because of its relation to the occurrence of metabolic and cardiovascular diseases in adult humans, as well as its relevance to growth efficiency, health, and productivity in livestock species. The normal developmental processes of the placenta and fetus, as well as hormonal and nutritional influences of fetal growth are discussed in the first section of this chapter. This is followed by a discussion of maternal undernutrition during the periconceptional period, from early- to mid-gestation, throughout gestation, and during late gestation. The final section of this chapter briefly reviews the epigenetic effects of maternal undernutrition-induced intrauterine growth restriction (IUGR).

Fetal and Placental Development

Early Embryonic and Placental Development

Within the first two weeks of gestation a fertilized ovine egg will undergo several phases of embryonic cleavage prior to implanting in the uterine lining (Rowson and Moor 1966). Once fertilized in the oviduct, the embryo cleaves into a morula and by day 4 is present in the uterus. The embryo will continue to divide and a blastocyst forms by day 6. The zona pellucida, which surrounds the blastocyst, will begin to shed at day 8 and be completely absent by day 11. A basement membrane, separating the trophoblasts and layer of endodermal cells is present at day 10, thus fetal and maternal tissue and circulation are separate. Additionally, the embryonic cleavage results in a distinctly separate inner cell mass which gives rise to the yolk sac,

allantois, amnion, and the embryo itself (Wintenberger-Torres and Flechon 1974; Gilbert 2003; Spencer *et al*. 2004).

Lengthening of the trophectoderm of the spherical blastocyst begins at day 11 of gestation (Wintenberger-Torres and Flechon 1974; Spencer *et al*. 2004). Lengthening of the trophectoderm is key to the production of interferon-τ, which is the signal for maternal recognition of pregnancy. Interferon-τ sends an antiluteolytic signal produced by trophoblasts to the mother that prolongs progesterone production (Farin *et al*. 1989; Bazer 1992). Estradiol-17β is elevated and triggers increased blood flow to the uterine horn ipsilateral to the ovary containing a corpus luteum, which may aid in the transport of the signal for maternal recognition of pregnancy (Reynolds *et al*. 1984).

Embryonic attachment is divided into three phase, which are precontact, apposition, and adhesion. In the precontact phase, days 14 to 15, the conceptus is immobilized and maternal caruncular epithelial cells begin to develop protrusions. Apposition, which occurs at days 15 to 18, is characterized by close association of the trophectoderm and uterine luminal epithelium. This interdigitation of protrusions from uterine and trophoblastic microvilli contributes to the association. Adhesion at days 16 to 22 is accomplished by the interdigitation of trophoblasts and endometrial luminal epithelium (Guillomot *et al*. 1981; Dey and Hyunjung 2006; Wooding 2008).

The allantois is developing and visible around days 15 to 17, vasculature in the allantois begins to develop immediately. The allantois will fuse with the chorion around day 30 (Bryden *et al*. 1972a). The ovine placenta is characterized by placentomes, the site of hormone and nutrient transfer between fetal and maternal tissues, each containing a fetal cotyledon and a maternal caruncle (Burton *et al*. 2006). For a brief period connections between the luminal epithelium and trophoblasts will

develop on areas of the uterine epithelium in between caruncles. Beginning at day 14 the trophectoderm begins producing binucleate cells that fuse with uterine epithelium forming trinucleate cells. By day 20 to 24, these fused hybrid cells have contributed to the formation of a syncytial plaque that partially covers the caruncular surface (Wooding 1984). Along with contributing to the syncytial plaque the binucleate cells will serve a major purpose of secreting hormones, such as placental lactogen, and other modifiers to make the uterine environment suitable for a developing conceptus (Wooding 2008).

A response to the conceptus can be seen in the gravid uterine horn by day 24 changes include increased uterine lumen diameter and decreased endometrial thickness. Microvascular density in deep caruncular tissues is elevated by day 24 and microvascular growth continues in parallel with the needs of the growing conceptus (Reynolds and Redmer 1992). Fetal villi elongate and push towards the surface of the caruncle. Crypts develop on the caruncular surface and push in the opposite direction of the fetal villi. The resulting interdigitation provides a secure attachment of the chorioallantoic placenta to the uterine epithelium and increased surface area for nutrient transfer (Assheton 1905; Wimsatt 1950; Wooding 2008).

Since the transfer of nutrients is confined to occur only at the cotyledon-caruncle interface, an elaborate vascular network develops on the maternal side to deliver said nutrients. In 1946 Barcroft and Barron published the following observations of fetal cotyledon, maternal caruncle, and uterine blood vessels throughout gestation. At day 45 it is apparent that the uterine artery divides into secondary branches, which divide several times and eventually supply blood vessels to placentomes. These arteries then divide into an extensive capillary network and anastomose near the surface of the caruncle. Small veins then feed into larger veins that are not within the cotyledon to

carry blood away from the tissues. The fetal placental vasculature is also apparent at day 45. Two umbilical arteries divide after entering the chorionic mesoderm. These give rise to branches that divide and enter into the cotyledons; they further divide within the cotyledon and extend into the villi that have developed (Barcroft and Barron 1946). By day 63 the cotyledons have greatly increased in size (relative to day 45) due to the continuous branching of fetal villi, with dense capillary network on the tips, into the maternal caruncle. The density of vessels in the fetal villi has noticeably increased by day 101 and by day 131 the interdigitation of fetal villi with caruncular epithelium has reached the peak of its complexity (Barcroft and Barron 1946).

The basic structures of the organ systems of the fetus are formed very early in gestation. The neural ridge and neural tube are established by day 16, a heart bulge is present by day 17, and a hepatic bulge by day 18 (Bryden *et al*. 1972a). By day 19 the primitive esophagus, as well as the intestinal tube, has developed. The rudimentary stomach is present by day 18, the reticulorumen by day 22. Intestines growing at a rapid rate require extra space, thus the tube twists, contorts, and even herniates into the umbilical cord. By day 34 the four-chambered stomach is recognizable. The pancreas and bile duct are also present by day 34 (Bryden *et al*. 1972b). Since differentiation of most of the organ systems occurs early in gestation, the remainder of gestation consists mostly of the proliferation of those systems, as well as muscle growth and fat deposition.

Hormonal Dynamics of Fetal Growth

Insulin, insulin-like growth factor-I (IGF-I), growth hormone (GH), and ghrelin are involved in regulating different aspects growth and nutrient metabolism and are affected by nutritional status. For the fetal sheep, glucose is the main source of energy

and its availability plays an important role in regulating the level of IGF-I in circulation (Oliver *et al*. 1993; Liu *et al*. 1994; Wallace *et al*. 2000). Since placental development is influenced by IGF-I and IGF-II, and the placenta is responsible for providing nutrients to the fetus, undernutrition (UN) likely affects the fetus first via disturbing placental development (Kelly 1992; Reynolds *et al*. 1997; Wathes *et al*. 1998; Gadd *et al*. 2000). During postnatal life GH stimulates the secretion of IGF-I from the liver however because the receptor does not become functional until late gestation, GH production during early gestation has minimal influence on growth (Klempt *et al*. 1993; Pratt and Anthony 1995; Li *et al*. 1996). Placental lactogen is imperative to fetal growth and is produced by placental binucleate cells; it stimulates metabolic responses such as glycogen synthesis and IGF secretion by fetal tissues (Freemark and Handwerger 1984; Anthony *et al*. 1995).

Both IGF's are involved in fetal growth and are capable of binding to the IGF type-1 receptor, generally IGF-I acts locally and IGF-II regulates overall fetal growth (Owens *et al*. 1991; Anthony *et al*. 1995). In fetal tissues IGF-II mRNA is much higher than IGF-I mRNA, thus the former has a more evident role in fetal development (Owens *et al*. 1991; Anthony *et al*. 1995). While fetal hepatic IGF-I mRNA is lower during gestation and increases postnatally, hepatic IGF-II mRNA is the exact opposite, being higher during fetal development and reduced postnatally (Owens *et al*. 1991; Delhanty and Han 1993). Further, the abundance of IGF-I and –II mRNA is higher in the fetal brain, stomach, and pancreas than in those same adult tissues. Hepatic IGFBP-2 mRNA expression increases with gestational age and decreases slightly postnatally (Delhanty and Han 1993).

Nutritional Demands of the Fetus

An adequate supply of oxygen, glucose, and amino acids is essential for normal fetal growth and development (Bell *et al*. 1986). Although the placenta undergoes rapid growth during early gestation, changes in the surface area for exchange, blood flow, and nutrient transfer capacity occur to accommodate rapid fetal growth (Owens *et al*. 1991). Thus the utilization of oxygen and glucose can be markedly different depending on the stage of gestation. The placenta can consume up to 80% of the oxygen and glucose taken up by the uterus (Bell *et al*. 1986). Facilitated diffusion of glucose from maternal circulation to the placenta and subsequently to fetal circulation transpires via glucose transporters 1 and 3 (Glut 1 and 3, respectively) in the placenta (Hay 2006). In order for glucose to move across the placenta the concentration of glucose in maternal circulation must be higher than in fetal circulation, because the glucose transporters in the placenta are not insulin dependent (Wooding *et al*. 2005). As discussed earlier, maternal-fetal nutrient transfer in the pregnant sheep occurs at placentomes, where maternal and fetal circulations are in very close proximity. Glut 1 is located in the uterine epithelium and both the apical surface microvilli and basal trophoblast membranes, which face maternal and fetal circulations, respectively (Wooding *et al*. 2005). Glut 3 is only located on the trophoblast membrane facing maternal circulation. It has been suggested that because of its low K_m and high affinity Glut 3 may help maintain glucose transport to the trophoblasts when maternal and fetal concentrations are low. Glut 1, on the other hand, is probably responsible for the majority of placental glucose transport. The requirements of glucose increase as the fetus grows rapidly during the second half of gestation (Hay 2006). Thus glucose transport across the placenta must increase via an increase in the trans placental

glucose gradient and an increase in glucose transport capacity of the placenta (Molina *et al*. 1991; Hay *et al*. 2003).

Amino acids are implicit for fetal growth and are utilized for carbon and nitrogen accretion in the fetus or for oxidative fuel in the placenta. As with glucose, the amino acid requirements of the fetus increase throughout gestation. In order to accommodate the increasing demands, changes in the concentrations of amino acid transporters as well as membrane potentials must occur. It has been suggested that the main determinant of the capacity of transport from maternal to fetal circulation is not the uteroplacental blood flow, but the transport capability of the basal trophoblast membrane (Battaglia and Regnault 2001). This is supported by the fact that uteroplacental blood flow will increase approximately six fold during pregnancy (Battaglia 1986). It is further derived from observations that leucine, isoleucine, valine, methionine and phenylalanine rapidly cross the trophoblast basal membrane, but lysine, threonine and histidine crossed slowly. The movement of amino acids across the placenta is facilitated by a variety of sodium-dependent and –independent systems that are specific to different amino acids. Similar to the localization of glucose transporters, some are found on the microvillous and the basal trophoblast membranes, while some are localized to one or the other. In general, neutral amino acids and cationic amino acids are taken up by the placenta from maternal circulation and subsequently delivered to fetal circulation. It is thought that the uptake of neutral amino acids and efflux of cationic amino acids occurs by an exchange method. In contrast, the placenta takes up glutamate and aspartate from maternal and fetal circulation, but they do not cross the placenta (Battaglia and Regnault 2001).

Midgestation Fetal and Placental Development

By midgestation, 70 – 80 dGA, the placenta is heavier than the fetus and the placenta of a late gestation ewe. The uteroplacental utilization of glucose, oxygen, and lactate are however low at midgestation and increase with gestation. Despite these low utilization rates, the uteroplacental tissues consume about 80% of the oxygen and glucose taken up by the gravid uterus at midgestation (Bell *et al*. 1986). These differences in size and nutrient utilization likely result from a combination of metabolic and cardiovascular changes that occur throughout pregnancy. Although the placenta may be heavier at midgestation, its functional capacity is relatively low and the amount of uterine blood shunted away from sites of placental exchange is relatively high (Rosenfeld *et al*. 1974; Wilkening *et al*. 1982; Bell *et al*. 1986).

Although during midgestation fetal weight increases about 20-fold; the rate of growth will concomitantly decrease as gestation progresses (Rattray *et al*. 1974). Further, though fetal heart rate and umbilical vascular resistance are high at midgestation, these will decrease later in gestation (Dawes 1968; Teasdale 1976; Bell *et al*. 1986). As with the placenta, the requirements and use of nutrients by the fetus progresses throughout gestation. Oxygen uptake at midgestation, for instance, was 40% greater on a wet weight basis or 2.5 times greater on a dry weight basis than that of a mature fetus (Battaglia 1978; Bell *et al*. 1986). Relatively high fetal body water, a higher fraction of metabolically active organs, and elevated protein synthesis may account for the increase in oxygen uptake (Meier *et al*. 1981; Bell *et al*. 1986). Of the total energy requirements for a fetus at midgestation, roughly one third is used for tissue accretion and the rest is used for oxidative metabolism. In addition, despite high umbilical glucose uptake, it only accounts for roughly two thirds of the energy required (Bell *et al*. 1986). Thus, it has been deduced that amino acid catabolism at

midgestation must be fundamental to meet the nutritional needs of the fetus (Battaglia 1978).

Late Gestation Fetal and Placental Development

By late gestation (130 – 145 dGA) the ovine fetus is four to six times larger than the placenta (Owens *et al*. 1991). The perfusion and functional capacity of the placenta in late gestation are both greater than in midgestation (Bell *et al*. 1986). Uteroplacental tissues in late gestation utilize much of the glucose and oxygen taken up by the uterus (Sparks *et al*. 1983; Bell *et al*. 1986). High nutrient utilization may be attributed to placental production of lactate, which is distributed to maternal and fetal circulation (Meschia *et al*. 1980; Sparks *et al*. 1983; Owens *et al*. 1991). Additionally, adequate oxygen supply to the placenta and fetus is met by increasing uterine blood flow during the final weeks of gestation (Meschia *et al*. 1980). During late gestation the conceptus is competing with the mother for available nutrients, since the uterus consumes a third of the maternally produced glucose (Hay *et al*. 1983). Placental glucose transfer and uptake is independent of insulin levels in maternal and fetal circulation, but is regulated by the maternal-fetal glucose gradient and fetal blood glucose (Hay *et al*. 1983; Owens *et al*. 1991).

While the placenta is utilizing a great deal more of the available glucose than the fetus, umbilical uptake of amino acids is relatively high. Neutral and basic amino acids are supplied mostly via maternal circulation, whereas glutamate is supplied by umbilical circulation (Meschia *et al*. 1980). The fetal liver takes in large amounts of gluconeogenic amino acids, as well as glucose and lactate. It in turn produces glutamate and pyruvate; the placenta converts glutamate to glutamine that is circulated back to the fetus (Battaglia 2007). The placenta also takes up serine from

maternal circulation for the production of glycine that is provided to the fetus (Owens *et al*. 1991).

Maternal Undernutrition and Intrauterine Growth Restriction

During the first month of gestation establishment and development of the fetal organ systems and placenta occurs and nutritional status of the ewe during early gestation influences development (Reynolds and Redmer 1995). Maternal UN is a method used to induce intrauterine growth restriction (IUGR) on a developing fetus that impacts fetal and maternal nutrient supply and demand, as well as uteroplacental blood flow. Low birth weight and inordinately large placenta are associated with maternal UN (Barker 1995; Vonnahme *et al*. 2003). Adaptations of fetal body composition, organ development, endocrine status, and cardiovascular function also occur as a result of maternal UN (Barker 1995; Godfrey and Robinson 1998; Godfrey and Barker 2000; Anthony *et al*. 2003). The "fetal origins" hypothesis describes that a fetus developing in a sub-optimal uterine environment (i.e. IUGR), has as a result an altered trajectory of growth and development. These adaptations can result in modification of function, physiology, and metabolism that may be temporary or permanent and potentially predispose the offspring to cardiovascular, metabolic, and endocrine diseases (Barker 1995). The term "fetal programming" refers to the compensatory mechanisms that a fetus adopts in order to survive in the face of IUGR (Lucas 1991).

Twin lambs are generally born lighter than singleton lambs (Gardner *et al*. 2005; Ford *et al*. 2007). Other differences include lighter cerebellum, pituitary, liver, heart, and spleen in twin lambs versus singleton lambs (Koritnik *et al*. 1981). Twin fetuses have lower arterial pO_2 and oxygen saturation than singleton fetuses (Edwards and

McMillen 2001). Smith *et al*. (2010) also found that twin lambs have lower plasma glucose and insulin than singleton lambs when measured prior to their first feeding. Additionally, one study reported that twinning results in elevated 11beta-hydroxysteroid dehydrogenase isozymes which may change the production or transportation of glucocorticoids across the placenta and potentially alter hypothalamic-pituitary-adrenal axis activation (Connor *et al*. 2009). These results indicate that differences may exist in the metabolic function of a twin versus a singleton fetus, simply as a result of the ewe carrying one or two fetuses. Thus it is possible that the measureable responses of fetal programming to maternal UN might vary depending on fetal number (twin or singleton pregnancy). Further the extent and duration of maternal UN could produce different results depending on fetal number. In light of this, the following review of maternal UN has been divided by the phase of gestation in which the ewe was subject to nutrient deprivation and as available the different impacts of maternal UN on twin or singleton pregnancies are highlighted.

Maternal Undernutrition in the Periconceptional Period

The periconceptional (PC) period is of interest because offspring from the Dutch Winter Famine who were exposed to UN during early gestation have higher incidences of coronary heart disease, elevated lipids and obesity (Ravelli *et al*. 1999; Roseboom *et al*. 2000a; Roseboom *et al*. 2000b). Additionally, as previously discussed, in the sheep the first third of gestation encompasses rapid embryonic cell proliferation, as well as development of the placenta. In rats the proliferation of cells in the inner cell mass and trophectoderm of the blastocyst are impaired, potentially hindering growth and development of the embryo, as a result of insufficient dietary protein during the PC period (Kwong *et al*. 2000).

In sheep maternal UN during the PC period resulted in increased twin fetal arterial blood pressure in late gestation, but did not impact singleton fetuses (Edwards and McMillen 2001). It is possible that PC-UN perturbs the development of the cardiovascular system through altered gene expression, since in the mouse, alterations in intrauterine concentrations of oxygen and glucose can modify the embryonic expression of genes involved in vascular development (Maltepe and Simon 1998). Regardless of maternal body condition, PC-UN offspring have an increased adrenal growth and female lamb cortisol response to stress (Zhang *et al*. 2010). Furthermore, Zhang *et al*. (2010) also showed that the changes associated with PC-UN might correlate to epigenetic programming of the adrenal gland via changes in adrenal IGF2 mRNA and methylation of the IGF2 gene. Premature increases in fetal ACTH and cortisol concentrations result from PC-UN and are indicative of early development of the pituitary-adrenal axis (Bloomfield *et al*. 2003). Periconceptional UN further results in reduced placental activity of 11β-HSD-2, which converts cortisol to cortisone, but does not impact 11β-HSD-1 activity, which converts cortisone to cortisol, these together result in an increase in the fetal cortisol to cortisone ratio at mid-gestation (Connor *et al*. 2009). The increase in fetal ACTH resulting from PC-UN may program cardiovascular development leading to the increase in blood pressure in late gestation twin sheep fetuses (Edwards and McMillen 2002b).

The development of the permanent mesonephros and metanephros in the kidney begins around 17 and 27 days, respectively (Moritz and Wintour 1999). Lambs exposed to PC-UN and twin control lambs both exhibit and increase in IGF-I and decrease in IGF-I receptor mRNA expression in renal tissue, which may limit the impact of IGF-I on kidney development. In twin lambs mRNA expression of IGF2 increased and IGF2R decreased and may indicate increased IGF2 action on kidney

development. Kidney development in the ovine fetus responds to glucocorticoid exposure, one theory is that this causes early completion of nephrogenesis, thus lower nephron numbers which may contribute to the occurrence of hypertension in adulthood (Moritz *et al*. 2003). It has also been suggested that twin fetal lambs may be more susceptible to PC-UN because glucocorticoid receptor expression is elevated, but 11b-HSD-2 mRNA expression is unchanged and may be at a maximum because of the elevated renal glucocorticoid activity (MacLaughlin *et al*. 2010). These results indicate that PC-UN impacts kidney development in its early stages and has the potential to permanently change kidney function.

Further, fetal nutrient supply during late gestation is altered, in that uterine blood flow in late gestation is higher following PC-UN (Rumball *et al*. 2008). Begum *et al*. (2012) recently reported that PC-UN, as well as twinning reduces methylation of the fetal hypothalamic proopiomelanocortin (POMC) and glucocorticoid receptors (GR), which could modify energy balance regulation. Twinning also alters the response of a fetus to maternal UN in late gestation and the response of insulin to glucose both in late gestation and postnatal life (Harding *et al*. 1997; Oliver *et al*. 2001; Todd *et al*. 2009). Additionally it has been shown that PC-UN alters postnatal growth via modifying nutrient intake and hormonal axes involved in regulating growth (Jaquiery *et al*. 2011a). Interestingly, other have shown that a decrease in muscle fiber density in response to maternal nutrient restriction from day 0 through 31 (periconceptional) that may suggest that in the face of maternal UN, the fetus could prioritize tissues and partition nutrients (Costello *et al*. 2008).

Maternal Undernutrition in Early- to Mid-gestation

From early to mid-gestation (30 – 80 dGA) placental development is at its highest level and thus proper maternal nutrition is imperative (Sebert *et al*. 2009). Contrasting results have been reported that maternal UN from early to mid-gestation causes a reduction in fetal weight at mid-gestation when ewes were fed 50% of their requirements, but no difference when ewes were fed 60% or 70% (Clarke *et al*. 1998; Vonnahme *et al*. 2003; Kwon *et al*. 2004; Luther *et al*. 2007). Furthermore, others have also reported no difference in fetal weight in both twin and singleton pregnancies, that maternal UN from 28 through 78 dGA or from 14 days pre-breeding through 70 dGA (Heasman *et al*. 1998; Hawkins *et al*. 2000; Kwon *et al*. 2004; Ford *et al*. 2007). Maternal UN during this period reduces offspring crown-rump length and placental weight near term (Mellor and Murray 1982; Clarke *et al*. 1998; Heasman *et al*. 1998; Luther *et al*. 2007).

Although underfeeding during early gestation disturbs metabolic function it is noteworthy that in response to acute restriction (between day 83 and 90 of gestation) maternal insulin and IGF-I increase. Furthermore the ewe will also mobilize lipids to maintain the supply of glucose being provided to the fetus (McMullen *et al*. 2005). Postnatal offspring from maternal UN during early gestation have higher basal glucose levels at 63 d postnatal (Ford *et al*. 2007). The response to glucose administration was also altered, in that the area under the curve of glucose was greater at 63 and 250 d postnatal (Ford *et al*. 2007). A hyperinsulinemic insulin response of lambs from nutrient restricted ewes was observed at 63 d postnatal, which may indicate abnormal regulation of pancreatic development and a hypoinsulinemic response at 250 d postnatal, which may indicate altered pancreatic β-cell function

(Ford *et al*. 2007). Together these results infer that maternal UN can program an impaired response of the pancreas to elevated blood glucose that is present in postnatal offspring (Ford *et al*. 2007).

Increases in liver weight relative to total body weight have been observed in 78-day fetuses from ewes underfed from early- to mid-gestation and might be related to the early onset of gluconeogenic function in the fetal liver (Hay *et al*. 1981; Vonnahme *et al*. 2003). Maternal UN of singleton bearing ewes from early to mid-gestation alters fetal hepatic and muscle IGF-I, IGF-II, and GH expression (Brameld *et al*. 2000). Additionally it has been suggested that although maternal UN during early gestation may not alter postnatal appetite, it can alter the response of a lamb to an obesogenic environment in later life (Sebert *et al*. 2009). Testosterone administration to a developing fetus results in fetal body modifications that mimic IUGR such as decreased body weight and length. In this model an increase in IGFBPs was observed only in the first month of life, subsequently in months 2 - 4 testosterone treated lambs exhibited significant catch-up growth. These results suggest that a reduction in free IGF-I (because of increased circulating IGFBPs) may contribute to growth retardation (Manikkam *et al*. 2004). An adaptation of increased vascular resistance in the placenta in response to maternal undernutrition may result in compensation by the fetal heart in the form of cardiac ventricular hypertrophy, modified cardiac gene expression, and elevated blood pressure postnatally (Hawkins *et al*. 2000; Vonnahme *et al*. 2003; Han *et al*. 2004).

Further, Rooke *et al*. (2010) found that lambs (mixed fetal numbers) born to Suffolk ewes, which are a lowland breed, but not Blackface ewes, which are a hill breed, restricted from 1 to 90 dGA were born lighter. In the same study it was suggested that increased plasma cortisol and NEFA levels in Blackface ewes could

indicate higher mobilize of adipose tissue during pregnancy in this breed. These factors may indicate reduced nutrient supply in pregnant Suffolk ewes versus Blackface ewes, and perhaps even reduced placental efficiency (Rooke *et al*. 2010). Since maternal UN in Blackface ewes did not impact birth weight of lambs versus control, it is also possible that Blackface ewes adapt more readily to maternal UN (Rooke *et al*. 2010). These results indicate that the response of offspring to maternal UN from early to mid-gestation is variable depending on the severity of undernutrition, the number of fetuses being carried, and perhaps even breed.

Prolonged Maternal Undernutrition

Nutrient restriction throughout the majority of gestation has been proven to impact fetuses in a manner somewhat similar to nutrient restriction during early gestation. Prolonged maternal undernutrition reduces lamb birth weight (singletons from ewes receiving 70 - 75% NR 4 dGA - term, twins & singletons from ewes receiving 50% NR from 28 dGA - term, or twins from ewes 70% NR from 8 dGA – term; (Edwards and McMillen 2002a; Kwon *et al*. 2004; Luther *et al*. 2007; Wallace *et al*. 2011). At birth lambs born to continuously undernourished ewes have reduced liver, kidney, lung, and spleen weights (Luther *et al*. 2007). While kidney fat mass increases in singletons, kidney mRNA levels of glucocorticoid, GH, IGF- and -II receptors, as well as IGF-I and -II were lower in twin than singleton fetuses following prolonged underfeeding (Brennan *et al*. 2005). Prolonged maternal nutrient deprivation also results in elevated fetal blood GH concentration (Bauer *et al*. 1995). It is likely that the shift in birth weight, as well as kidney fat mass, and gene expression may be altered as a result of nutrient restriction, specifically, the alteration in supply of glucose and amino acids that reach the developing fetus (Kwon *et al*. 2004).

Maternal Undernutrition in Late Gestation

As a result of underfeeding during the last month of gestation birth weight was unchanged, but plasma glucose and fetal arterial blood pressure were elevated (Edwards and McMillen 2001). It has also been shown that underfeeding during late gestation can result in reduced fetal growth rate, as well as reduced birth, brain, pituitary, thyroid, heart, liver, spleen, and kidney weights (Koritnik *et al*. 1981; Gao *et al*. 2007).

The brain:liver weight ratio, which is an indicator of asymmetric growth, (Dawkins; as quoted by (Dawes 1968), being lower in lambs born to ewes undernourished during late gestation suggests partitioning of nutrients to critical tissues (Koritnik *et al*. 1981). Underfeeding during late gestation can result in increased insulin receptor, glucose transporter-4, and type 1 insulin-like growth factor receptor mRNA in muscle tissue, as well as elevated GH concentrations (Koritnik *et al*. 1981; Costello *et al*. 2008). These provide evidence for a temporary adaptation to glucose metabolism, which could have long-term postnatal metabolic repercussions. Further, a test at 1 year postnatal revealed that singleton lambs born to ewes underfed during late gestation have altered glucose tolerance and insulin resistance, which is supported by reduced expression of GLUT4 protein in adipose (Gardner *et al*. 2005).

Postnatal Impacts of IUGR

Survivability of newborn lambs is both an economic and a welfare related concern; it was reported that roughly 15% of lambs born in the UK die prior to weaning (DEFRA 2004). Neonatal morbidity and mortality are higher in low birth weight lambs, which is often a byproduct of nutrient restriction induced-IUGR (Wu *et*

al. 2006). Breed, maternal age, timing, duration and extent of maternal UN, as well as fetal number, pre-and post-natal housing, and post-natal diet likely confound differences in the postnatal response of a lamb to maternal UN.

Despite a reduction in lamb birth weight (mixed pregnancies) in Suffolk sheep fed .75 energy requirements from 1 to 90 dGA, there was no effect of maternal undernourishment on lamb body weight at 1 or 2 years of age (Rooke *et al*. 2010). Others reported that Suffolk lambs had lower body temperature and plasma T3 and T4 for the first 3 d of life than Blackface lambs, which they ascribed to lower thyroid and brown adipose function (Dwyer and Morgan 2006). Rooke et al., (2010) reported that elevated plasma T3 in restricted lambs might be an adaptation of the fetus in response to IUGR for increased thermogenic abilities.

Another confounding factor on the response to maternal UN could also be fetal number, whether the ewe carried one, two, or multiple fetuses. Twin lambs are generally more vulnerable to parasitism than singleton lambs (Wolf *et al*. 2008). Rooke *et al*. (2010) thus measured the effects of maternal UN on fecal egg count to assess the general healthiness of twin lambs and reported increased counts in Suffolk lambs during the first of life.

As with the increase in morality associated with small birth weight, others have reported that restricted ewes were less able to successfully raise their lambs than control ewes (Rooke *et al*. 2010). In conjunction, others reported that lambs from restricted mothers had more difficulty identifying their mother in the first d of life than control, implying behavioral impairments as a result of nutrient restriction (Coombs and Dwyer 2008; Rooke *et al*. 2010). Nordby *et al*. (1987) also reported ewes nutritionally restricted during gestation have reduced milk production, smaller lambs and increased neonatal mortality.

The impact of maternal UN potentially goes beyond the pre-weaning period and into adolescent and adult life, when carcass characteristics and quality are of concern. Muscle fiber types and numbers are established during prenatal growth, thus maternal UN could alter the muscle fiber proportions and in turn growth potential of the lamb (Nordby *et al*. 1987). Proceeding from this knowledge, many researchers have examined the impact of the level of maternal nutrition on carcass composition in lambs. Nordby *et al*. (1987) stunted the growth of lambs by restricting maternal nutrients to 70% of requirements from -30 to 100 dGA, when muscle fiber hyperplasia is significant.

Epigenetics and Maternal Undernutrition

Histone modification and DNA methylation are genomic modifications that occur to ensure gene information can be duplicated in each generation of cells, these epigenetic states are likely vulnerable to various influences (Pinney and Simmons 2011). Histone methylation is associated with activation and repression of transcription. Decreases and increases in histone acetylation are commonly associated with activation and repression of transcription, respectively (Bannister and Kouzarides 2005; Pinney and Simmons 2011). Another mechanism of epigenetic regulation is DNA methylation, which in normal gene sequences modifies the binding affinity of transcription factors and thus functions as a regulatory element (Pinney and Simmons 2011). In normal development DNA methylation is associated with X-chromosomal inactivation, genomic imprinting, and tissue-specific gene regulation (Schubeler *et al*. 2000; Gopalakrishnan *et al*. 2008).

Nutrient restriction during the periconceptional period (-60 to +30 dGA) results in increased histone H3K9 acetylation and decreased methylation in the gene promoters

of proopiomelanocortin and glucocorticoid receptor (GR), as well as increased hypothalamic GR expression (Stevens et al. 2010). Further it was more specifically determined that in twins and singletons from maternal undernutrition, as well as in fed twins, there is a decrease in POMC and GR promoter methylation (Begum et al. 2012). These results indicate that twinning in addition to undernutrition can elicit epigenetic changes in POMC and GR. The alteration of GR expression in conjunction with the epigenetic changes to POMC and GR in the hypothalamus may contribute to changes in feed intake and metabolism in adult life, since POMC is an appetite-regulating neuropeptide.

Summary

Insufficient nutrient provision during gestation seems, initially, to be detrimental to the developing fetus because it impacts the supply of nutrients that are available for transportation across the placenta (Moores *et al*. 1993; Anthony *et al*. 2003; Vonnahme *et al*. 2003). Extensive research on maternal UN during gestation depicts its negative impact on birth weight, organ development, fetal growth trajectory, and even placental development. It is apparent that the impact of maternal UN reaches beyond gross body and organ weights and into the mechanisms of metabolism, alterations that likely carry over into adulthood.

Table 1. Timing of maternal undernutrition and impact on fetal body and organ weights

Time	Fetal #	Body	Liver	Brain	Heart	Lung	Kidney	Adrenal	Brain:Liver	Ref.
Periconception	1	↔ at 55 dGA and term	.	.	.	.	↔ at term	↔ at 128 dGA ↑ 85 dPP	.	b,d, n, p, s
	2	↔ at 55 dGA and term	.	.	.	.	↔ at term	.	.	b, f, n
	1 & 2	↓ at 50 dGA	↔ at 128 dGA and 85 dPP	.	↔ at 128 dGA and 85 dPP	↔ at 128 dGA and 85 dPP	↔ at 128 dGA and 85 dPP	↔ at 128 dGA ↓ at 85 dGA ↑ at 85 dPP	.	e, i
Early to Mid	1	↔ at 80 dGA, term and 1 year ↓ at 78 and 130 dGA	↔ at 140 and 145 dGA	↓ at 80dGA, ↔ 145 dGA	↔ at 130 and 145 dGA	↔ at 130 and 145 dGA	↔ at 130 and 145 dGA, and term	.	↔ at 130 dGA	a, b, c, h, j, m, q
	2	↔ at term	.	.	.	.	↔ at term	.	.	b
	1 & 2	↓ at 78 dGA and term* ↔ at 135 dGA ↑ at 280 dPP	↓ at 78dGA ↑ at 78 dGA per unit body weight	.	↑ at 78 dGA per unit body weight	↓ at 78dGA	↓ at 78dGA	.	.	l, o, q
Early to term	1	↓ at 130 dGA ↔ at term, weaning, and 6 mo	↓ at 130 dGA	↔ at 130 dGA	↔ at 130 dGA	↓ at 130 dGA	↓ at 130 dGA, ↔ at term	.	↑ at 130 dGA	b, m, r
	2	↓ at term or ↔ at term	.	.	.	.	↔ at term	.	.	b, f
	1 & 2	↓ at 135 dGA	.	.	.	.	.	.	.	l
Late	1	↔ at term and 1 year					↔ at term			b, h
	2	↔ at term					↔ at term			b
	1 & 2	↓ at term	↓ at term	↓ at term	↓ at term		↓ at term	↔ at term	↑ at term	g, k

*Offspring birth weight was reduced at term as a result of undernutrition during gestation in Suffolk ewes, but not in Blackface ewes

[a]Brameld et al., 2000; [b]Brennan et al., 2005; [c]Clarke et al., 1998; [d]Connor, 2009a; [e]Connor, 2009b; [f]Edwards and McMillen, 2002; [g]Gao et al., 2007; [h]Gardner et al., 2005; [i]Hawkins et al., 2000; [j]Heasman et al., 1998; [k]Koritnik et al., 1981; [l]Kwon et al., 2004; [m]Luther et al., 2007; [n]McLaughlin et al., 2010; [o]Rooke et al., 2010; [p]Rumball et al., 2008; [q]Vonnahme et al., 2003; [r]Wallace et al., 2011; and [s]Zhang et al., 2010.

Chapter III. Duration of maternal undernutrition differentially alters fetal growth, development and blood gases in twin sheep pregnancies.

Summary

The objective of this experiment was to examine the impact of duration of maternal undernutrition (UN) during gestation on twin sheep pregnancies. Ewes were either fed 100% (C), or 50% of their nutrient requirements from 28 to 78 d gestational age (dGA) and readjusted to 100% beginning at 79 dGA (LC), or 50% from 28 to 110 dGA, followed by a 5% increase at 5 d intervals until 135 dGA (LL). At 135 dGA fetuses were surgically removed for collection of organs and blood. Fetal body weight of the LC fetuses was greater than the LL fetuses ($P = 0.0132$). Brain, liver, and intestine weights were higher in LC than LL fetuses at 135 dGA ($P \leq 0.05$). In the umbilical vein, LC fetuses tended to have greater ($P = 0.1041$) glucose concentrations than LL. The umbilical vein:umbilical artery glucose gradient was greater ($P = 0.0172$) in LC fetuses than LL fetuses. Umbilical vein:umbilical artery O_2 content differences tended to be greater ($P = 0.0852$) in C than LC and LL fetuses. Thus maternal UN followed by realimentation beginning at 79 dGA may induce twin fetuses to undergo compensatory growth during late gestation, resulting in heavier body and organ weights near-term. While continuous restriction results in lambs near-term with reduced body and organ weights compared to maternal UN from early- to mid-gestation.

Introduction

Fetal, maternal and environmental factors influence fetal growth and development. Proper nutrient supply during early stages of gestation is critical for organ development and fetal growth trajectory can be altered by maternal UN (Reynolds and Redmer 1995; Ford *et al*. 2007). Maternal UN can induce intrauterine growth restriction (IUGR), and low birth weights have been related to increased incidences of adult metabolic diseases (Barker *et al*. 1993; Barker 1995; Ravelli *et al*. 1999). The process whereby a growing fetus adapts in order to compensate for a sub-optimal developmental environment within the uterus is referred to as fetal programming (Lucas 1991). The "thrifty phenotype" hypothesis indicates that altered glucose utilization, insulin secretion and responsiveness may result from maternal UN during development (Hales *et al*. 1991). These compensatory measures, which may be significant for fetal survival and development, might be detrimental to the offspring and contribute to the development of adult metabolic diseases (Stein *et al*. 1996; Barker 1998).

Previous studies have shown that early- to mid-gestation maternal UN in sheep leads to reduced fetal body size, reduced blood glucose concentrations, as well as left ventricular hypertrophy at 78 dGA (Vonnahme *et al*. 2003; Han *et al*. 2004; Ford *et al*. 2007). However, nutrient restriction during early gestation followed by realimentation has no effect on fetal body weight near term (Brameld *et al*. 2000) or birth weight (Heasman *et al*. 1998) in singleton pregnancies. Normal birth weights of lambs from nutrient-restricted realimented pregnancies that were a mixture of singleton and twins have also been reported (Ford *et al*. 2007). In another model of IUGR in sheep, insulin sensitivity and action, glucose production, and promotion of glucose utilization seem to indicate a compensatory adaptation of metabolic processes

(Limesand *et al*. 2007). Similarly, one recent study reported that hepatic gluconeogenesis is initiated in the fetus in the circumstance of late-gestation hypoglycemia; further indicating altered metabolic mechanisms to compensate for the insults maternal UN to the fetus (Rozance *et al*. 2008).

Twin pregnancy in sheep may predispose the fetus to naturally occurring IUGR (Cleal *et al*. 2007; Ford *et al*. 2007; Quigley *et al*. 2008). In addition, prolonged nutrient restriction from early to late gestation can cause significant reductions in fetal and placental weights of mixed pregnancies (Mellor and Murray 1982). However, information is limited regarding the impact of maternal diet restriction, during early gestation and prolonged restriction, on twin pregnancies. We hypothesized that early gestational maternal UN followed by realimentation during late gestation would result in compensatory growth of twin fetuses, whereas continuous maternal UN would result in fetal growth restriction, with control fed twin pregnancies presenting an intermediate phenotype.

Experimental methods

Animal Model

Only multiparous, western whiteface ewes carrying twin pregnancies (assessed at 135 d gestational age; dGA) were used for this study (n = 19). The ewes had an average pre-experiment weight of 75.63 ± 7.11 kg. Procedures for this study complied with guidelines of the United States Department of Agriculture. Protocols for care and use of the animals were approved by the Colorado State University Institutional Animal Care and Use Committee. Prior to initiation of the experiment ewes were group housed and offered feed ad libitum. The experimental diet consisted of pelleted beet pulp (77.8% total digestible nutrients [TDN], 90.1% dry matter [DM], and 9.9%

crude protein) and a vitamin-mineral mixture to meet additional requirements. Ewes were treated with controlled intra-vaginal drug release devices (EAZI-BREED™ CIDR® Sheep and Goat Device, Pfizer, New York) for 7 days followed by two 5 mg doses of $PGF_{2\alpha}$ (Lutalyse, Pfizer, New York) 4 hours apart. A ram was introduced 48 hrs after the first dose of $PGF_{2\alpha}$, breeding was supervised and a ewe having been visually confirmed as bred two or more times qualified as having been bred. At 21 dGA, ewes were placed in individual pens and fed the experimental diet to meet 100% of nutrient requirements for early pregnant ewes (NRC, 2006 recommended requirements) on an individual weight basis. Beginning on 28 dGA ewes were randomly assigned to C (n = 7; 100% of nutrient requirements) or LL (n = 7) and LC (n = 5), with both treatment groups receiving 50% of nutrient requirements. For all treatments, beginning on 79 dGA, NRC recommended requirements for late pregnant ewes were used to calculate feed requirements. Beginning on 79 dGA and continuing through 133 dGA, LC ewes were fed 100% nutrient requirements. On 79 dGA LL ewes were maintained on 50% of nutrient requirements, however to avoid the incidence of gestational ketosis a 5% increase over 5 day increments began at 110 dGA. Ewes were weighed weekly and the ration was adjusted according to changes in body weight (Figure 1).

Tissue Collection

At 135 ± 1 dGA, ewes were anesthetized by i.v. administration of sodium pentobarbital (27 mg/kg); intubated and maintained on 1-3% isoflurane, while a midventral laparotomy was performed to remove products of conception for tissue harvest. Umbilical artery and vein blood was collected from each fetus; a uterine artery blood sample was collected from a subsample of ewes (C, n = 4; LC, n = 4; and

LL, n = 3) prior to removal of the fetus. The fetus was euthanized while under anesthesia by i.v. administration of sodium pentobarbital (90 mg/kg). Following removal from the dam, fetal body weight, crown-rump length, abdominal circumference, and gender were recorded. Adrenals, brain, heart, intestines, kidneys, liver, pancreas and spleen were removed from the fetus and weighed individually. In a subsample of ewes (C, n = 4; LC, n = 4; and LL, n = 4) the uterus was drained of fluid and weighed, all placentomes were dissected from the uterus, and weights and numbers of type A, B, C, and D type placentomes were recorded (Vatnick *et al*. 1991).

Blood Analysis

Following collection, blood was immediately analyzed for tCO_2, pCO_2, pO_2, sO_2, hematocrit, and hemoglobin concentration using an iSTAT blood analyzer (Abbott Laboratories, Abbott Park, IL). Blood was collected into tubes containing 30 mg sodium fluoride and centrifuged for 10 min at 1,500 × *g* at 4°C to harvest plasma. Plasma was removed from the pellet and stored at -20°C for later analysis. Plasma glucose and lactate concentrations were measured with an YSI model 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH).

Calculations and Statistical Analysis

Fetal body mass index (BMI) was calculated as fetal weight (kg)/crown-rump length (m^2) and ponderal index was calculated as fetal weight (kg)/crown-rump length (m^3). Blood oxygen capacity was calculated as the product of hemoglobin concentration (g Hb/dL blood) and the oxygen carrying capacity of hemoglobin (1.34 mL O_2/g Hb). Blood oxygen content was calculated as the sum of Hb-bound O_2 (O_2

saturation, %/100 x 1.34 mL O_2/g Hb x g Hb/dL) and dissolved O_2 (0.003 mL/mL blood x pO_2 mmHg). Placental efficiency was calculated as fetal body weight (g) divided by total placentome weight (g).

Each ewe was treated as one experimental unit and twin fetuses within the ewe were treated as repeated measurements. We hypothesized that nutrient restriction followed by realimentation would induce faster fetal growth; whereas continuous nutrient restriction would induce fetal growth restriction and control fed pregnancies would present an intermediate phenotype at 135 dGA. Therefore data were analyzed by the following preplanned orthogonal contrasts: C vs. LC & LL and LC vs. LL. Any interaction by fetal sex observed is reported. All data are presented as least-square means ± S.E.M using the PROC MIXED model of SAS (SAS Institute, Cary, NC, USA). Statistical significance was set at $P \leq 0.05$ and statistical trends at $P \leq 0.10$.

Results

Ewe Feed Intake and Body Weight

Mean ewe body weights throughout gestation are depicted in Figure 1. At the beginning of the experiment, body weights of C, LC and LL ewes were 75.0 ± 3.0, 75.0 ± 3.5 and 73.8 ± 3.0 kg, respectively (C vs. LC & LL: $P = 0.8784$; LC vs. LL: $P = 0.8028$). Body weights at 135 dGA were 98.6 ± 3.5, 92.5 ± 4.2 and 79.3 ± 3.5 kg for C, LC, and LL ewes respectively (C vs. LC & LL: $P = 0.0117$; LC vs. LL: $P = 0.0270$). Average daily gain for early and late gestation is depicted in Table 1. Average daily gain during early gestation (28 dGA through 78 ± 3 dGA) for C, LC and LL ewes was 0.14 ± 0.03, -0.12 ± 0.04, and -0.07 ± 0.03, respectively (C vs. LC & LL: $P < 0.01$; LC vs. LL: $P = 0.3178$) respectively. During late gestation, from 79 ± 3 dGA through 133 ± 3 dGA, the average daily gain for C, LC and LL ewes was

0.31 ± 0.03, 0.50 ± 0.05, and 0.21 ± 0.05 kg d^{-1}, respectively (Control vs. LC & LL: P = 0.5377; LC vs. LL: P = 0.0011).

Fetal Body and Organ Weights

Mean fetal body weights for each treatment group are presented in Figure 2, and fetal body weights, empty fetal carcass weights, crown-rump length, abdominal circumference, and organ weights are presented in Table 2. Body weight of C fetuses was not different (P = 0.9375) when compared to LC and LL, while LC fetuses were heavier (P = 0.0132) than LL fetuses. Treatment effects on individual organ weights and body measurements were only evident in the statistical comparison of LC vs. LL fetuses ($P \leq 0.05$). Body weight, empty carcass weight, crown-rump length, abdominal circumference, brain, liver and intestine weights were greater ($P < 0.05$) in the LC vs. LL fetuses. Adrenal, heart, kidney, lung and pancreas weights were not affected ($P \geq 0.05$) by maternal UN. Fetal body mass index, ponderal index, and fetal brain:liver weight ratio, which is an index of asymmetric growth, were not affected by dietary treatment.

Male fetuses were heavier than female fetuses (4.51 ± 0.11 kg vs. 4.2 ± 0.10 kg; P = 0.0361). Empty carcass weight (3.00 ± 0.07 kg vs. 2.81 ± 0.07 kg; P = 0.0430), liver weight (116 ± 4.3 g vs. 104 ± 4.0 g; P = 0.0534), head weight (492 ± 10.4 g vs. 460 ± 9.7 g; P = 0.0302), abdominal circumference (37.46 ± 0.43 cm vs. 35.82 ± 0.40 cm; P = 0.0087), and pancreas weight (2.29 ± 0.20 g vs. 1.76 ± 0.20 g; P = 0.0273) were greater in male versus female fetuses. A treatment x gender interaction (P = 0.0338) for intestine weight was observed. Intestines from male fetuses were heavier in C (180.85 ± 8.51 g) than LC (163.29 ± 10.37 g) and LL (165.41 ± 7.71 g)

pregnancies, whereas intestines from female fetuses were heavier in LC (199.42 ± 10.37 g) than C (161.14 ± 9.56 g) and LL (155.41 ± 9.04 g) pregnancies.

Maternal blood

Uterine artery glucose concentrations did not differ (P = 0.3538),. To determine the maternal to fetal glucose gradient the difference between uterine artery and umbilical vein glucose concentrations was calculated (Table 3). There was no difference in the uterine artery to umbilical vein glucose gradient based on dietary treatment.

Fetal blood

Of the individual blood gas parameters, only pCO_2 was affected by dietary treatment (Table 4). Lower pCO_2 was observed in C ($P \leq 0.05$) compared to LC and LL in both umbilical vein and artery blood. Blood glucose concentration was not altered in either the umbilical vein or artery; however, the glucose concentration gradient between the umbilical vein and artery was lower in LL fetuses (0.26 ± 0.03 mmol/L) than LC fetuses (0.39 ± 0.04 mmol/L; Figure 3, P = 0.0174). The O_2 gradient from umbilical vein to umbilical artery tended to be lower (P = 0.0852) in LC and LL fetuses compared to C (Table 3). There was no impact of treatment on the umbilical vein to artery difference in pCO_2 or O_2 capacity.

Placentome Data

The count and weight distribution of type A, B, C, and D placentomes are given in Table 5. Placentae from LC pregnancies have a greater (P =0.0533) total weight of placentomes than LL (429.4 ± 37.6 g vs. 320.6 ± 37.6 g). Type D placentomes tended

to be more abundant ($P = 0.0778$) and account for more weight ($P = 0.0878$) in LC vs. LL placentae. Calculated placental efficiencies for Control, LC and LL pregnancies were 13.5 ± 1.57, 11.0 ± 1.57, and 13.0 ± 1.57 respectively (Control vs. LC & LL: $P = 0.4698$ and LC vs. LL: $P = 0.4082$).

Discussion

Understanding the impact of inadequate nutrient provision to a gestating ewe on the growth and development of a fetus is pertinent to livestock efficiency and quality. Twin pregnancy induces IUGR when ewes are fed their nutrient requirements during gestation (Cleal *et al*. 2007; Ford *et al*. 2007; Quigley *et al*. 2008). The reduction of fetal weight in twins ranges from 8-15% to 22-45% in mixed (twin and singleton bearing) pregnancies (West 1996; Anthony *et al*. 2003; Ford *et al*. 2007; Quigley *et al*. 2008; Sebert *et al*. 2010). In the present study only twin pregnancies were utilized, therefore some degree of natural fetal growth restriction likely existed across all treatments as a consequence of the ewes carrying twins. The lack of effect of nutrient restriction (LC and LL) on fetal body or organ weights, relative to the Control pregnancies, is likely explained by the aforementioned naturally occurring growth restriction in twin pregnancies. Consistent with others, in our study, fetal body weights of Control twins were approximately 10% lighter than Control singletons (data not shown) (Cleal *et al*. 2007; Ford *et al*. 2007). Similarly, pre-conception through near-term (60% maintenance, 133 dGA) undernourishment resulted in fetuses with reduced CRL and liver weight; however other major organs were unaffected, though these ewes carried singleton pregnancies (Quigley *et al*. 2008). Maternal restriction (60 and 70% maintenance) from early gestation through near-term in mixed pregnancies resulted in decreased gut, kidney, heart, thymus, pancreas and

liver weights, as well as thoracic and umbilical girths (Osgerby *et al*. 2002; Scheaffer *et al*. 2004). Interestingly, in the present study, despite long-term nutrient deprivation the brain to liver weight ratio was not affected by treatment. Fetal brain to liver weight ratio is a calculated indicator of asymmetric growth retardation (Dawes 1968). No difference in indicators of asymmetric growth restriction supports the concept that the Controls were also growth restricted as a result of twin pregnancies, and that if these data were compared to singleton data (comparable breed and size composition), differences in indicators of asymmetric fetal growth may have been observed.

Nutrient restriction during early gestation has been extensively studied, producing a variety of results. In mixed (both singletons and twins) pregnancies, body weights of fetuses from ewes restricted to 50% of maintenance requirements were reduced at 78 dGA, but not at 135 dGA, when compared to control-fed pregnancies (Vonnahme *et al*. 2003; Kwon *et al*. 2004). Nutrient restriction throughout most of gestation resulted in a 15% reduction in body weight of fetuses from mixed pregnancies (Kwon *et al*. 2004), while we observed only a 6% reduction in body weight in LL pregnancies when compared to Controls. This suggests that continuous maternal UN in singleton pregnancies results in greater relative IUGR than it does in twin pregnancies. In the present study nutrient restricted-realimented (LC) fetuses had greater body, brain, intestine, liver, adrenal, and lung weights compared to LL fetuses. In addition, AC and CRL were greater in LC fetuses when compared to LL fetuses. It has been reported that in singleton pregnancies, nutrient restriction during early gestation followed by realimentation to adequate nutrition does not affect fetal body weight (Heasman *et al*. 1998). Furthermore, nutrient restriction (ewes received 85% maintenance) beginning before conception and lasting through 70 dGA did not result in altered near-term fetal body weights (Steyn *et al*. 2001). Sub-optimal diets during

early gestation may condition the fetus to a more efficient metabolic rate, allowing for survival on the low supply of nutrients, which may be more pronounced in twin pregnancies. Consequently, if fetal metabolic rate is set sufficiently low, the provision of adequate nutrition later in gestation (realimentation) could result in compensatory growth resulting in greater relative fetal body weights near-term. Additionally, late-gestation nutrient restriction has been shown to cause reduced body weight or have no effect on body weight when comparing both singleton and twin pregnant ewes (Leury *et al*. 1990; West 1996; Edwards and McMillen 2001; Rae *et al*. 2001; Gao *et al*. 2007). Maternal dietary restriction (received 60 and 70% maintenance) throughout the entire length of gestation resulted in a reduction in fetal body weight near-term in twin and singleton pregnancies (Osgerby *et al*. 2002; Scheaffer *et al*. 2004). Similar results were observed when diet was restricted to maintain maternal live body weight(Luther *et al*. 2007). Further, a report contrasting four different periods of maternal nutrient restriction to a control diet resulted in no effect on fetal body weights (Rae *et al*. 2001). Clearly there has not been a consistent effect of maternal UN on near-term fetal body weight, but our results suggest that twin pregnancies may respond differently than singleton pregnancies do.

The various beginning and end points of maternal nutrient restriction, in addition to the range in magnitudes of maternal nutrient restriction most certainly accounts for the assortment of results regarding fetal body and organ weights. In the present study fetal body, brain, liver, intestine, adrenal and lung weights of fetuses from nutrient restricted-realimented (LC) dams were greater than for the continuously restricted (LL) fetuses. This may suggest metabolic alterations that occur as a result of a restricted supply of nutrients that facilitate fetal growth in a sub-optimal uterine environment (Quigley *et al*. 2008). Although maternal blood glucose concentrations

were not statistically different between our treatments at 135 dGA, LC dams showed elevated blood glucose relative to Control and LL dams. Furthermore, maternal glucose concentrations in Control and LL pregnancies were similar at 135 dGA, although the diets were still quite different. While our sample size was limited, these numerical differences might suggest that maternal UN during early- through mid-gestation could program an enhanced maternal peripheral insulin resistance and/or pancreatic sensitivity to an insulinotropic stimulus.

Heasman et al., (1998) reported that nutrient restriction from early to mid-gestation lowers pre-feeding plasma glucose concentrations at mid gestation, but does not impact glucose concentrations in those ewes near-term in singleton pregnancies. In contrast, nutrient restriction throughout gestation results in reduced maternal plasma glucose concentrations in singleton bearing ewes (Brameld *et al*. 2000; Gao *et al*. 2007; Quigley *et al*. 2008). In our study, twin pregnancy with continuous restriction did not change the maternal blood glucose concentrations near-term, nor was the uterine artery-umbilical vein glucose gradient impacted. This further suggests adaptation of nutrient restricted twin bearing ewes is different from singleton bearing ewes in an effort to maintain energy supply to the fetuses.

Other investigators have shown that fetal blood parameters such as pO_2, pCO_2 and glucose, are influenced by intrauterine growth restriction (Wallace *et al*. 2000; Quigley *et al*. 2008; Thorn *et al*. 2009). In our twin pregnancies, there were not as many differences between the Control and continuously undernourished fetuses as might be expected, which could reflect naturally occurring impaired fetal growth in Control twin pregnancies. The umbilical vein-umbilical artery glucose concentration gradient was lower in LL than LC fetuses. Lower glucose concentration gradients in LL fetuses could be an indication of lower utilization of glucose by the fetus,

suggesting that metabolic rates of these fetuses have shifted to survive on an inadequate plane of nutrition. Furthermore, there was a tendency for lower umbilical vein-umbilical artery differences in blood O_2 content, which supports the concept of lower metabolic rates in these fetuses. Reductions in metabolic set points within these offspring may well impact postnatal growth. In placental insufficiency induced-IUGR it has been demonstrated that oxygen delivery to the fetus is impacted by blood flow, placental oxygen consumption, and blood oxygen carrying capacity among others, which directly relates to the alteration of fetal glucose utilization and uptake rates (Barry *et al*. 2008). The umbilical vein-umbilical artery differences in blood glucose and O_2 content suggest a lower metabolic rate in LL fetuses, but since umbilical blood flow rates were not obtained, calculation of extraction rates by the fetus was not possible.

The fetal to placentome weight ratio was not impacted by treatment. The total weight of placentomes was greater in LC when compared to LL fetuses. The number and weight of type D placentomes tended to be greater in LC vs. LL placentae. Short-term nutrient deprivation during early and mid-gestation, as well as long-term nutrient restriction (22 through 135 dGA) shifted placentome distribution towards everted D-type placentomes (Clarke *et al*. 1998; Osgerby *et al*. 2002; McMullen *et al*. 2005). Since placentome number is determined in early gestation it is feasible that nutrient restricted-realimented placenta compensate for lack of nutrition in early gestation in the form of increased type D placentation (Wallace 1948). Alternatively, the increase in nutrient availability beginning at 79 dGA may instigate excessive growth of the type D placentomes, a form of compensation for earlier maternal UN. Indeed, it has been determined that although the different morphological placentome types do not necessarily be important, size does influence the vascularity and transfer of nutrients

across the placenta (Vonnahme *et al*. 2008). Thus the present results, total placentome weight, but not count, is higher in the LC treatment, could indicate placental overgrowth that occurred upon realimentation. Further, this overgrowth may have been a futile adaptation, if development of the placentomes did not increase surface area for transfer near the maternal facing cotyledon surface. Regardless of any potential functional significance of placental morphology, umbilical vein pCO_2 was elevated in both LL and LC pregnancies at 135 dGA, suggesting greater metabolic rates of the placenta relative to the Control pregnancies. This suggests a long-term shift in placental function, initiated during early- to mid-gestation in response to maternal UN in twin pregnancies.

As we initially hypothesized, in twin sheep pregnancies, maternal UN followed by realimentation resulted in compensatory fetal growth, whereas continuous maternal UN resulted in slower fetal growth and metabolism, with control-fed pregnancies presenting an intermediate phenotype. While a considerable amount of research on the impact of maternal UN has been conducted in singleton and mixed pregnancies, to our knowledge this is the first direct comparison of the two dietary regimens in twin pregnancies only. While further investigation is needed, our maternal and fetal glucose and blood gas data suggest that twin pregnancies may respond somewhat differently than singleton pregnancies. It appears that early- to mid-gestation maternal UN may program maternal metabolism, such that realimentation could result in greater maternal glucose, and continuous maternal UN results in glucose concentrations not dissimilar from control-fed pregnancies. Furthermore, based on umbilical vein pCO_2 values, it appears that placental metabolism may be impacted whether the ewe is realimented or not. The study of twin pregnancies, as a natural

model of IUGR, can provide insight into maternal-fetal interactions that may not be attainable with the study of singleton or mixed pregnancies alone.

Tables and Figures

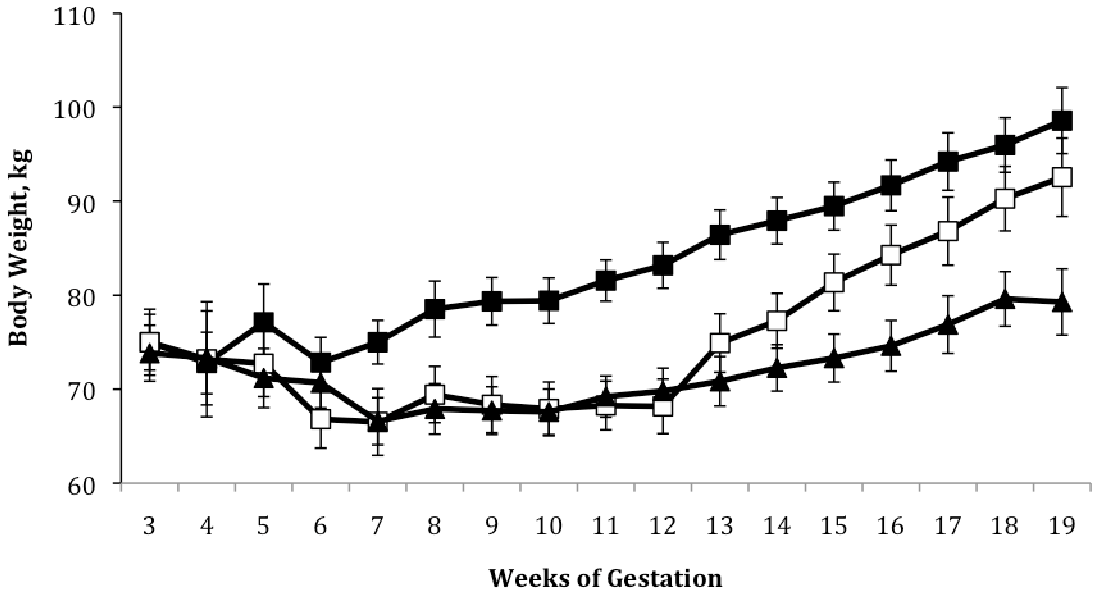

Figure 1. Mean maternal body weight throughout gestation. Control ewes were fed 100% of their NRC (2006) recommended requirements (■), LC were fed 50% of their requirements from day 28 through 78 of gestation followed by 100% through the remainder of gestation (□), and LL were fed 50% of their requirements from day 28 through the remainder of gestation (▲).(Trt*Week of gestation, $P < 0.0001$)

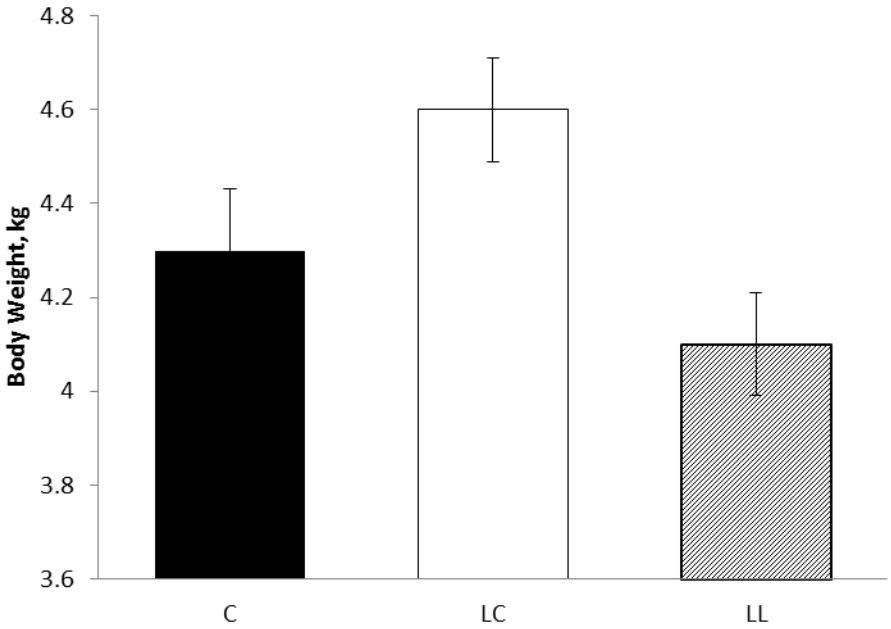

Figure 2. Mean fetal body weight by treatment at 135 days of gestation. Control (C) ewes were fed 100% of their NRC (2006) recommended requirements, LC were fed 50% of their requirements from day 28 through 78 of gestation followed by 100% through the remainder of gestation, and LL were fed 50% of their requirements from day 28 through the remainder of gestation. (Control vs. LC and LL: P = 0.94; LC vs. LL: P = 0.01).

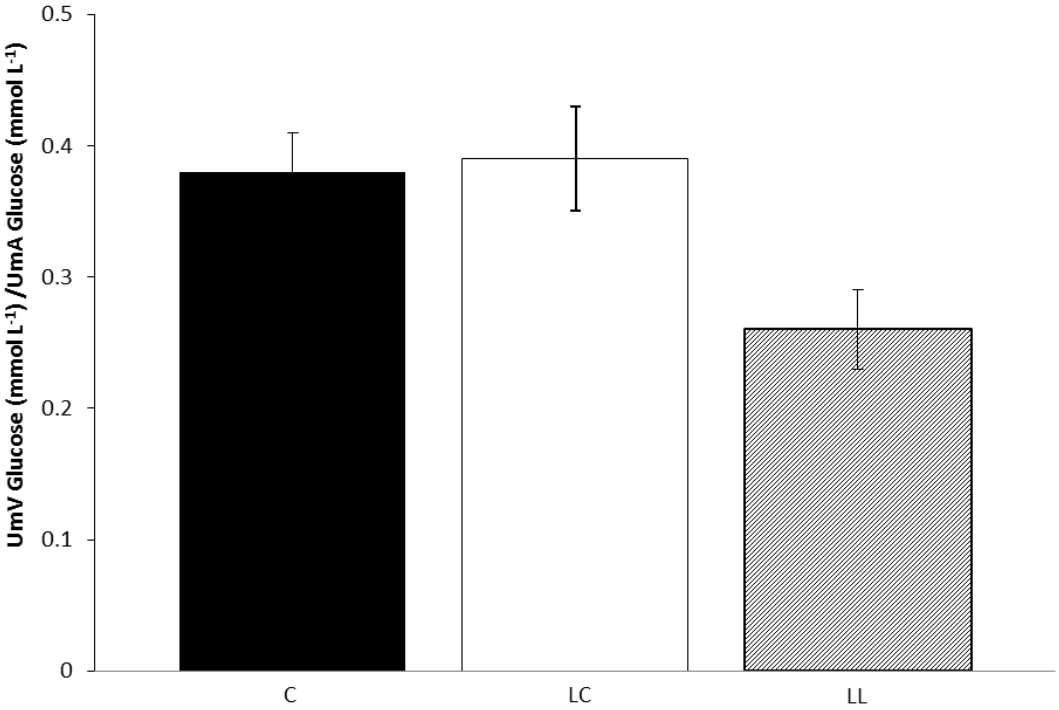

Figure 3. Umbilical vein (UmV) to umbilical artery (UmA) glucose gradient at 135 days of gestation. Control (C) ewes were fed 100% of their NRC (2006) recommended requirements, LC were fed 50% of their requirements from day 28 through 78 of gestation followed by 100% through the remainder of gestation, and LL were fed 50% of their requirements from day 28 through the remainder of gestation. (C vs. LC and LL P=0.21; LC vs. LL P=0.02).

Table 2. Maternal average daily gain of ewes receiving a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through 133 of gestation (LL).

	Gestational intake			*P* values	
	C (n = 7)	LC (n = 5)	LL (n = 7)	C vs. LC, LL	LC vs. LL
Early gestation (kg d^{-1}; day 28 to 78)	0.14 ± 0.03	-0.13 ± 0.04	-0.07± 0.30	< 0.0001	0.3178
Late gestation (kg d^{-1}; day 79 to 133)	0.31 ± 0.31	0.50 ± 0.05	0.21 ± 0.05	0.5377	0.0011

Data are the mean ± s.e.m.

Table 3. Fetal body and organ measurements at day 135 of gestation in relation to maternal diet; ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through 133 of gestation (LL).

	Gestational intake			*P* values	
	C (n = 7)	LC (n = 5)	LL (n = 7)	C vs. LC, LL	LC vs. LL
Body weight (kg)	4.35 ± 0.13	4.64 ± 0.15	4.09 ± 0.13	0.9375	0.0132
Carcass weight (kg)	2.90 ± 0.09	3.14 ± 0.11	2.69 ± 0.09	0.8820	0.0060
CRL (cm)	57.46 ± 0.75	59.53 ± 0.88	56.50 ± 0.75	0.5671	0.0184
AC (cm)	36.56 ± 0.48	37.60 ± 0.55	35.76 ± 0.47	0.8494	0.0224
Brain (g)	57.90 ± 1.55	62.34 ± 1.84	54.62 ± 1.55	0.7725	0.0054
Intestine (g) ^	170.99 ± 5.90	181.36 ± 6.57	160.41 ± 5.63	0.9882	0.0314
Liver (g)	106.53 ± 5.04	123.03 ± 5.77	101.51 ± 4.89	0.3760	0.0125
Kidney (g)	23.10 ± 1.07	26.71 ± 1.27	23.98 ± 1.07	0.1174	0.1201
Adrenals (g)	0.39 ± 0.04	0.44 ± 0.05	0.33 ± 0.04	0.8975	0.0791
Heart (g)	25.78 ± 1.50	28.40 ± 1.82	26.07 ± 1.50	0.4620	0.3402
Lung (g)	133.40 ± 5.56	141.22 ± 6.58	124.56 ± 5.56	0.9429	0.0711
Pancreas (g)	2.12 ± 0.27	1.87 ± 0.30	2.09 ± 0.27	0.6835	0.6054

Data are the mean ± s.e.m.
CRL, crown rump length; AC, abdominal circumference.
† Trt*gender $P = 0.0338$

Table 4. Uterine artery:umbilical vein and umbilical vein:umbilical artery differences in glucose, pCO_2 and oxygen content at 135 days of gestation in relation to maternal diet; ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through 133 of gestation (LL).

	Gestational intake			*P* values	
	C (n = 7)	LC (n = 5)	LL (n = 7)	C vs. LC, LL	LC vs. LL
Uterine artery:umbilical vein gradient					
Glucose	2.89 ± 0.24	3.51 ± 0.26	2.95 ± 0.28	0.2769	0.1548
Umbilical vein:umbilical artery gradient					
Glucose	0.38 ± 0.03	0.39 ± 0.04	0.26 ± 0.03	0.2502	0.0174
pCO_2	-3.92 ± 0.88	-4.27 ± 1.04	-3.61 ± 0.94	0.9857	0.6457
O_2 content	5.02 ± 0.43	4.43 ± 0.47	3.41 ± 0.47	0.0852	0.1713

Data are the mean ± s.e.m.

Table 5. Umbilical vein and artery blood gas measurements at 135 days of gestation in relation to maternal diet; ewes either received a control diet (C), 50% of nutrient requirements from day 28 through 78 of gestation (LC), or 50% of nutrient requirements from day 28 through 133 of gestation (LL).

Blood Source	Umbilical Vein Gestational intake			*P* values		Umbilical Artery Gestational intake			*P* values	
	C (n = 7)	LC (n = 5)	LL (n = 7)	C vs. LC, LL	LC vs. LL	C (n = 7)	LC (n = 5)	LL (n = 7)	C vs. LC, LL	LC vs. LL
Glucose (mmol L^{-1})	1.54 ± 0.18	1.91 ± 0.20	1.43 ± 0.18	0.5742	0.1041	1.16 ± 0.17	1.52 ± 0.19	1.19 ± 0.17	0.3913	0.2233
Lactate (mmol L^{-1})	3.65 ± 0.68	3.94 ± 0.75	3.63 ± 0.68	0.8752	0.7594	3.57 ± 0.71	3.85 ± 0.78	3.61 ± 0.71	0.8559	0.8182
TCO_2 (mmol L^{-1})	27.08 ± 0.87	29.21 ± 0.97	28.71 ± 0.83	0.1096	0.7543	28.92 ± 0.90	30.20 ± 0.96	29.96 ± 0.82	0.3265	0.7734
Hct (%PCV)	35.08 ± 1.46	34.57 ± 1.63	33.45 ± 1.39	0.5635	0.6094	35.50 ± 1.45	34.80 ± 1.58	34.54 ± 1.38	0.6604	0.9246
Hb (g dL^{-1})	11.93 ± 0.46	11.53 ± 0.50	11.39 ± 0.44	0.4128	0.8335	12.08 ± 0.50	11.85 ± 0.54	11.75 ± 0.50	0.6648	0.9059
pCO_2 (mmHg)	57.86 ± 2.42	66.80 ± 2.71	63.48 ± 2.46	0.0311	0.3797	61.78 ± 2.48	71.17 ± 2.71	66.85 ± 2.53	0.0351	0.2633
pO_2 (mmHg)	31.00 ± 3.19	32.04 ± 3.30	30.33 ± 3.30	0.9638	0.7221	16.10 ± 1.90	17.30 ± 1.91	18.00 ± 1.94	0.5208	0.8012
HCO_3(mmol L^{-1})	25.93 ± 0.85	27.07 ± 0.94	26.67 ± 0.80	0.3844	0.7511	27.12 ± 0.89	28.14 ± 0.97	27.89 ± 0.89	0.5020	0.6724
SO_2, %	49.10 ± 6.75	46.58 ± 6.97	44.07 ± 6.97	0.6614	0.8037	17.30 ± 3.66	18.50 ± 3.85	19.80 ± 3.70	0.7021	0.8165
O_2 Content	7.84 ± 0.96	7.15 ± 0.99	6.54 ± 0.99	0.4181	0.6704	2.82 ± 0.54	2.93 ± 0.60	3.00 ± 0.55	0.8457	0.9313
O_2 Capacity	15.99 ± 0.61	15.45 ± 0.68	15.26 ± 0.59	0.4128	0.8335	16.18 ± 0.68	15.88 ± 0.72	15.75 ± 0.69	0.6648	0.9059

Data are the mean ± s.e.m. TCO_2, total CO_2; HCO_3, bicarbonate; Hct, hematocrit; Hb, hemoglobin; pCO_2, carbon dioxide partial pressure; and pO_2, oxygen partial pressure.

Table 6. Placentome type and weight distribution at 135 days of gestation in relation to maternal diet; ewes either received a control diet (C), 50% of nutrient requirements from day 28 through 78 of gestation (LC), or 50% of nutrient requirements from day 28 through 133 of gestation (LL).

Type	Gestational intake			*P* values	
	C (n = 4)	LC (n = 4)	LL (n = 4)	C vs. LC, LL	LC vs. LL
Total count	38.4 ± 3.2	43.3 ± 3.2	36.4 ± 3.2	0.7183	0.1443
A	18.4 ± 3.7	12.5 ± 3.7	12.0 ± 3.7	0.1909	0.9248
B	12.8 ± 2.7	14.5 ± 2.7	14.1 ± 2.7	0.6414	0.9227
C	6.88 ± 2.5	6.38 ± 2.5	8.88 ± 2.5	0.8081	0.4855
D	0.37 ± 3.2	9.88 ± 3.2	1.38 ± 3.2	0.2002	0.0778
Total wt (g)	353.1 ± 37.6	429.4 ± 37.6	320.6 ± 37.6	0.6393	0.0533
A (g)	128.1 ± 31.7	72.5 ± 31.7	85.6 ± 31.7	0.2208	0.7729
B (g)	139.4 ± 27.3	148.8 ± 27.3	117.5 ± 27.3	0.8537	0.4281
C (g)	78.1 ± 28.0	71.9 ± 28.0	98.8 ± 28.0	0.8361	0.5051
D (g)	7.5 ± 46.4	136.3 ± 46.4	18.8 ± 46.4	0.2317	0.0878

Data are the mean ± s.e.m.

Chapter IV. The impact of maternal undernutrition on lamb growth and blood glucose in twin sheep pregnancies.

Summary

The experimental objective was to determine the impact of duration of maternal undernutrition during gestation in twin pregnancies on lamb growth and development. Ewes were randomly assigned to one of three treatments and acclimation to individual pens (7 d) began at 21 d of gestational age (dGA). Ewes were fed 100% (C; n = 8), 50% of their nutrient requirements from 28 to 78 dGA and readjusted to 100% beginning at 79 dGA (LC; n = 10), or 50% from 28 to term (LL; n = 9). Lambs were birthed naturally and harvested at 18 wk postnatal (PN). Blood serum was collected from ewes throughout gestation and until 10 weeks postpartum and postnatally from lambs through 18 weeks PN. Lambs born to LC ewes were heavier (P = 0.0168) than LL lambs. Crown-rump length (CRL) of LC lambs tended (P = 0.0933) to be greater than LL. Body weight of LC lambs was greater (P < 0.05) than LL lambs at 2 and 3 wk PN and the same trend (P = 0.0654) was observed at 4 wk. At 4, 16 and 17 wk PN body weight tended (P < 0.10) to be greater in C than LC & LL lambs. At 18 wk PN brain weight (P = 0.0331) and rear leg length (P = 0.0287) of LC lambs was greater andCRL tended (P = 0.0882) to be greater than that of LL. At 18 wk PN, brain weight was greater (P = 0.0331) in LC than LL lambs. Glucose (GLU) concentrations at 49 and 77 dGA were greater (P < 0.05) in C vs. LC & LL ewes; the same trend (P < 0.10) occurred at 63 dGA. At 105, 112, 126, and 147 dGA GLU was greater (P < 0.05) in LC vs. LL ewes. At 2 wk PP ewe GLU was greater (P = 0.0327) in LC & LL than C. At 8 wk PP blood glucose was greater (P = 0.0251) in

LC & LL than C. Lamb GLU was reduced (P = 0.0173) in LC vs. LL at 14 wk PN. Elevated body weight and CRL of LC lambs at birth may indicate a shift in fetal metabolism to compensate for nutrient restriction during early gestation. Minimal differences between C and nutrient restricted treatments indicate naturally occurring restriction in twin pregnancies. Elevated post-weaning GLU in LL lambs may indicate increased insulin resistance in these lambs.

Introduction

Intrauterine growth restriction (IUGR) can be effectively induced by maternal undernutrition (UN) and result in low birth weight lambs (Barker *et al*. 1993; Barker 1995; Osgerby *et al*. 2002; Anthony *et al*. 2003). Organ development and the fetal growth trajectory are highly vulnerable to maternal UN during the early stages of gestation (Reynolds and Redmer 1995; Osgerby *et al*. 2002; Ford *et al*. 2007). In the case of sub-optimal developmental conditions in-utero fetal programming occurs to increase the prospect of fetal survival (Lucas 1991). Further, a "thrifty phenotype" hypothesis indicates that maternal UN results in modified glucose utilization, insulin secretion and responsiveness during development (Hales *et al*. 1991). Although fetal programming may be crucial for continued growth of the IUGR fetus, it has potentially deleterious effects on offspring and may even contribute to the development of adult metabolic diseases in humans (Stein *et al*. 1996; Barker 1998). Maternal UN may result from natural fluctuations in forage availability and quality which could have negative impacts on offspring growth, metabolic function, and carcass quality (Black 1983; Bell 1992; Anderson 1993; Kelley *et al*. 1996; Del Curto *et al*. 2000).

In sheep, twin pregnancy induces IUGR even when adequate nutrients are available to the ewe (Cleal *et al*. 2007; Ford *et al*. 2007; Quigley *et al*. 2008). Overall neonatal lamb survival decreases following maternal UN in mixed pregnancies presumably because of smaller, weaker lambs in combination with reduced milk production capacity of the ewe (Nordby *et al*. 1987). Although metabolic adaptations that result from maternal UN may contribute to increased neonatal mortality, some adaptations may not become apparent until the offspring reaches adulthood. In one study, it was reported that in singleton lambs birth weight was related more to glucose tolerance, IGF-I and blood pressure than to maternal diet in late gestation (Oliver *et al*. 2002); however it has been reported that periconceptional maternal UN alters offspring glucose tolerance (Todd *et al*. 2009; Smith *et al*. 2010). Maternal UN during early gestation results in increased hepatic fat accumulation when the offspring are reared in an obesogenic environment. Depending on the duration of maternal UN the function of the hypothalamic-pituitary-adrenal axis in the adult offspring from singleton pregnancies may also be down regulated (Bloomfield *et al*. 2003). Further, maternal UN can impair development of the kidney (Lloyd *et al*. 2012) and even affect vascular function in the adult offspring (Torrens *et al*. 2009).

The composition and quality of the lamb carcass are subject to fetal adaptations that result from maternal UN. In one study, maternal UN in a mixture of twin and singleton pregnancies resulted in offspring with lower average daily gain and took longer to reach slaughter weight (Mellor 1983; Nordby *et al*. 1987). In another study, it was shown that a high plane of nutrition following maternal UN can elicit significant catch-up growth and increase carcass fat content (Lawlor and Hopkins 1981). Maternal UN also increases muscle fiber diameter, decreases sarcomere length, and increases total weight of the

semitendinosus muscle at slaughter (Nordby *et al*. 1987). Jaquiery *et al*. (2011b) have suggested that a disconnection between fetal growth and its regulators might be responsible for adverse metabolic events in adulthood, even though birth weight and growth trajectory may not be affected.

The post-natal impact of maternal UN from early- to mid-gestation or throughout gestation in the case of twin pregnancies is of interest since twinning is commonplace in sheep production. Therefore, the objective of this experiment was to determine the impact of maternal UN from 28 to 78 dGA and from 28 to parturition on the growth trajectory, biometric measurements at birth and slaughter, and organ weights at slaughter of the lambs. We hypothesized that early gestational maternal undernutrition followed by realimentation during late gestation would enhance lamb growth, but prolonged undernutrition would suppress lamb growth.

Experimental methods

Animals

Only multiparous, western whiteface ewes carrying twin pregnancies (assessed at birth) were used for the study (n = 27; 78.43 ± 2.86 kg). The ewes had an average pre-experiment weight of 78.43 ± 2.86 kg. Procedures for this study were consistent with guidelines of the United States Department of Agriculture. Protocols for care and use of the animals were approved by the Colorado State University Institutional Animal Care and Use Committee. Prior to initiation of the experiment, ewes were group housed and offered feed ad libitum. The diet consisted of pelleted beet pulp, which included a

vitamin-mineral mixture (77.8% total digestible nutrients, 90.0% dry matter, and 9.4% crude protein) to meet requirements.

Ewes were treated with controlled intra-vaginal drug release devices (EAZI-BREED^{TM} CIDR$^{®}$ Sheep and Goat Device, Pfizer, New York) for 7 d followed by two 5 mg doses of $PGF_{2\alpha}$ (Lutalyse, Pfizer, New York) 4 hr apart. A ram was introduced 48 hr after the first dose of PGF_2. Breeding was observed and as an indicator of insemination. At 21 dGA, ewes were placed in individual pens and fed the experimental diet to meet 100% of nutrient requirements for early pregnant ewes (NRC, 2006 recommended requirements) on an individual weight basis. Beginning on 28 dGA ewes were randomly assigned to C (n = 8; 100% of nutrient requirements) or LL (n = 9) and LC (n = 10), with both treatment groups receiving 50% of nutrient requirements. For all treatments, beginning on 79 dGA, NRC recommended requirements for late pregnant ewes were used to calculate feed requirements. Beginning on 79 dGA and continuing through parturition, LC ewes were fed 100% nutrient requirements. On 79 dGA LL ewes were maintained on 50% of nutrient requirements through parturition. During gestation, ewes were weighed weekly and the ration was adjusted according to changes in body weight. To enable close monitoring of labor ewes were moved into an enclosed, heated barn at 143 dGA. Following parturition at 151 ± 3 dGA all ewes were gradually adjusted to ad libitum alfalfa hay.

Lambs were allowed to suckle their dams freely or supplemented with a commercial milk replacer if no milk was produced by their respective dam (Merrick's, Inc., Middleton, WI). Beginning at 2 wk PN, lambs had free choice access to lamb creep pellets and alfalfa hay (All*American Show Lamb Creep, Ranch-Way Feeds, Fort

Collins, CO). At 10 wk PN lambs were removed from their mothers and gradually adjusted to a complete feed that was offered at 150% of the recommended feeding rate to allow ad libitum intake (All*American Show Lamb Complete, Ranch-Way Feeds, Fort Collins, CO). Lambs were weighed weekly and the ration was adjusted according to changes in body weight.

Biometric Measurements

Each week throughout gestation and following parturition until 10 wk PP ewe body weight was recorded. At birth and at 18 wk PN body weight, crown-rump length, abdominal circumference, thoracic circumference, hip girth (only at 18 wk PN), front and rear leg length, and gender were recorded. Ewes and lambs were weighed weekly following parturition. At 18 wk PN the lambs were harvested at a local abbatoir. Immediately following exsanguination the adrenals, brain, heart, intestine, kidneys, liver, pancreas and spleen were weighed individually.

Blood Collection and Analysis

Blood samples were collected from the ewes 1-2 hr before daily feeding by jugular venipuncture at 21, 35, 49, 63, 77, 91, 98, 105, 112, 119, 126, 133, 140 and 147 dGA. Jugular vein blood samples were also collected 1-2 hr before daily feeding from ewes at 2, 4, 6, 8 and 10 wk PP and from lambs at 2,4 6, 8, 10, 12, and 16 wk PN.

For the determination of glucose and lactate concentration, blood was collected into tubes containing 30 mg sodium fluoride and centrifuged for 10 min at 1,500 × *g* to harvest plasma. Plasma was removed from the pellet and stored at -20°C for later

analysis. Plasma glucose and lactate concentrations were measured with an YSI model 2700 Select Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH).

Milk Collection

At 6 wk PP each ewe was separated from her lambs for the estimation of milk volume. A protocol utilizing Oxytocin and hand-milking similar to that used by Gardner and Hogue (1964) was implemented. At the beginning and end of a 2-hour period a 1 mL subcutaneous injection of Oxytocin (20 USP units per mL; Vet One, Bimeda-MTC Animal Health Inc., Ontario, Canada) was administered to induce milk let down. Each udder was completely stripped of milk following the Oxytocin injection at the beginning and end of the 2-hour period. The volume of milk produced at the end of the 2-hour period by the left and right halves of the udder was recorded and a subsample of milk was collected from this milking and frozen for crude protein, fat, and lactose analysis.

Statistical Analysis

All data are presented as LSmeans ± S.E.M. Means were separated at a significant level of $P < 0.05$ and considered as a trend at a level of $P < 0.10$. Each ewe was treated as one experimental unit and twin fetuses within the ewe were treated as repeated measurements. We hypothesized that nutrient restriction followed by realimentation would induce faster fetal growth; whereas continuous nutrient restriction would induce fetal growth restriction and C pregnancies would present an intermediate phenotype at birth. Therefore, fetal and lamb blood data were analyzed by preplanned orthogonal contrasts: C vs. LC & LL and LC vs. LL. Any interaction by fetal sex observed is

reported. Repeated measurements of ewe data were analyzed using the PROC MIXED model of SAS (SAS Inst. Carry, NC). Ewes or lambs that did not survive through the entire project were not included in analysis.

Results

Maternal Body Weight

Mean ewe body weights throughout gestation are depicted in Figure 4. At the beginning of the experiment, body weights for C, LC and LL ewes were 77.73 ± 2.65, 77.86 ± 2.90, and 79.70 ± 3.04 kg, respectively (C vs. LC & LL: $P = 0.7581$; LC vs. LL: $P = 0.6670$). Body weights at term (144 ± 3 dGA) were 102.46 ± 2.68, 92.19 ± 2.80, and 79.65 ± 3.09 kg for C, LC, and LL ewes respectively (C vs. LC & LL: $P < 0.0001$; LC vs. LL: $P = 0.0054$). Average daily gain for early and late gestation is depicted in Table 6.

Offspring Body Measurements

Mean lamb birth weights for each treatment are given in Figure 5. Body weight, crown-rump length, head circumference, abdominal circumference, thoracic girth, front and rear leg lengths are given in Table 7. Birth weights of LC lambs (5.41 ± 0.28 kg) were greater than LL lambs (4.34 ± 0.29 kg; $P = 0.0168$). Crown-rump length (CRL) tended ($P = 0.0933$) to be greater in LC (17.90 ± 0.54 cm) than LL (16.38 ± 0.68 cm) lambs. Abdominal and head circumferences, thoracic girth, front and rear leg lengths were not affected by treatment ($P \geq 0.05$).

Mean lamb body weights from birth through 18 wk PN are depicted in Figure 6. Body weight of LC lambs was greater than LL lambs at 2 ($P = 0.0316$; 8.52 ± 0.51 kg vs. 4.34

± 0.29 kg) and 3 wk PN (P = 0.0248; 9.79 ± 0.53 kg vs. 7.94 ± 0.53 kg) and the same trend (P = 0.0654) was observed at 4 wk PN (11.63 ± 0.70 kg vs. 9.51 ± 0.81 kg). Body weight tended to be greater (P < 0.10) in C vs. LC and LL lambs at 4, 17, and 18 wk PN. Pre- and post-weaning average daily gains of lambs were not impacted (Table 8) by maternal gestational treatment.

Mean lamb body measurements at 18 wk PN are given in Table 9. At 18 wk PN rear leg length was greater (P = 0.0287) in LC (63.92 ± 1.30 cm) than LL (61.54 ± 1.26 cm) lambs. At 18 wk PN CRL of LC (114.46 ± 2.82 cm) lambs tended (P = 0.09) to be greater than LL (107.30 ± 3.72 cm) lambs. Male lambs were heavier than female lambs (47.00 ± 1.32 kg vs. 41.19 ± 1.32 kg; P = 0.0023). Abdominal circumference (102.81 ± 1.52 cm vs. 98.31 ± 1.50 cm; P = 0.0455) and front leg length (55.32 ± 0.98 cm vs. 52.26 ± 0.98 cm; P = 0.0315) of male lambs were greater than female lambs. A treatment x gender interaction (P = 0.0295) for hip girth was observed. Hip girth of male lambs was greater in C (99.69 ± 2.79 cm) than LC (91.71 ± 2.46 cm) and LL (91.87 ± 2.57 cm), whereas hip girth of female lambs was greater in LC (97.29 ± 2.99 cm) than C (92.19 ± 2.22 cm) and LL (95.09 ± 3.62 cm).

Mean lamb organ weights at 18 wk PN are given in Table 10. At 18 wk PN, brain weight was greater (P = 0.0331) in LC (107.98 ± 2.61 cm) than LL (99.74 ± 2.47 cm) lambs. The weights of the hot carcass (21.80 ± 0.66 kg vs. 18.93 ± 0.66 kg; P = 0.0012), head (2575.32 ± 79.1 g vs. 2120.02 ± 79.1 g; P = 0.0002), liver (960.42 ± 27.9 g vs. 781.64 ± 27.1 g; P < 0.0001), adrenals (1.79 ± 0.15 g vs. 2.17 ± 0.16 g; P = 0.0694), intestines (10603 ± 303 g vs. 8662.8 ± 330 g; P < 0.0001), pancreas (51.26 ± 4.14 g vs. 38.21 ± 4.04 g; P = 0.339), spleen (87.93 ± 3.63 g vs. 77.33 ± 3.58 g; P = 0.0379), lungs

(557.37 ± 21.88 g vs. 445.51 ± 21.89 g; $P = 0.0005$), heart (204.18 ± 5.84 g vs. 179.61 ± 5.83 g; $P = 0.0034$), right ventricle (49.24 ± 1.63 g vs. 41.58 ± 1.63 g; $P = 0.0009$), left ventricle (105.74 ± 3.45 g vs. 92.85 ± 3.45 g; $P = 0.0069$), and septum (25.45 ± 0.96 g vs. 22.82 ± 0.95; $P = 0.0633$) were greater in male lambs than female lambs. A treatment x gender interaction ($P = 0.0306$) for the weight of adrenals was observed. The adrenals of male lambs were heavier in LL (2.27 ± 0.27 g) than C (1.51 ± 0.28 g) and LC (1.60 ± 0.25 g), whereas the adrenals of female lambs were heavier in LC (2.71 ± 0.30 g) than C (1.95 ± 0.24 g) and LL (1.87 ± 0.31 g).

Milk Volume and Composition

Mean milk volume produced, crude protein and fat percentage, and lactate concentration were not affected by treatment ($P \geq 0.10$) and are given in Table 11.

Blood Glucose

Mean maternal glucose concentrations during gestation and postpartum are depicted in Figures 7 and 8, respectively. At 49 and 77 dGA glucose concentration was greater ($P < 0.05$) in C vs. LC & LL ewes; the same trend ($P < 0.10$) occurred at 63 dGA. At 105, 112, 126, and 147 dGA glucose concentration was greater ($P < 0.05$) in LC vs. LL ewes. At 2 wk PP ewe glucose concentration was greater ($P = 0.03$) in LC (3.60 ± 0.17 mM) and LL (3.29 ± 0.17mM) than C (2.99 ± 0.16 mM).

Mean lamb blood glucose concentrations from 2 through 16 wk PN are depicted in Figure 9. At 8 wk PN blood glucose concentration was greater ($P = 0.0251$) in LC (4.83 ± 0.12 mM) & LL (4.81 ± 0.11 mM) than C (4.48 ± 0.11 mM). Blood glucose

concentration at 14 wk PN was reduced (P = 0.0173) in LC (4.12 ± 0.09 mM) vs. LL (4.52 ± 0.09 mM).

Discussion

To our knowledge this is the first study to directly compare the growth response of twin lambs following early gestation (LC) versus prolonged (LL) maternal UN and maternal UN (LC & LL) versus C. We report that the weight and morphometric measurements at birth of twin lambs from maternal UN (LC & LL) were not different from C lambs; this is due to C being intermediate between the two undernourished treatments. These results are consistent with our findings that the impact of maternal UN (LC & LL) relative to C twin pregnancies on weight and morphometric measurements of near term lambs is negligible (Chapter III). These results also support the speculation that C lambs provide and intermediate phenotype because of naturally occurring growth restriction as a consequence of the ewe carrying multiple lambs (Gardner *et al*. 2005; Cleal *et al*. 2007; Ford *et al*. 2007; Quigley *et al*. 2008). Consistent with our results, others have reported that maternal UN in singleton, twin, or mixed pregnancies during early (Heasman *et al*. 1998; Gardner *et al*. 2005; Daniel *et al*. 2007; Ford *et al*. 2007; Kotsampasi *et al*. 2009) or late gestation (Edwards and McMillen 2001; Yuen *et al*. 2002; Gardner *et al*. 2005) does not affect lamb birth weight. Further, it has also been shown in mixed pregnancies that a fetus at 78 dGA from maternal UN (50% of maintenance requirements 28 to 78 dGA) will have reduced body weight (Vonnahme *et al*. 2003). In contrast, underfeeding during late gestation in mixed and twin pregnancies has also resulted in lambs of smaller birth weight (Koritnik *et al*. 1981; Tygesen *et al*. 2008; Sebert *et al*. 2011). Together this supports our hypothesis that maternal UN from 28 to 78

dGA results in acceleration of fetal growth upon realimentation to adequate nutrients, so that the difference in body weight at birth is not significantly impacted. However, we also report that maternal UN followed by realimentation (LC) results in greater body weight and crown-rump length at birth (151 ± 3 dGA) than prolonged maternal UN (LL). These findings correspond with our previous observation that at 135 dGA, LC lamb body and organ weights are greater than LL (Chapter III). Mellor (1983) also reported similar findings that prolonged maternal UN in mixed pregnancies results in lambs of smaller birth weights. In another study, prolonged nutrient restriction (69 days total, from 31 through 100 dGA) did not affect lamb birth weight (Kotsampasi *et al*. 2009). In our study the period of prolonged maternal UN treatment (LL) was considerably longer (123 days total from 28 through 151 dGA) and resulted in lower birth weights than lambs from the LC treatment. These results also support our hypothesis that prolonged maternal UN would induce fetal growth restriction and result in lambs of lower birth weight.

Despite the differences in body weight at birth maternal UN during gestation did not impact the postnatal growth trajectory in terms of average daily gain in either the pre- or post-weaning period (birth through 10 wk PN or 10 wk PN through 18 wk PN, respectively). These results are consistent with those of Kotsampasi *et al*. (2009) who reported that neither early (first 30 d) nor prolonged maternal UN (31 to 100 d) during gestation impacted rate of growth. Ford *et al*. (2007) reported an increased postnatal weight gain in offspring from early gestation maternal UN followed by realimentation in mixed pregnancies, though birth weights were similar. In contrast, restriction (receiving 50%) from 30 to 70 dGA resulted in reduced overall lamb average daily gain when lambs were grown to 24 wk of age, while restriction from 30 to 85 dGA did not alter average

daily gain when lambs were only grown to 17 wk of age (Daniel *et al*. 2007). Sebert *et al*. (2011) also reported that twin lambs from late-gestation maternal UN had higher post-weaning (3 to 7 mo of age) rates of gain than twin lambs reared as singletons (forcing accelerated early post-natal growth). From these results it seems that despite the response of compensatory growth of twin lambs in late gestation to maternal UN from early- to mid- gestation, this adaptation is not noticeable in the first 18 wk PN. This response may be imputed to the occurrence of twin pregnancies, since there was no difference in the growth rate when comparing C to maternal UN (LC & LL) or when comparing the duration of maternal UN.

In the present study we report that at 18 wk PN the brain weight of LC was greater than LL lambs, perhaps indicating that mechanisms adopted by the fetus to protect brain development have carried over into PN growth (McMillen *et al*. 2001). In another study Daniel *et al*. (2007) reported that growth restricted twin lambs (maternal UN from 30 to 70 dGA) had reduced liver, heart, lung, and body weights at 24 wk PN. Whereas in the same study, growth restricted twin lambs (maternal UN from 30 to 85 dGA) exhibited an increase in kidney weight, but no difference in slaughter weight (Daniel *et al*. 2007). It is possible that as with the differences in rate of gain, the impact of early- to mid-gestation maternal UN on organ development and body measurements may become more defined at a later stage in growth. Interestingly, in the current study we report that at 18 wk PN lambs from maternal UN (LC & LL) had shorter rear leg length than C lambs, which supports the idea that carcass characteristics vary with nutritional management (Thomas and Kott 1995). Further, we also report that lambs from prolonged maternal UN (LL) had reduced crown-rump and rear leg lengths at 18 wk PN compared to LC lambs. Ford *et al*.

(2007) reported that heavier lambs from early gestation maternal UN also had higher carcass fat content than C lambs at 280 d PN. Together these results could indicate that maternal UN may have adverse impacts on offspring organ functionality and overall body composition, but that these may not become evident until the offspring reaches adulthood (Bloomfield *et al*. 2003; Torrens *et al*. 2009; Lloyd *et al*. 2012).

Here we report that blood glucose concentrations, but not insulin (Chapter V) were greater in lambs from maternal UN (LC & LL) than from C at 8 wk PN. The results of elevated pre-weaning (8 wk PN) blood glucose in maternal UN offspring may correlate to the elevated insulin and blood glucose following a glucose tolerance test reported in early gestation maternal UN offspring at 63 d PN (9 wk PN; (Ford *et al*. 2007). Whereas blood glucose concentrations, but not insulin (Chapter V) were reduced in LC lambs at 14 wk PN compared to LL. Others have reported that 12 mo old offspring from late-gestation maternal UN exhibit glucose intolerance (Gardner *et al*. 2005).. Additionally, we found that the insulin to glucose ratio was increased over time more rapidly in the LC lambs, which indicates that insulin may be becoming less responsive to glucose with age (Green *et al*. 2011). Ford *et al*. (2007) also reported elevated glucose, but lower insulin in response to a glucose tolerance test of post-weaning (250 d PN) lambs from early gestation maternal UN. Together these results suggest that there may be a different regulation of glucose metabolism during the pre- and post- weaning periods as a result of maternal UN and that this may be differentially altered by the duration of maternal UN.

Blood glucose concentrations, were reduced as a result of undernutrition, which is another report that following maternal UN during early gestation, at 78 dGA, both maternal and fetal blood glucose concentrations were reduced (Vonnahme *et al*. 2003).

Maternal blood glucose concentrations in UN ewes were not different from C ewes during late gestation. It has been reported elsewhere that maternal plasma glucose concentrations are reduced during nutrient restriction (35 to 78 dGA) relative to C, but that following realimentation blood glucose increased to match C levels. In addition, in conjunction with the level of nutrient intake, glucose concentrations were generally greater in realimented UN ewes (LC) than LL following realimentation which began at 79 dGA. Further, the elevated blood glucose at 2 wk PP in UN ewes (LC & LL) could signify a carryover effect of maternal UN into the early post-partum period involving modified nutrient partitioning for adequate milk production.

Realizing the impact of inadequate maternal nutrition during gestation on lamb growth and development post-partum is pertinent to livestock production and to further understanding the origins of adult metabolic disorders in humans. It is further relevant to understanding the developmental mechanisms of metabolic diseases in adult humans.

Tables and Figures

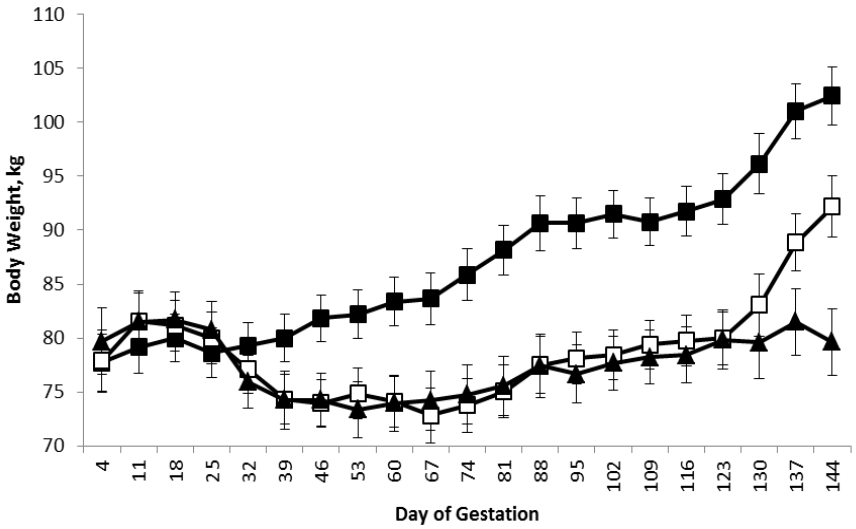

Figure 4. Mean maternal body weight by treatment throughout gestation. Control ewes were fed 100% of their NRC (2006) recommended requirements (■), LC were fed 50% of their requirements from day 28 through 78 of gestation followed by 100% through the remainder of gestation (□), and LL were fed 50% of their requirements from day 28 through the term (▲).(Trt*day of gestation, $P < 0.0001$).

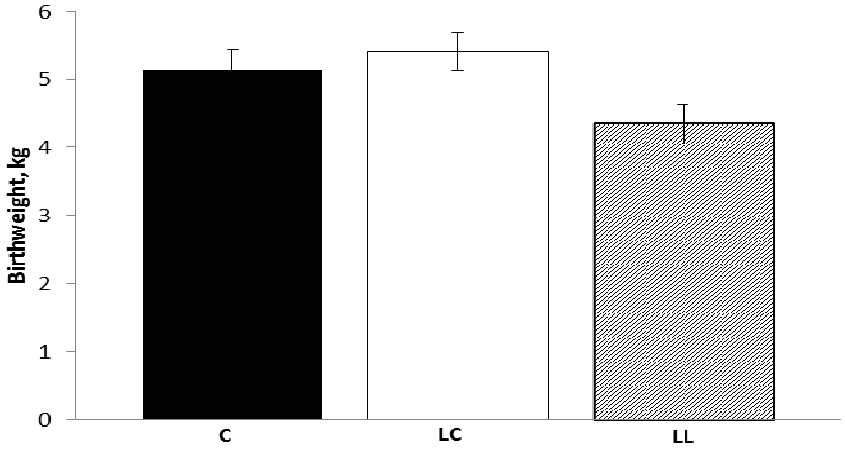

Figure 5. Mean lamb body weight by treatment at birth. Control (C) ewes were fed 100% of their NRC (2006) recommended requirements, LC were fed 50% of their requirements from day 28 through 78 of gestation followed by 100% through the remainder of gestation, and LL were fed 50% of their requirements from day 28 through term. (C vs. LC and LL: P = 0.46; LC vs. LL: P = 0.02).

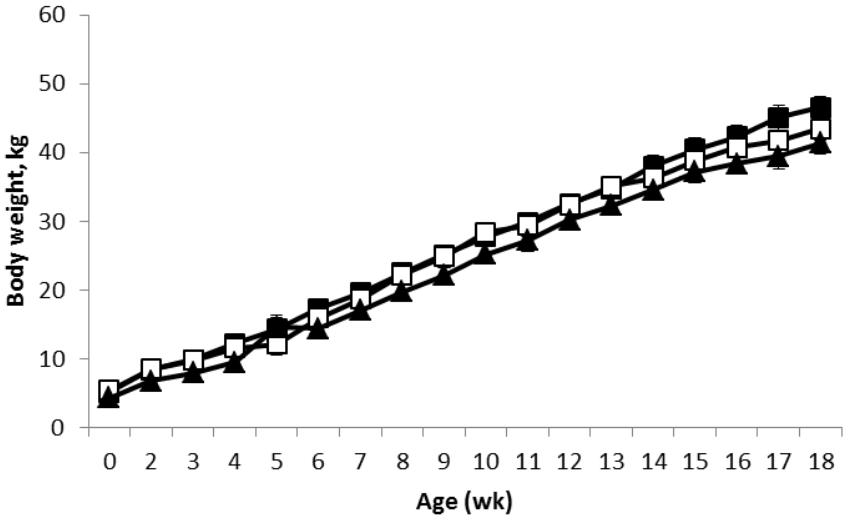

Figure 6. Mean weekly lamb body weights by treatment. Control ewes were fed 100% of their NRC (2006) recommended requirements (■), LC were fed 50% of their requirements from day 28 through 78 of gestation followed by 100% through the remainder of gestation (□), and LL were fed 50% of their requirements from day 28 through term (▲). (Trt*wk, $P > 0.10$).

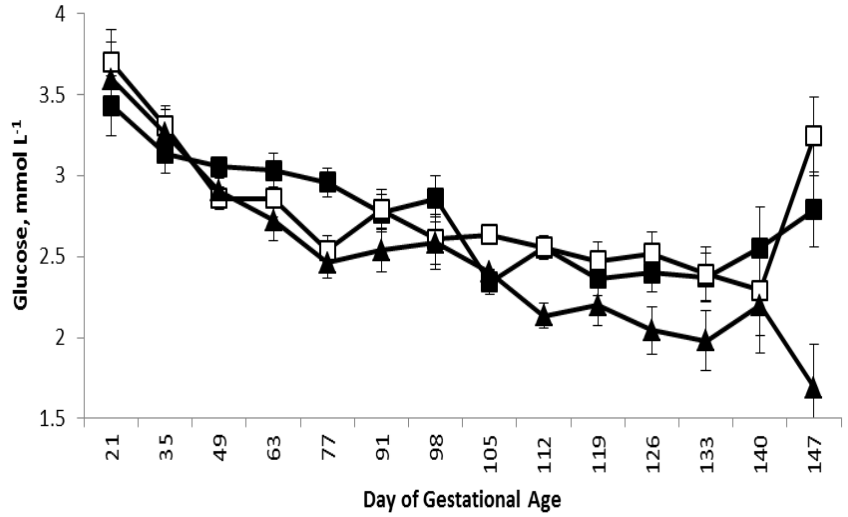

Figure 7. Mean maternal blood glucose concentrations by treatment throughout gestation. Control ewes were fed 100% of nutrient requirements (■), LC were fed 50% of nutrient requirements from day 28 through 78 of gestation followed by 100% for through the remainder of gestation (□), and LL were fed 50% of their nutrient requirements from day 28 through term (▲). (Trt*day of gestation, $P < 0.05$).

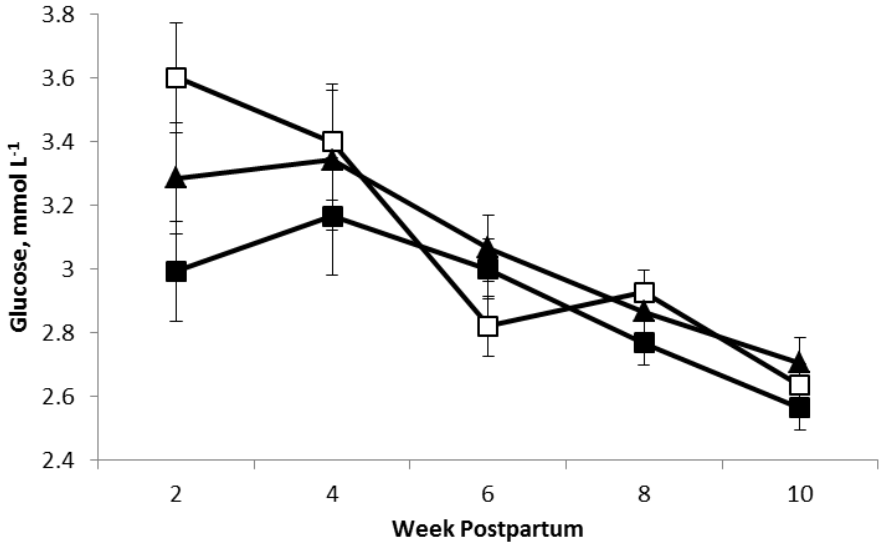

Figure 8. Mean maternal blood glucose concentrations by treatment from 2 wk to 10 wk postpartum. Control ewes were fed 100% of their NRC (2006) recommended requirements (■), LC were fed 50% of their requirements from day 28 through 78 of gestation followed by 100% through the remainder of gestation (□), and LL were fed 50% of their requirements from day 28 through the term (▲). (Trt*week, $P < 0.05$).

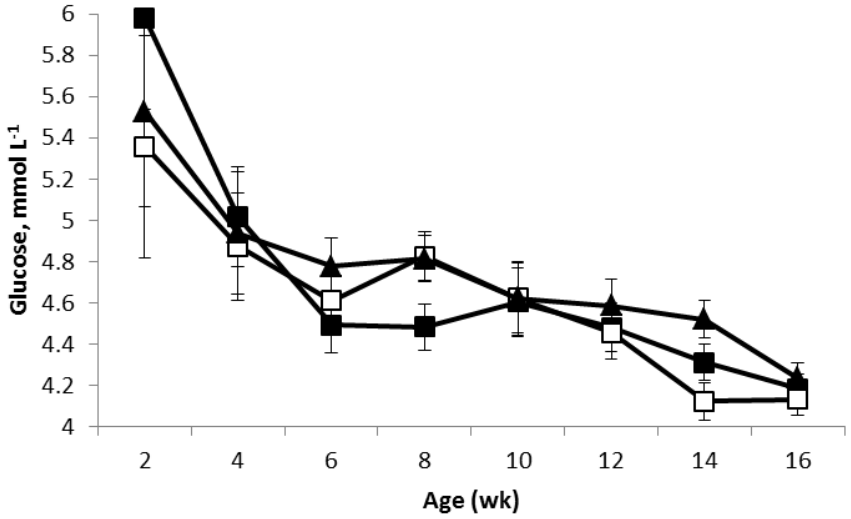

Figure 9. Mean weekly lamb blood glucose concentration by treatment. Control ewes were fed 100% of their NRC (2006) recommended requirements (■), LC were fed 50% of their requirements from day 28 through 78 of gestation followed by 100% through the remainder of gestation (□), and LL were fed 50% of their requirements from day 28 through term (▲). (Trt*wk, $P > 0.10$).

Table 7. Maternal average daily gain of ewes receiving a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through term (LL).

	Gestational intake			*P* values	
	C (n = 8)	LC (n = 10)	LL (n = 9)	C vs. LC, LL	LC vs. LL
Early gestation (kg d^{-1}; d 28 to 78)	0.11 ± 0.02	-0.13 ± 0.02	-0.12 ± 0.02	<0.0001	0.8434
Late gestation (kg d^{-1}; d 79 to 147)	0.27 ± 0.03	0.26 ± 0.03	0.07 ± 0.03	0.0043	<0.0001

Data are the mean ± s.e.m.

Table 8. Fetal body measurements at birth in relation to maternal diet; ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through term (LL).

	Gestational intake			*P* values	
	C (n = 8)	LC (n = 10)	LL (n = 9)	C vs. LC, LL	LC vs. LL
Birthweight, kg	5.14 ± 0.29	5.41 ± 0.28	4.34 ± 0.29	0.4639	0.0168
CRL, cm^1	16.93 ± 0.60	17.90 ± 0.54	16.38 ± 0.68	0.7839	0.0933
HC, cm	26.02 ± 0.69	26.48 ± 0.74	25.89 ± 0.77	0.8489	0.5910
TG, cm^2	15.43 ± 0.46	15.38 ± 0.41	14.61 ± 0.52	0.4442	0.2584
AC, cm	14.99 ± 0.54	15.35 ± 0.49	14.23 ± 0.61	0.7727	0.1636
Front leg, cm	37.77 ± 1.05	38.36 ± 1.07	35.84 ± 1.17	0.6172	0.1280
Rear leg, cm	44.26 ± 1.24	43.88 ± 1.26	43.11 ± 1.38	0.6285	0.6822

Data are the mean ± s.e.m.
CRL, crown rump length; HC, head circumference; TG, thoracic girth; AC, abdominal circumference.

Table 9. Lamb average daily gain relation to maternal diet; ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through term (LL).

	Gestational intake			*P* values	
	C (n = 8)	LC (n = 10)	LL (n = 9)	C vs. LC, LL	LC vs. LL
Pre-weaning (kg d^{-1}; birth to wk 10)	0.32 ± 0.02	0.33 ± 0.02	0.30 ± 0.02	0.8185	0.3628
Post-weaning (kg d^{-1}; wk 10 to 18)	0.32 ± 0.02	0.28 ± 0.02	0.29 ± 0.02	0.3248	0.7293
Overall (kg d^{-1})	0.32 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.3240	0.5014

Data are the mean ± s.e.m.

Table 10. Lamb body measurements at 4 mo in relation to maternal diet; ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through term (LL).

	Gestational intake			*P* values	
	C (n = 8)	LC (n = 10)	LL (n = 9)	C vs. LC, LL	LC vs. LL
Weight, kg	46.05 ± 1.68	44.56 ± 1.80	41.67 ± 1.71	0.1793	0.2590
CRL, cm	108.61 ± 2.61	114.46 ± 2.82	107.30 ± 2.72	0.4978	0.0882
HC, cm	47.00 ± 1.14	48.57 ± 1.23	46.86 ± 1.20	0.6219	0.3306
TG, cm	87.12 ± 1.63	87.99 ± 1.76	84.84 ± 1.67	0.7325	0.2078
AC, cm	101.88 ± 1.77	101.82 ± 1.89	97.99 ± 1.81	0.3852	0.1582
HG, cm	95.94 ± 1.92	94.50 ± 2.05	93.48 ± 2.33	0.4403	0.7466
Front leg, cm	53.13 ± 1.19	53.44 ± 1.28	54.80 ± 1.21	0.5171	0.4487
Rear leg, cm	67.20 ± 1.22	63.92 ± 1.30	61.54 ± 1.26	0.0186	0.0287

Data are the mean ± s.e.m.

CRL, crown rump length; HC, head circumference; TG, thoracic girth; AC, abdominal circumference; HG, hip girth.

Table 11. Lamb organ measurements at 4 mo in relation to maternal diet; ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through term (LL).

	Gestational intake			*P* values	
	C (n = 8)	LC (n = 10)	LL (n = 9)	C vs. LC, LL	LC vs. LL
HCW, kg	21.40 ± 0.91	20.95 ± 0.98	18.75 ± 0.92	0.1876	0.1183
Head, g	2477.98 ± 103	2378.36 ± 111	2186.68 ± 105	0.1467	0.2236
Brain, g	103.94 ± 2.43	107.98 ± 2.61	99.74 ± 2.47	0.9788	0.0331
Liver, g	872.34 ± 29.53	897.64 ± 31.41	843.12 ± 30.61	0.9588	0.2298
Kidney, g	118.94 ± 4.69	129.45 ± 5.11	119.71 ± 5.01	0.3548	0.1935
Adrenal, g	1.73 ±0.21	2.16 ± 0.22	2.07 ± 0.21	0.1598	0.7712
Gut, g	9908.38 ± 384	9611.20 ± 412	9379.45 ± 465	0.4214	0.7113
Pancreas, g	45.55 ± 4.83	46.84 ± 5.00	41.82 ± 4.98	0.8409	0.4850
Spleen, g	84.03 ± 4.45	84.69 ± 4.91	79.16 ± 4.52	0.7114	0.4189
Lung, g	491.86 ± 27.18	507.66 ± 29.13	504.80 ± 29.74	0.6815	0.9459
Heart, g	198.42 ± 7.39	197.09 ± 7.93	180.19 ± 7.51	0.3038	0.1376
Left Atrium, g	12.21 ±0.61	12.71 ± 0.66	11.43 ± 0.70	0.8516	0.1981
Right Atrium, g	12.94 ± 0.69	12.11 ± 0.75	11.66 ± 0.79	0.2481	0.6855
Right Ventricle, g	47.13 ± 2.12	46.37 ± 2.27	42.72 ± 2.14	0.3409	0.2558
Left Ventricle, g	101.37 ± 4.48	100.95 ± 4.80	95.58 ± 4.54	0.5850	0.4262
Septum, g	24.45 ± 1.13	25.08 ± 1.21	22.86 ± 1.15	0.7372	0.2003

Data are the mean ± s.e.m.
HCW, hot carcass weight.

Table 12. Milk production at 6 wk PP of ewes receiving a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through term(LL).

	Gestational intake			*P* value	
	C (n = 8)	LC (n = 10)	LL (n = 9)	C vs. LC, LL	LC vs. LL
Right Volume, mL	111.14 ± 19.20	94.83 ± 20.73	81.60 ± 22.71	0.3660	0.6731
Left Volume, mL	167.57 ± 34.66	152.83 ± 37.43	107.50 ± 37.34	0.4037	0.4045
Total Volume, mL	278.71 ± 40.83	247.67 ± 44.11	175.50 ± 44.11	0.2098	0.2643
Protein, %	24.25 ± 2.00	25.60 ± 2.16	22.48 ± 2.16	0.9354	0.3219
Crude Fat, %	30.02 ± 2.52	30.22 ± 2.72	26.70 ± 2.72	0.6274	0.3733
Lactose, g/L	42.01 ± 1.88	42.18 ± 2.03	45.56 ± 2.03	0.4405	0.2564

Data are the mean ± s.e.m.

Chapter V. Blood insulin, insulin-like growth factor (IGF)-I, ghrelin, and growth hormone (GH) concentrations in response to duration of maternal undernutrition in twin sheep pregnancies.

Summary

Maternal undernutrition (UN) during gestation perturbs the developmental environment of a fetus and can predispose the offspring to metabolic abnormalities. We investigated the impact of maternal UN on insulin (INS), IGF-I, GH, and ghrelin (GRL) concentrations in uterine and umbilical blood at 135 d of gestational age (dGA) in experiment 1 (E1) and in maternal and lamb blood in experiment 2 (E2). Ewes carrying twins were randomly assigned to treatment and placed in individual pens at 21 dGA for acclimation. Ewes were either fed 100% (C; E1, n = 7; E2, n = 8), 50% from 28 to 78 dGA and readjusted to 100% at 79 dGA (LC; E1, n = 5; E2, n = 10), or 50% of nutrient requirements from 28 to 135 dGA (LL; E1, n = 7; E2, n = 9). Uterine and umbilical blood was collected during cesarean section before delivery of the fetuses in E1. Maternal blood was collected during gestation and postpartum (PP) until weaning, lamb blood was collected postnatal (PN) until slaughter in E2. Uterine artery IGF-I, GH and INS were not impacted by treatment. Umbilical vein IGF-I was greater ($P = 0.01$) in LC than LL. Umbilical artery IGF-I and INS were also greater ($P \leq 0.05$) in LC than in LL. Insulin was greater ($P \leq 0.05$) in C vs. LC & LL ewes at 63, 77, 98, 105, 112, 119, 126, 133, and 140 dGA, while at 112 and 147 maternal INS was greater ($P < 0.05$) in LC vs. LL. Maternal IGF-I was greater ($P < 0.05$) at 112, 119, 126, 133, 140, and 147 dGA in C vs.

LC & LL. Maternal IGF-I was greater ($P = 0.01$) at 147 dGA in LC vs. LL, with a similar trend ($P = 0.08$) at 2 wk PP. Maternal GH was greater ($P < 0.05$) at 49 and 140 dGA in LC & LL vs. C, with a similar trend ($P < 0.10$) at 119 dGA. At 35 dGA GH was greater ($P = 0.03$) in LC vs. LL, whereas at 49 ($P = 0.01$) and 112 dGA ($P = 0.08$) GH was greater in LL vs. LL. Ghrelin was greater ($P = 0.02$) in LC & LL vs. C ewes at 140 dGA, with a similar trend ($P < 0.10$) at 4 wk PP. Lamb INS was greater ($P = 0.04$) in C vs. LC & LL and greater ($P = 0.02$) in LC than LL at 2 wk PN. Lamb IGF-I tended to be greater ($P = 0.08$) in C vs. LC & LL at 4 wk PN. Lamb GRL was greater ($P = 0.06$) in LC & LL vs. C at 2 wk PN. Umbilical IGF-I and insulin in LC indicate a shift in IGF-I and accelerated fetal growth as a result of realimentation following maternal UN. Prolonged and early- to mid-gestation maternal UN results in diminished INS and elevated GRL in early post-natal life. Further, elevated maternal GRL in the postpartum period may indicate a carryover impact of UN during gestation.

Introduction

Naturally occurring periods of maternal undernutrition are regular consequences of forage fluctuation in the High Plains and Intermountain West of the United States (Anderson 1993; Thomas and Kott 1995; Del Curto *et al*. 2000). It is well established that maternal undernutrition during gestation can impact fetal growth and development (Reynolds and Redmer 1995; Ford *et al*. 2007). Low birth weights are also associated with increased incidences of adult metabolic diseases (Barker *et al*. 1993; Barker 1995; Ravelli *et al*. 1999). The process whereby a growing fetus adapts in order to compensate for a sub-optimal developmental environment within the uterus is referred to as fetal

programming (Lucas 1991). The "thrifty phenotype" hypothesis indicates that altered glucose utilization, insulin function and secretion may result from undernutrition during development (Hales *et al*. 1991). These compensatory measures, which may be significant for fetal survival and development, might be detrimental to the fetus and contribute to the development of adult metabolic diseases (Stein *et al*. 1996; Barker 1998).

The availability of nutrients to a gestating ewe and thus availability of nutrients to the fetus greatly influence the growth of a fetus (Owens *et al*. 1991). Bassett and Gluckman (1986) determined GH concentrations are substantial in fetal circulation although GH receptor (GHR) abundance is low in fetal tissues. Hepatic GHR mRNA is detectable by mid-gestation there is minimal binding of GH to the receptor (Klempt *et al*. 1993; Pratt and Anthony 1995). Binding of GH to the functional receptor increases in late gestation and postnatal life (Gluckman *et al*. 1990; Klempt *et al*. 1993). Further, as GHR expression increases in late gestation, IGF-I expression also increases in the fetal liver (Li *et al*. 1996). The postnatal impact of GH is carried out largely through stimulation of hepatic IGF-I secretion. Additionally, the abundance of (Khandwala *et al*. 2000) the IGF-I receptor is highest during fetal and early postnatal life (Hyatt *et al*. 2007).

Nutritional status is the primary regulator of the somatotropic axis; in young steers undernourishment causes peripheral GH insensitivity and decreased IGF-I in circulation (Bauer *et al*. 1995). It has also been shown that fetal sheep exposed to maternal undernutrition become hypersomatotrophic (de Zegher *et al*. 1990). Since energy status can effectively influence hepatic GHR levels maternal undernutrition might alter the

GH/IGF axis in the developing fetus and resulting offspring (Weller *et al*. 1994; Min *et al*. 1996; O'Sullivan *et al*. 2002).

The post-natal impact of short and long-term maternal undernutrition in the case of twin pregnancies is of interest since twinning is commonplace in sheep production. Therefore, the objective of this experiment was to determine the impact of undernutrition from 28 to 78 dGA and from 28 to parturition in the ewe on the GH/IGF axis and insulin in the fetus and young growing lamb, as well as on the gestating and postpartum ewe. We hypothesized that early to mid-gestation maternal undernutrition would result in increased fetal IGF-I concentration, whereas prolonged maternal undernutrition would reduce insulin and IGF-I concentrations. We further hypothesized that short and long-term maternal undernutrition would induce different GH and IGF-I concentrations depending on length of undernourishment and in relation to C fetuses and postnatal lambs.

Materials and Methods

Umbilical and Uterine Blood Hormone Study

Multiparous, western whiteface ewes carrying twin pregnancies were used for the study (n = 19; 75.63 ± 7.11 kg). Procedures for this study complied with guidelines of the United States Department of Agriculture. Protocols for care and use of the animals were approved by the Colorado State University Institutional Animal Care and Use Committee. Prior to being moved to individual pens, ewes were group housed and offered feed ad libitum. The diet consisted of pelleted beet pulp (77.8% total digestible nutrients [TDN], 90.1% dry matter [DM], and 9.9% crude protein) and a vitamin-mineral

mixture to meet additional requirements. Ewes were treated with Controlled intra-vaginal drug release devices (EAZI-BREED™ CIDR® Sheep and Goat Device, Pfizer, New York) for 7 days followed by two 5 mg doses of $PGF_{2\alpha}$ (Lutalyse, Pfizer, New York) 4 hours apart. A ram was introduced 48 hrs after the first dose of $PGF_{2\alpha}$, breeding was supervised and a ewe having been visually conformed as bred two or more times qualified as having been bred. At 21 d gestational age (dGA), ewes were placed in individual pens and fed 100% of nutrient requirements for early pregnant ewes (NRC recommended requirements) on an individual weight basis. Beginning on 28 dGA ewes were randomly assigned to C (n = 7; 100% of nutrient requirements, NR) or LL (n = 7) and LC (n = 5) both treatments received 50% of NR. For all treatments, beginning on 79 dGA, NRC recommended requirements for late pregnant ewes were used to calculate feed requirements. Beginning on 79 dGA and continuing through 133 dGA, LC ewes were fed 100% NR. On 79 dGA LL ewes were maintained on 50% of NR, however to avoid the incidence of gestational ketosis a 5% increase over 5 day increments began at 110 dGA. Ewes were weighed weekly and the ration was adjusted according to changes in body weight.

Umbilical and uterine blood was collected at 135 ± 1 dGA. Ewes were anesthetized by i.v. administration of sodium pentobarbital (27 mg/kg), the ewes were intubated and maintained on 1-3% isoflurane, and a midventral laparotomy was performed to remove products of conception for tissue harvest. Umbilical artery and vein blood was collected from each fetus; a uterine artery blood sample was collected from a subsample of ewes (n = 11) prior to delivery of the fetus. Blood was collected into tubes containing 30 mg sodium fluoride and centrifuged for 10 minutes at 1,500 × *g* at 4°C to harvest plasma.

Plasma was removed from the pellet and stored at -20°C for later analysis. Fetal body and organ weights and blood metabolite parameters were also measured and have been presented in Chapter III.

Maternal and Lamb Jugular Vein Hormone Study

Multiparous, western whiteface ewes carrying twin pregnancies were used for the study (n = 27; 78.43 ± 2.86 kg). Ewes were randomly distributed into dietary treatments identical to that in the umbilical and uterine blood hormone study (C, n = 8; LC, n = 10; and LL, n = 9), except that the diets were maintained until natural parturition occurred. An increase in feed in the LL treatment to avoid ketosis was not implemented in this study, because urine ketone testing did not indicate any incidinces in ketosis. Ewes were moved into an enclosed, heated barn at 143 dGA. Ewes in the LL treatment were housed in individual lambing pens until they had been adjusted to ad libitum alfalfa hay following lambing. All ewes were gradually adjusted to ad libitum alfalfa hay intake following parturition. Ewes were weighed weekly and the ration was adjusted according to changes in body weight.

Lambs were allowed to suckle their dams freely or supplemented with a commercial milk replacer if no milk was produced by their respective dam (Merrick's, Inc., Middleton, WI). Beginning at 2 weeks of age, lambs had free choice access lamb creep pellets and alfalfa hay (All*American Show Lamb Creep, Ranch-Way Feeds, Fort Collins, CO). Lambs were weaned from their mothers at 10 weeks of age and gradually adjusted to a complete feed that was offered at 150% of the recommended feeding rate to allow ad libitum intake (All*American Show Lamb Complete, Ranch-Way Feeds, Fort

Collins, CO). Lambs were weighed weekly and the ration was adjusted according to changes in body weight.

Maternal jugular vein blood was collected during gestation at 21, 35, 49, 63, 77, 91, 98, 105, 112, 119, 126, 133, 140 and 147 dGA. Postpartum maternal blood samples were collected from ewes at 2, 4, 6, 8 and 10 weeks post-partum (PP). Postnatal (PN) blood samples were collected from lambs at 2, 4, 6, 8, 10, 12, 14, and 16 weeks.

Blood Hormone Analysis

The concentration of plasma insulin was determined by rapid solid-phase RIA at New Mexico State University (Reimers *et al*. 1982). The inter-assay CV for umbilical and uterine artery blood samples was 5.6% and 8.5%, respectively. The inter-assay CV and intra-assay CV for the maternal and lamb blood samples were 8.4 % and 6.8 %, respectively.

The concentration of plasma IGF-I in umbilical and uterine blood was determined by RIA at New Mexico State University (Berrie *et al*. 1995). The inter-assay CV for uterine and umbilical artery blood samples was 12.3%. The concentration of plasma IGF-I in maternal and lamb blood was determined by RIA at the University of Missouri the inter-assay and intra-assay CV for maternal and lamb blood samples were both less than 10 %(Morrison *et al*. 2002).

The concentration of GH in serum was determined by RIA at the University of Missouri (Powell and Keisler 1995; Lalman *et al*. 2000). Inter- and intra-assay CV for umbilical and uterine artery blood samples were 5.3% and 9.8%, respectively. The inter- and intra-assay CV for maternal and lamb blood samples were both less than 10 %.

Plasma ghrelin concentrations were measured by RIA specific for the biologically active form of ghrelin (Catalog # GHRA-88HK, Linco Research, St. Charles, MO). Serial dilutions (1:1, 1:2, 1:4, 1:8, and 1:16) of pooled ovine plasma samples were made in duplicate, those produced curves that were parallel to the standard curve (slopes of -15.55 and -12.71 for the standard curve and serially diluted ovine samples, respectively). Recovery was tested by adding the reconstituted standard ghrelin provided in the kit to the pooled ovine plasma sample with known exogenous ghrelin concentration. Recovery of exogenous ghrelin was 106%. The inter-assay CV averaged 4.13% (over 2 assays) and the intra-assay CV was 5.72%.

Statistical Analysis

All data are presented as LSmeans ± S.E.M. Means were separated at a significant level of $P < 0.05$ and considered as a trend at a level of $P < 0.10$. Each ewe was treated as one experimental unit and twin fetuses within the ewe were treated as repeated measurements. We hypothesized that restriction followed by realimentation would induce faster fetal growth, therefore, fetal and lamb blood data were analyzed by preplanned orthogonal contrasts: C vs. LC & LL and LC vs. LL. Any interaction by fetal sex observed is reported. Repeated measurements of the ewe were analyzed using the PROC MIXED model of SAS (SAS Inst. Carry, NC). Ewes or lambs that did not survive through the entire project were not included in analysis.

Results

Umbilical Blood

The concentrations of insulin, IGF-I, GH, and ghrelin are given in Table 12. Umbilical arterial insulin was higher (P = 0.0360) in LC than LL fetuses. Umbilical venous (P = 0.0068) and arterial (P = 0.0518) IGF-I concentrations were higher in LC than LL fetuses. Umbilical arterial GH and ghrelin concentrations were not impacted by treatment.

Uterine Artery Hormone Concentrations

The concentrations of insulin, IGF-I, GH, and ghrelin in uterine artery blood were not impacted by treatment (Table 13).

Maternal Hormone Concentrations

The concentrations of insulin, IGF-I, and GH in maternal blood during gestation and postpartum until weaning are depicted in Figure 10. Blood insulin concentrations were higher in C vs. LC & LL ewes at 63 (P = 0.0060), 77 (P = 0.0107), 98 (P = 0.0239), 105 (P = 0.0002), 112 (P = 0.0173), 119 (P = 0.0196), 126 (P = 0.0053), 133 (P = 0.0362), and 140 (P = 0.0470) dGA. Blood insulin concentrations were higher in LC vs. LL ewes at 112 (P = 0.0435) and 147 (P = 0.0301) dGA. Postpartum blood insulin concentrations were not impacted by treatment. Blood IGF-I concentrations were lower (P = 0.0222) in C vs. LC & LL ewes at 35 dGA. Blood IGF-I concentrations tended (P = 0.0927) to be lower in LC vs. LL ewes at 91 dGA, but were higher (P = 0.0116) in LC vs. LL at 147 dGA. Blood IGF-I concentrations were higher in C vs. LC & LL ewes at 98 (P = 0.1043),

112 (P = 0.0047), 119 (P = 0.0179), 126 (P = 0.0036), 133 (P = 0.0060), 140 (P = 0.0253), and 147 (P = 0.0008) dGA. Postpartum blood IGF-I concentrations tended to be greater (P = 0.0766) in LC vs. LL ewes at 2 wk PP. Blood GH concentrations were greater (P = 0.0335) in LC vs. LL ewes at 35 dGA, whereas the LL GH was greater 49 dGA (P = 0.0094) and that trend (P = 0.0753) was also observed at 112 dGA. At 49 (P = 0.0368) and 140 dGA (P = 0.0347) GH was greater in LC & LL vs. C ewes, a similar trend (P = 0.0633) was observed at 119 dGA. Postpartum GH concentrations were not impacted by treatment. The concentration of ghrelin in maternal blood at 21 and 140 dGA, and 4 wk PP is given in Figure 11. Blood ghrelin concentrations were greater in LC & LL vs. C ewes at 140 dGA (P = 0.0184) and 4 wk PP (P = 0.0014).

Lamb Hormone Concentrations

The concentrations of insulin, IGF-I, and GH in lamb circulation are depicted in Figure 12. Blood insulin concentrations were higher (P = 0.0407) in C vs. LC & LL lambs at 2 wk PN and higher (P = 0.0160) in LC vs. LL lambs at 2 wk PN. Blood insulin concentrations were higher in male lambs at 2 (P = 0.0160) and 14 (P = 0.0503) wk PN, the same trend was observed at 12 (P = 0.0584), and 16 (P = 0.0557) wk PN. Blood IGF-I concentrations tended to be higher (P = 0.0750) in C vs. LC & LL lambs at 4 wk PN. Blood IGF-I concentrations tended to be higher (P = 0.0749) in LC vs. LL at 8 wk PN. Blood IGF-I concentrations were greater in male lambs at 8 (P = 0.0178), 10 (P = 0.0050), 12 (P = 0.0002), 14 (P = 0.0003), and 16 (P = 0.0232) wk PN. Blood GH concentrations were higher (P = 0.0009) in male lambs at 8 wk PN. The concentration of

ghrelin in lamb blood at 2 and 16 wk PN is given in Figure 13. Blood ghrelin concentrations tended to be greater ($P = 0.0581$) in LC & LL vs. C lambs at 2 wk PN.

Discussion

To our knowledge this is the first study to directly compare the effect of two different nutrient restriction regimens during gestation in sheep carrying twin fetuses on the level of insulin, IGF-I, GH, ghrelin in umbilical and uterine blood, maternal blood during gestation and postpartum through weaning, and lamb blood postnatally. In the present study at 135 dGA, maternal undernutrition did not result in altered uterine artery plasma insulin concentrations while, maternal plasma insulin concentrations were reduced at 90 and 130 dGA in singleton-bearing ewes maintained on low dietary intake throughout gestation (Luther *et al*. 2007). Here we also report that nutrient restricted-realimented ewes (LC) had elevated plasma insulin at 147 dGA, and that glucose concentrations were elevated in LC ewes (Chapter IV). Osgerby *et al*. (2002) also reported that underfeeding, where ewes carrying singletons were receiving 70% NR from 26 through 135 dGA, resulted in elevated plasma insulin in maternal circulation. The concentrations of insulin, as well as the responsiveness of glucose to insulin decrease with gestational age in a normal sheep pregnancy (Blom *et al*. 1976; Hove and Blom 1976; Regnault *et al*. 2004). However, the elevations in glucose and insulin at 147 dGA, which are similar to those demonstrated in overnourished pregnant ewes, might suggest that nutrient restricted-realimented ewes are exhibiting a greater level of insulin resistance near term than the continuously undernourished ewes (Peel *et al*. 2012).

Reduced plasma insulin, glucose, and IGF-I near-term have been reported in singleton fetuses from long term undernourished ewes (embryo transfer through 130 dGA and 26 through 135 dGA), these parameters may have the potential to impair fetal growth (Osgerby *et al*. 2002; Luther *et al*. 2007). Luther *et al*. (2007) also reported that singleton fetuses from nutrient-restricted realimented ewes had higher plasma insulin concentrations than those from long-term nutrient-restriction ewes. We report similar findings of elevated insulin concentration in LC umbilical arterial blood at 135 dGA and lamb blood 2 wk PP compared to LL, while glucose concentrations were elevated only at 135 dGA in umbilical vein (Chapter III). Increased levels of insulin at 2 wk PP, without elevated glucose concentrations, as well as increasing insulin to glucose ratio over time (not reported) may indicate that these lambs are developing insulin resistance. Ford *et al*. (2007) reported that lambs from nutrient-restricted ewes had altered insulin response to glucose, at 63 d PN the response was hyperinsulinemic, but at 250 d PN the response was hypoinsulinemic. Their results indicated that nutrient restriction during early gestation perturbs normal pancreatic development and can lead to glucose intolerance (Ford *et al*. 2007). Together with our results it is clear that maternal undernutrition during early gestation perturbs insulin function and secretion in response to glucose and that this paradigm may evolve with age.

Short term maternal undernutrition in late gestation increases GH concentrations in both maternal and fetal circulation in singleton pregnancies (Koritnik *et al*. 1981; Bauer *et al*. 1995). We also report that maternal undernutrition, regardless of duration, increases maternal blood GH concentrations at 49, 119, and 140 dGA. In the first study maternal undernutrition did not impact GH concentration in either uterine or umbilical blood at

135 dGA. Here we present similar results to those indicating that late gestation nutrient restriction results in increased GH concentrations in maternal circulation, but not in uterine, umbilical or postpartum offspring circulation. Insulin-like growth factor hormones regulate pre- and postnatal growth and IGF-I is positively correlated with lamb body and organ weights at term (Owens *et al*. 1991; Cohick and Clemmons 1993; Heasman *et al*. 1998). Further, IGF-I is critical for fetal development, but its secretion is susceptible to nutritional status in that fetal and maternal plasma IGF-I concentrations decrease in response to maternal undernutrition (Bassett *et al*. 1990b; Powell-Braxton *et al*. 1993; Bauer *et al*. 1995; Gicquel and Le Bouc 2006). In the present study IGF-I concentrations were lower in nutrient restricted (LC & LL) ewes compared to C from 112 dGA through term. Undernutrition thus seems to disrupt the stimulating effect of GH on IGF-I secretion, since GH is unchanged, while IGF-I seems to be responsive to nutritional status.

Ghrelin stimulates the secretion of GH and plasma concentrations of ghrelin generally peak prior to feeding (Kojima *et al*. 1999; Gualillo *et al*. 2002). Ghrelin is primarily secreted by oxyntic cells, which line the stomach, however its receptors are found in a variety of tissues. Ghrelin can stimulate gastric acid secretion and gut motility in rats, as well as stimulate feed intake independent of its relationship with GH (Kojima *et al*. 1999). In the present study we did not find differences in umbilical ghrelin concentrations at 135 dGA. However, at 140 dGA and at 4 wk PP we report that ghrelin concentrations were higher in nutrient restricted ewes (LC & LL) compared to C ewes. Despite realimentation to 100% NR, both LC and LL ewes maintained elevated ghrelin concentrations. Additionally, we report that plasma ghrelin concentrations tend to be

elevated in lambs from nutrient restricted ewes (LC & LL) compared to control lambs at 2 weeks PN (Figure 13). Although the terms small for gestational age and IUGR are not synonymous, sometimes IURG fetuses may be born as small for gestational age and it has been reported that umbilical blood ghrelin concentrations are elevated in small for gestational age (Farquhar *et al*. 2003). Further, strong evidence exists to support the theory that ghrelin is involved in energy metabolism, specifically in the pregnant ewe where treatment with ghrelin increased dry matter intake and lower non-esterified fatty acid and insulin concentrations (Melendez *et al*. 2006). It is possible that maternal undernutrition permanently alters the GH/IGF system, including the growth hormone secretagogue, ghrelin, in the resulting offspring (Hayashida *et al*. 2001). In twin bearing sheep, further research is needed to investigate the potential impacts of maternal undernutrition on energy metabolism and growth performance of mature, replacement animals as it relates to livestock production.

Tables and Figures

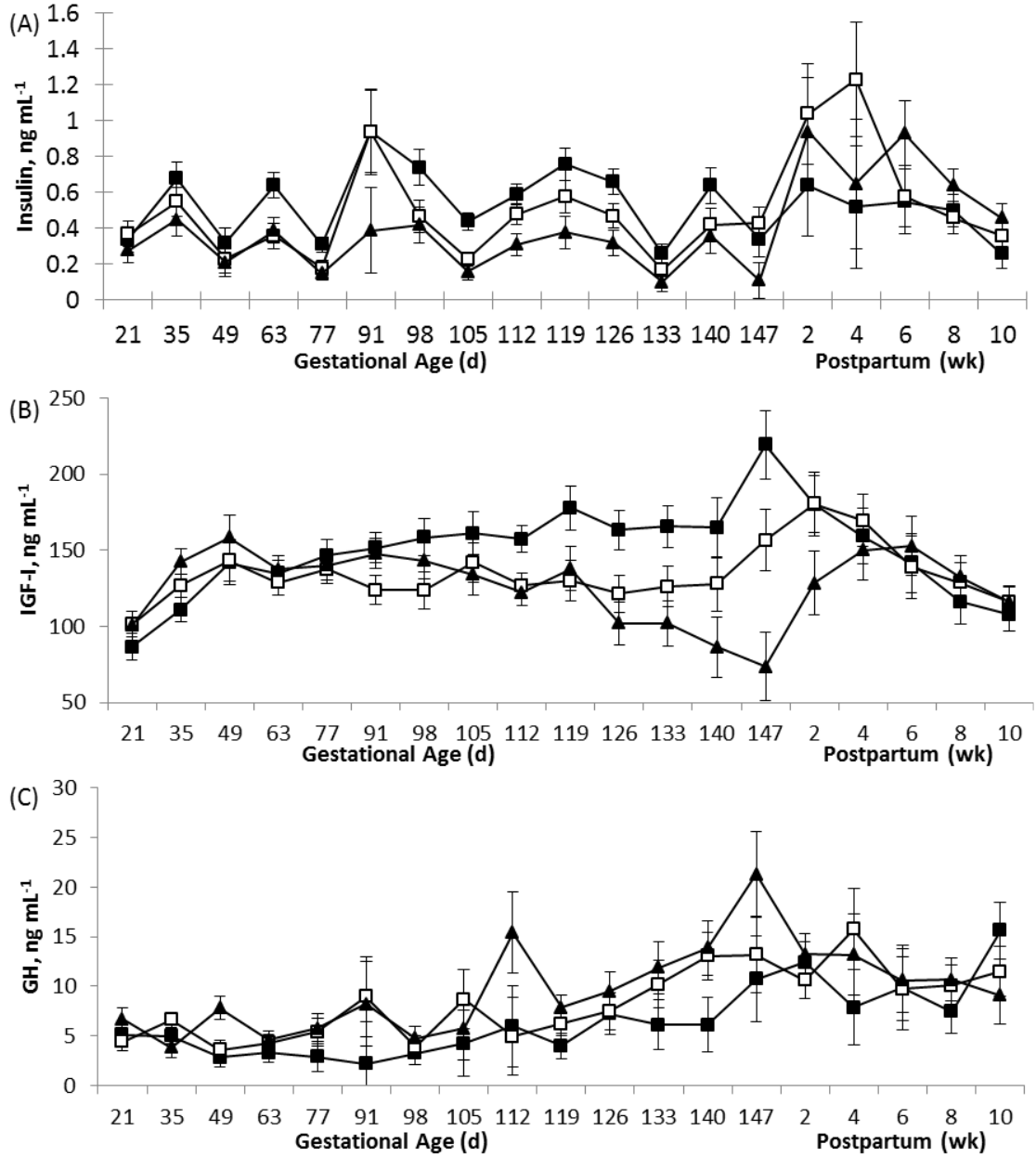

Figure 10. Mean maternal blood concentrations of (A) insulin, (B) IGF-I, and (C) GH by treatment throughout and following gestation. Ewes either received a control diet (■; 100% of their NRC 2006 recommended requirements), 50% of their requirements from 28 through 78 dGA of gestation followed by 100% through term (□), or 50% of their requirements from 28. dGA through term (▲). (A: Trt*gestational age $P > 0.10$, Trt*postpartum wk $P > 0.10$; B:

Trt*gestational age $P < 0.05$, Trt*postpartum wk $P > 0.10$; C: Trt*gestational age $P > 0.10$, Trt*postpartum wk $P > 0.10$).

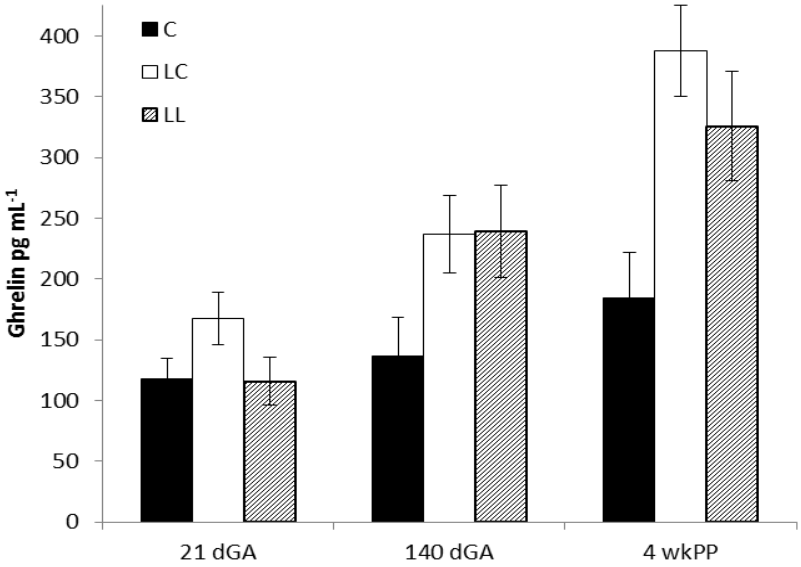

Figure 11. Concentration of ghrelin in maternal blood at 21 and 140 days of gestational age (dGA) and 4 wk postpartum (wk PP). Ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from 28 through 78 dGA of gestation followed by 100% through term (LC), or 50% of their requirements from 28 dGA through term (LL). (140 dGA, C vs. LC and LL: $P = 0.02$; 4 wk PP, C vs. LC and LL: $P < 0.01$).

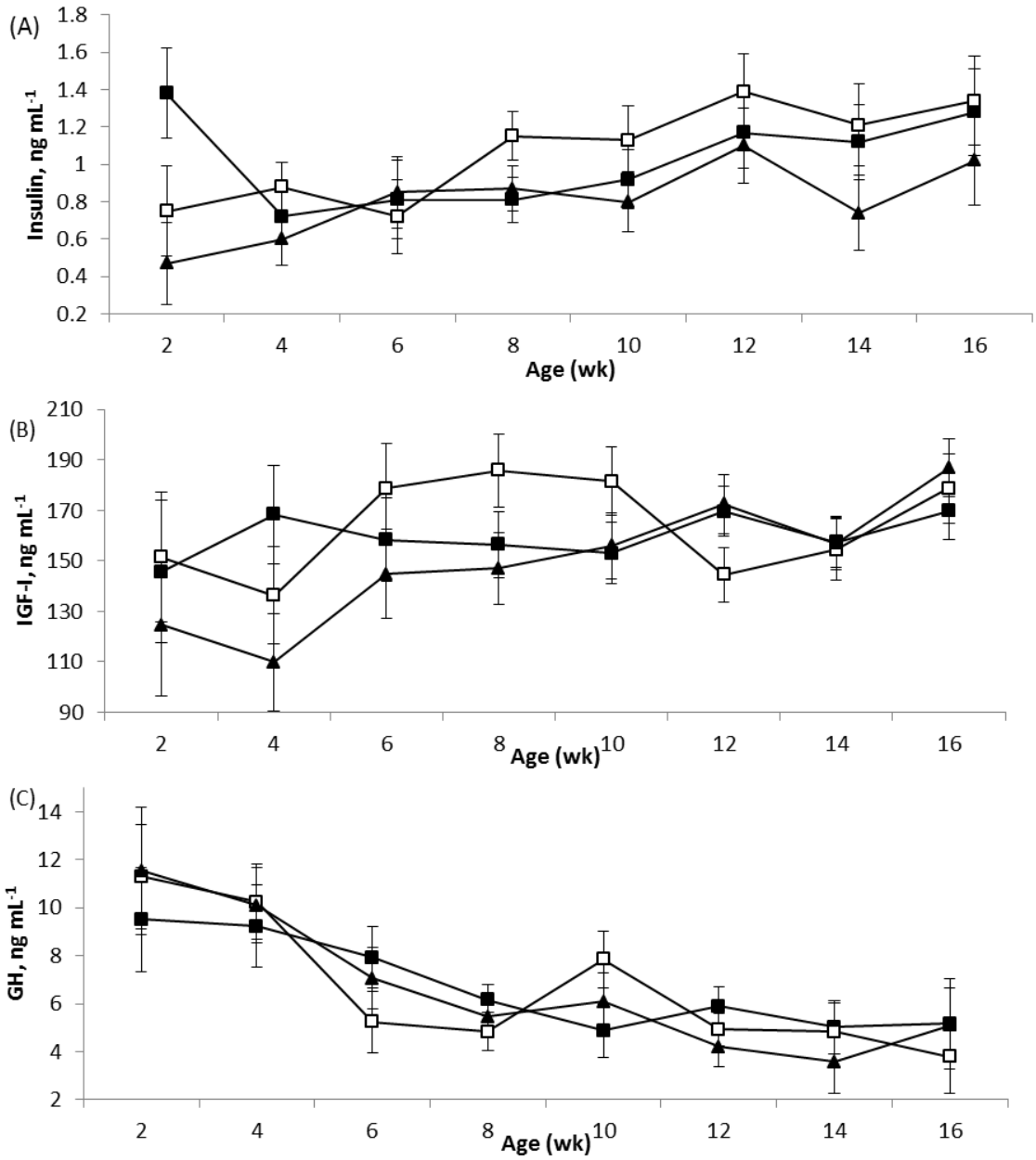

Figure 12. Concentration of (A) insulin, (B) IGF-I, and (C) GH in lamb circulation from wk 2 through 16 of age. Ewes either received a control diet (■;100% of their NRC 2006 recommended requirements), 50% of their requirements from 28 through 78 dGA of gestation followed by 100% through term (□), or 50% of their requirements from 28 dGA through term (▲).(A: Trt*wk, $P > 0.10$; B: Trt*wk, $P > 0.10$; C, Trt*wk, $P > 0.10$).

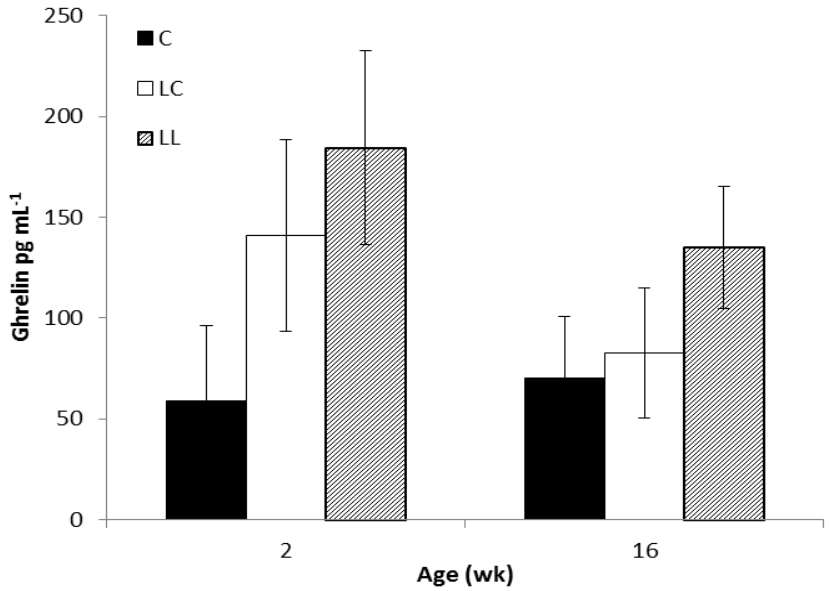

Figure 13. Concentration of ghrelin in lamb blood at 2 and 16 wk of age. Ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from 28 through 78 dGA of gestation followed by 100% through term (LC), or 50% of their requirements from 28 dGA through term (LL). (Wk 2, Control vs. LC and LL: P = 0.06).

Table 13. Umbilical vein and artery blood concentrations of insulin, insulin-like growth factor (IGF)-I, growth hormone (GH) and ghrelin at day 135 gestation in relation to maternal diet; ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through term (LL).

Blood Source	Umbilical Vein					Umbilical Artery				
	Gestational intake			*P* values		Gestational intake			*P* values	
	C (n = 7)	LC (n = 5)	LL (n = 7)	C vs. LC, LL	LC vs. LL	C (n = 7)	LC (n = 5)	LL (n = 7)	C vs. LC, LL	LC vs. LL
Insulin (ng mL^{-1})	.	.	.	.	.	0.30 ± 0.14	0.70 ± 0.15	0.24 ± 0.13	0.3385	0.0360
IGF-I (ng mL^{-1})	50.9 ± 5.93	74.3 ± 6.71	46.7 ± 5.62	0.2129	0.0068	67.9 ± 8.14	91.4 ± 8.97	66.6 ± 7.51	0.2845	0.0518
GH (ng mL^{-1})	.	.	.	.	.	31.6 ± 6.77	40.1 ± 7.42	39.8 ± 6.60	0.3318	0.9775
Ghrelin (pg mL^{-1})	25.3 ± 8.01	23.0 ± 8.77	31.8 ± 7.5	0.8301	0.4537	29.6 ± 11.1	24.5 ± 22.2	44.5 ± 10.3	0.7244	0.2293

Data are the mean ± s.e.m.

Table 14. Uterine artery blood concentrations of insulin, insulin-like growth factor (IGF)-I, growth hormone (GH) and ghrelin at day 135 gestation in relation to maternal diet; ewes either received a control diet (C; 100% of their NRC 2006 recommended requirements), 50% of their requirements from day 28 through 78 of gestation (LC), or 50% of their requirements from day 28 through term (LL).

	Gestational intake			*P* values	
	C (n = 7)	LC (n = 5)	C (n = 7)	C vs. LC, LL	LC vs. LL
Insulin (ng/mL)	0.69 ± 0.19	0.62 ± 0.19	0.28 ± 0.21	0.3283	0.2633
IGF-I (ng/mL)	127.0 ± 21.8	152.4 ± 21.8	106.4 ± 25.2	0.9342	0.2050
GH (ng/mL)	13.54 ± 5.66	11.21 ± 6.54	25.08 ± 6.54	0.5483	0.1771

Data are the mean ± s.e.m.

Chapter VI. Summary

Here we directly compared the impact maternal undernutrition from early- to mid-gestation and from early gestation until term on the growth and development of fetal and postnatal twin offspring in sheep. Additionally we determined the impact of maternal undernutrition during the aforementioned periods in gestation on the circulating concentrations of insulin, IGF-I, ghrelin and GH, which are important regulators of growth and metabolism. We specifically investigated the effect of maternal undernutrition in twin pregnancies, which is valuable, since information specifically detailing the response of twin pregnancy to undernutrition is limited. Further, twin pregnancies are commonplace in sheep production and it is well established that twin and singleton fetuses are different in terms of body and organ weights at birth (Ford *et al*. 2007; Koritnik *et al*. 1981). Maternal undernutrition (UN) during gestation can have impacts on birth weight, organ development, fetal growth trajectory, and even placental development depending on fetal number, timing of undernutrition, and the degree of nutrient restriction. However the impacts of maternal UN also alter the development and mechanisms of metabolism, such as insulin resistance, heart disease, and diabetes, may not become evident until adult life (Moores *et al*. 1993; Anthony *et al*. 2003; Vonnahme *et al*. 2003).

The first half of gestation is a critical period in fetal organ development in sheep and undernutrition during this period impairs body and organ development (Ford *et al*. 2007). We observed that realimentation following undernutrition from early – to mid-gestation (LC) in comparison to prolonged undernutrition (LL) resulted in compensatory growth of the fetuses, so that LC lambs were larger near-term and at

birth. Elevations in brain, liver, adrenal, lung and intestine weights of nutrient restricted-realimented fetuses, further implies that compensatory growth occurs upon realimentation. We also observed a higher gradient of glucose from umbilical vein to umbilical artery at 135 dGA in LC blood compared to LL, indicating that the LC fetus is utilizing more of the glucose that is supplied from the placenta. The amount of glucose being supplied to the LC fetus was higher (greater umbilical vein glucose concentration) and in response to increased glucose, insulin was elevated (greater umbilical artery insulin concentration). These results support our hypothesis that nutrient restriction programs fetal metabolism, so that upon realimentation the fetus compensates for maternal insult in early gestation. Interestingly, pCO_2 levels in umbilical vein and artery increased, while the gradient oxygen content decreased as a result of undernutrition. These results suggest that undernutrition, regardless of timing, alters fetal metabolism to survive on a lower plane of nutrition, since the fetuses are using less oxygen. Umbilical vein and artery IGF-I concentrations were elevated in nutrient restricted-realimented blood, but there was no change in GH concentrations. This is in contrast to our hypothesis that maternal undernutrition followed by realimentation would result in elevated glucose, IGF-I, insulin, and GH. These results may depict a disconnection between GH and IGF-I programmed during maternal undernutrition earlier in gestation for LC fetuses, so that near-term glucose has greater influence over IGF-I concentrations. Our results also demonstrate that maternal undernourishment (LC & LL) results in lambs of similar body weights to the control group, others have also reported that body weight is not impacted by dietary treatment in twin pregnancies (Brennan *et al*. 2005; Edwards and McMillen 2002). Since all of the ewes carried twin pregnancies, birth weights were intermediate to the two levels of undernourishment.

During the second study we collected samples of maternal blood throughout gestation. In late gestation (147 dGA) LC ewes, compared to LL, had increase in glucose, with increased insulin, IGF-I, and ghrelin, but growth hormone remained unchanged. These results imply that glucose has more of a regulatory impact on IGF-I near-term than GH. Similar to the results of near-term twin fetal lambs in Chapter III, greater body weight and larger CRL was also observed in lambs born to nutrient restricted-realimented ewes at birth and at weeks 2 and 3 PN (Chapter IV). This supports our hypothesis that compensatory growth would continue postnatally, however the impact did not continue past 3 weeks PN. At 18 wk PN we did not observe differences in body weight, but did find that CRL and rear leg length were greater in LC lambs. Additionally, at the same time point rear leg length was reduced as a result of undernourishment. Together these results imply that compensatory growth adaptations acquired during undernutrition may contribute to postnatal body conformation. Additionally that undernutrition, regardless of timing, may be detrimental to conformation of the adult offspring. Further, brain weight was also elevated in lambs from nutrient restricted-realimented ewes at 18 weeks postpartum, perhaps indicating that compensatory mechanisms to spare the crucial organ in undernutrition are conserved in postnatal life. At 2 weeks PN blood insulin concentrations were reduced in undernourished lambs (LC & LL), but glucose concentrations did not differ. Further, at 14 weeks PN blood insulin concentrations were not different, but glucose was reduced in LC compared to LL lambs. Together these results may indicate that the reduced rate of metabolism incurred from insult in early- to mid-gestation may manifest later in adult life in terms of insulin secretion or sensitivity to glucose. Interestingly, at 2 weeks PN blood ghrelin concentrations were increased in lambs from undernourished ewes, but there was no increase in glucose,

IGF-I or GH, again indicating a disconnection in the GH/IGF-I axis as a result of undernutrition.

Our results highlight that maternal undernutrition, regardless of timing, alters the GH/IGF-I axis so that glucose concentrations have more of a regulatory impact on IGF-I concentrations than GH. Further, our results indicate that maternal undernutrition followed by realimentation instigates compensatory fetal growth, which may carry into adult life at a metabolic level.

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APPENDIX A

List of Abbreviations

C	dietary treatment control group
CRL	crown rump length
CV	coefficient of variation
dGA	day of gestational age
GH	growth hormone
GR	glucocorticoid receptor
IGF-I	insulin-like growth factor-I
IGFBP	insulin-like growth factor binding protein
IUGR	intrauterine growth restriction
LC	short term undernutrition dietary treatment group
LL	prolonged undernutrition dietary treatment group
NR	nutrient requirements
PC-UN	periconceptional undernutrition
PN	postnatal
POMC	proopiomelanocortin
PP	postpartum
RIA	radioimmunoassay
UN	undernutrition