

INFLUENCE OF MATERNAL PLANE OF NUTRITION AND ARGININE
SUPPLEMENTATION ON MARES AND THEIR FOALS: GLUCOSE AND
INSULIN DYNAMICS

A Thesis

by

ANDREA ESTELLE HANSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2012

Major Subject: Animal Science

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Approved by:

Chair of Committee,
Committee Members,

Head of Department,

Josie Coverdale
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David Hood
H. Russell Cross

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ABSTRACT

Influence of Maternal Plane of Nutrition and Arginine Supplementation on Mares and Their Foals: Glucose and Insulin Dynamics. (August 2012)

Andrea Estelle Hanson, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Josie Coverdale

Thirty-two Quarter horse mares (468 to 668 kg BW; 3 to 19 yr) were utilized in a randomized complete block design. Animals were blocked by expected foaling date and randomly assigned to treatments within block. Treatments began 110 d prior to expected foaling date and were arranged as a 2 x 2 factorial consisting of two planes of nutrition, moderate (Mod; 0.5% BW as fed grain/d) or high (High; 1% BW as fed grain/d) and two levels of L-arginine supplementation, 0.21 g/kg BW/d (Arg) or no supplemental Arg (Con; L-alanine to maintain isonitrogenous diets). Mares were housed by block, allowed ad libitum access to water and coastal bermudagrass (*Cynodon dactylon*) hay, and fed commercial grain twice daily in individual stalls.

A modified frequent sampling i.v. glucose tolerance test (FSIGT) was performed on mares during the 11th month gestation and on foals at 5 and 30 d of age. Jugular catheters were placed 1 h before FSIGT, and horses were allowed ad libitum access to bermudagrass hay and water throughout. After a baseline plasma sample was collected, a glucose bolus of 0.3 g/kg BW was administered. Blood samples were collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min. At minute 20, an insulin bolus of 30 mU/kg BW was administered. Blood samples continued to be collected at 22, 23, 24, 25, 27, 30, 35,

40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min. Samples were placed into tubes containing sodium heparin, immediately placed on ice, and centrifuged within 20 min. Plasma was then collected, placed in microtubes and frozen at -20°C for later analysis.

Glucose concentrations were analyzed using a colorimetric assay and insulin concentrations determined using a commercial RIA kit. There was no influence of dietary treatment on mare glucose area under the curve (AUC_g) or peak glucose (PG) and insulin (PI) concentrations ($P \geq 0.55$). Mare insulin area under the curve (AUC_i) tended to be influenced by the interaction between nutritional plane and ARG supplementation ($P \leq 0.06$) with HighCon mares having greater AUC_i than ModCon ($P \leq 0.05$), and HighCon mares having greater AUC_i than mares fed HighArg ($P \leq 0.05$). Foal AUC_g , AUC_i , and PI were not influenced by maternal diet. However, PG concentration in foals tended to be influenced by mare AA supplementation with foals from Con mares having higher concentrations compared to Arg ($P \leq 0.09$). An influence of age was observed on foal AUC_g and AUC_i . Foal AUC_g was greater at 5 d compared to 30 d ($P \leq 0.003$). Foal AUC_i tended to be greater at 30 d compared to 5 d ($P \leq 0.08$). Data suggest maternal plane of nutrition and arginine supplementation can alter mare and foal glucose and insulin dynamics.

DEDICATION

I would like to dedicate this thesis to my friends and family who have provided me with encouragement, guidance, and wisdom.

ACKNOWLEDGEMENTS

A special thank you to my committee chair, Dr. Josie Coverdale for her excellent guidance and support throughout my graduate career. I would also like to thank my committee members Dr. Tryon Wickersham and Dr. David Hood. I was lucky to have such an amazing group of mentors who took interest in my learning as a student both in the classroom and in the field.

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Maternal metabolism effect on the developing fetus is an area of interest in many species, including the horse. Until 350 years ago, a horse's diet consisted primarily of forage material, but as pasture development improved and animal workload increased, grain supplementation became a common management practice (Kronfeld and Harris, 2003). Feeding cereal grains rich in non-structural carbohydrates (NSC) can provide greater quantities of digestible energy (DE) compared to forage. Increased dietary DE can enhance growth and performance of an animal, but over feeding grains can potentially induce or increase obesity, or lead to other metabolic disorders (Geor, 2008).

Obesity

Equine obesity is a growing concern as it has been estimated that nearly 50% of the equine population in Western civilization is obese (Sillence et al., 2002). Obesity may be defined as an excess of body fat significant enough to cause impairment to health (Dugdale et al., 2010). In humans, body mass index determines obesity. Although there is not a universal definition of obesity in the horse, Henneke et al. (1983) developed a body condition score (BCS) system as an evaluation tool for owners, veterinarians, and nutritionists to assign a numerical value to the amount of fat observed on an animal. BCS ranges from 1 to 9, with 1 representing an emaciated animal and 9 representing a morbidly obese animal. Each number is assigned based on appearance and physical

This thesis follows the style of Journal of Animal Science.

Palpation of 6 distinct locations on the body: over the ribs, along the neck, behind the shoulder, around the withers, above the tail head, and down the back. Ideal BCS depends upon use of the animal with a score of 5 being ideal for maintenance or performance horses, and 6 being ideal for reproductive performance. Animals with a BCS of 7 are overweight and greater than 8 is obese (Henneke et al., 1983). Excess body fat and obesity in the horse can eventually lead to other disorders such as hypertension, laminitis, equine metabolic syndrome, and insulin resistance (Kronfeld, 2003).

Insulin

Glucose metabolism and insulin response. Equine digestive systems are known primarily for hindgut fermenting capabilities which converts forage structural carbohydrates into volatile fatty acids (VFA) to meet energy requirements. However, equids also have capacity for small intestine digestion of NSC, which is metabolically more efficient in the utilization of energy (Parker, 2008). During foregut enzymatic digestion, soluble carbohydrates are hydrolyzed into glucose. Glucose is then transported out of the intestinal lumen via secondary active transport sodium and glucose transporters (SGLT), located within the brushy border, and into the blood stream via glucose transporter (GLUT), GLUT 2. Glucose is used as an energy source for all metabolic processes of the body (Sherwood, 2010).

Research has found approximately 50% of glucose use occurs in the brain, while the splanchnic area disposes of about 25%. Both tissues are considered insulin-independent tissue, meaning there is no reliance on hormones for absorption of glucose out of the blood stream and into tissue. The remaining 25% of glucose is dependent on

the hormone insulin for absorption (DeFronzo, 1988). Under normal physiological conditions, an increase in circulating blood glucose levels signals conversion of pro-insulin into an active peptide, insulin, through excitation coupling. Insulin is produced in the β -cells of the pancreas located within the islets of Langerhans. Insulin is then secreted along with its residual connecting peptide, C-peptide, into the blood stream (Horwitz et al., 1975). Human, porcine, and equine insulin differ by only two amino acids (Kim et al., 2006). Because of the close similarities in composition, exogenous human and porcine insulin is commonly used in equine research. Furthermore, studies have shown porcine and human insulin elicits similar response in humans (Greene et al., 1983). Metabolically, the role of insulin is to maintain whole body glucose homeostasis as it promotes glucose transport, metabolism, and uptake into tissue.

Glucose is transported through glucose transporters (GLUT) from the blood into the surrounding tissue. While there are fourteen known forms of GLUT, GLUT 4 is the primary transporter of glucose into insulin dependent tissues such as muscle and adipose tissue. GLUT 4 is located intracellular and only translocates to the plasma membrane in response to insulin binding (Sherwood, 2010). As insulin binds to transmembrane insulin receptors located on the plasma membrane, intracellular tyrosine kinase is activated. As tyrosine kinase is quickly phosphorylated at the intracellular portion of the receptor, the phosphatidylinositol 3-kinase (PI-3K) pathway is activated. This results in an insulin stimulated intracellular signaling pathway ultimately resulting in the translocation of intracellular glucose transporter GLUT 4 to the plasma membrane. GLUT 4 acts as a channel for glucose to enter the cell. Once inside the cell, glucose is

quickly phosphorylated into glucose-6-phosphate, preventing glucose from exiting the cell (Shepherd and Kahn, 1999).

In addition, to the activation of GLUT 4, insulin stimulates glycogen synthesis in skeletal muscle and liver tissue, which acts as a storage molecule for excess circulating blood glucose and down regulates gluconeogenesis in the liver (Shepherd and Kahn, 1999; Kronfeld et al., 2005). Insulin also stimulates nitric oxide (NO) production in the vascular endothelium, which releases a vasodilator through PI-3K pathway.

Simultaneously, insulin activates the release of endothelin-1 (ET-1) which is a vasoconstrictor released through the mitogen-activated protein kinase (MAPK) pathway (Kim et al., 2006). Furthermore, insulin inhibits the hydrolysis of triglycerides and increases adipose storage by preventing the breakdown of triglycerides in adipose tissue through inhibition of intracellular lipase. Insulin also stimulates the conversion of free fatty acids into fatty tissue and synthesis of glycerol (Frank et al., 2006; Sherwood, 2010).

Insulin resistance and sensitivity. Abnormal cellular response to circulating insulin can be generally described as insulin resistance (IR). More specifically, insulin resistance can be associated with a decreased responsiveness, decreased sensitivity, or a decrease of both responsiveness and sensitivity of tissue to circulating insulin.

Decreased insulin responsiveness is defined as a state where the maximum cellular response to normal amounts of insulin is decreased. Decreased sensitivity to insulin (SI) is defined as a state where maximum cellular response to insulin remains unchanged, while greater amounts of the hormone are required to elicit a normal response (Kahn,

1978). Because of the similarities, equine literature commonly uses the terms IR and SI interchangeably.

In human medicine, altered insulin response is defined as either type 1 or type 2 diabetes mellitus. Patients with type 1 diabetes fail to produce sufficient amounts of endogenous pancreatic insulin in response to elevated blood glucose. Characterized by continuously high levels of glucose in the blood stream, exogenous insulin must be administered for proper glucose up take into tissue. This type of impaired insulin production is uncommon in horses, and usually associated with gestational defects or injury to the pancreas (Roberts and Hill, 1973). In type 2 diabetes, adipose tissue, skeletal muscle, and liver cells fail to respond to insulin signaling. Type 2 diabetes is also coupled with β -cell exhaustion in humans (Stumvoll et al., 2005). In the horse, type 2 diabetes mellitus is rare, as compensation for reduced insulin response is overcome by an increase in pancreatic β -cells insulin production (Kronfeld et al., 2005).

Dysfunction in human cellular response is thought to occur at the PI-3K pathway which is the pathway associated with GLUT 4 translocation, while the MAPK pathway remains intact (Shepherd and Kahn, 1999; Kahn and Flier, 2000). In all animals, IR can be physiologically associated with starvation or pregnancy, or pathologically associated with obesity (Rijnen and van der Kolk, 2003).

Obesity, insulin resistance, and diet. Equine research has found a link between obesity, insulin resistance and diet. Hoffman et al. (2003a) used a minimal model to estimate glucose effectiveness and insulin sensitivity in obese and non-obese horses adapted to diets with varying NSC content. The minimal model has been primarily used in human diabetes research and distinguishes glucose disposal independent of insulin. It

is important to note, in this study BCS of each horse was maintained at a range of 5 to 8, with 5 to 5.9 labeled as non-obese, 6 to 6.9 as moderately obese, and 7 to 9 as obese. Animals were adapted to a forage only diet during period 1. During period 2, animals were divided into two separate groups with one group fed a diet high in starch and sugar and the other fed an isocaloric diet high in fat and fiber. Period 3 completed the cross over design. Modified frequent sampling i.v. glucose tolerance tests (FSIGT) were performed after adaptation to each of the three diets. This study was instrumental in demonstrating FSIGT as a useful tool in evaluating glucose and insulin dynamics in the horse. Results indicated obese insulin sensitivity was 80% lower than non-obese with decreased glucose tolerance. Furthermore, obese horses secreted larger quantities of endogenous insulin in response to blood glucose as compared to non-obese. Results indicate that horses fed grain meals rich in starch and sugar (SS) have a greater risk of developing insulin resistance. A decrease in β -cell production of endogenous insulin in response to exogenous glucose was seen in horses fed SS. This suggests animals became progressively less sensitive to the elevated blood glucose levels and required greater concentrations prior to pancreatic insulin secretion (Hoffman et al. 2003a). Similar studies support the conclusion that feeding diets high in NSC alters insulin response and contributes to the development of IR (Kronfeld et al., 2005). Additional data from Pratt et al. (2005) indicated a 30% decrease in insulin mediated glucose disposal and an increase in insulin production in horses fed high NSC diets for 6 wk.

Maternal Metabolism

Research suggests variations in maternal diet can alter physiological adaptations of equine metabolism during pregnancy. Hoffman et al. (2003b) evaluated the effect of

a high starch and sugar diet (SS) based on corn grain and molasses compared to a corn oil supplemented fat and fiber diet (FF), on pregnant mare metabolism. Although this study found mares fed SS had lower sensitivity to oral glucose and faster glucose removal, it demonstrated the ability to alter equine maternal metabolism through diet. More recent research by Winsco et al. (2011) studied the effects of DE manipulation on mare metabolism looking specifically at the glucose and insulin response. During late pregnancy, mares were adapted to either a forage and grain diet (FG) or forage only diet (F). During the 11th month of gestation, FSIGT were performed on the pregnant mares. Results concluded FG mares had greater circulating blood glucose and insulin concentrations, suggesting increased insulin resistance in FG mares. Metabolic adaptation occurred resulting in a decrease of glucose and insulin response in FG diets.

Natural metabolic changes occur during pregnancy, which prepare an animal for the increase in energy requirements for placental and fetal growth and development. These changes ensure a continuous supply of nutrients will reach the developing offspring independent of diet. Human research has considered late pregnancy a diabetogenic state because of a 50% decrease in insulin mediated glucose disposal in maternal metabolism (Zavalza-Gomez et al., 2008). Equine research has found similar characteristics during late stage pregnancy including an increased production of insulin in response to circulating glucose. Increased insulin secretion has been coupled with a decrease of insulin sensitivity in maternal metabolism, resulting in significantly slower glucose removal (Hoffman et al., 2003b; George et al., 2011). These normal adaptations to pregnancy are characterized by changes in pancreatic β -cell function, which contribute to pregnancy induced insulin resistance (Fowden et al., 1984).

Diet effect on intrauterine environment. Adaptations to maternal nutrition can alter maternal metabolism, and eventually, impact fetal growth and development through influence of the intrauterine environment. The intrauterine environment directly influences the structure, physiology, and metabolism of a developing fetus. The fetus can adapt to changes in the environment, a phenomenon known as “fetal programming” (Barker and Clark, 1997), but these adaptations may have immediate or life-long implications on the developing offspring (Wu et al., 2004).

Under normal physiological conditions, transfer of nutrients from maternal blood to fetal blood occurs across a thin placental barrier. Horses have a diffuse, microcotyledonous placenta, and makes full contact with the uterine wall, which increases fetal nutrient supply. Ovine placenta is a similar epithelochorial structure to the equine placenta. Some substance such as oxygen, carbon dioxide, water and electrolytes easily diffuse across the membrane. Amino acids are transferred from mother to fetus by a secondary active transport system (Wooding and Fowden, 2006).

Glucose is readily transported between maternal and fetal blood, but does require the establishment of a concentration gradient (Hay et al., 1984; Sherwood, 2010). This concentration gradient permits the diffusion of glucose through GLUT 1 transport proteins (Smith et al., 1992). The placenta utilizes a large portion of the glucose supply as research in sheep during late term pregnancy has shown the placenta accounts for 50-75% of uterine glucose uptake. This creates hypoglycemia in the fetus and contributes to an increase in the overall glucose concentration gradient between maternal and fetal blood (Hay, 1995).

The intrauterine programming of fetal metabolism occurs during late gestation as pancreatic insulin production begins to respond to available glucose (Fowden and Hill, 2001; Fowden et al., 2005). Glucose intolerance and other metabolic disorders can be induced during this period of growth. In the rat, maternal nutrient manipulation during the last 7 d of gestation caused adult glucose intolerance in the offspring (Holeman et al., 2003). Increased fetal β -cell insulin secretion was observed during late gestation in the horse (Fowden et al., 2005), and continued to increase past parturition (Holdstock et al., 2004). Winsco et al. (2011) indicated programming of foal metabolism can occur through maternal dietary manipulation. Grain supplementation in mares during the last third of pregnancy was seen to decrease insulin sensitivity and alter glucose clearance in the offspring.

The function of insulin in the fetus is to maintain optimal growth, and alter glucose and protein metabolism (Brown and Hay, 2006). In the event of continuous hyperglycemia, the fetal pancreas down regulates β -cell production of insulin in sheep (Jackson et al., 1993) and decreases GLUT 4 expression potentially limiting fetal growth and development (Carver et al. 1995). In contrast, human research indicated a greater fetal pancreatic release of insulin in response to elevated glucose levels from diabetic mothers (Baird and Farquhar, 1962). During maternal hypoglycemia, glucose utilization by the placenta decreases. This decrease in placental glucose supply will result in a decrease in placental weight. Changes in maternal glucose supply results in significant adaptations to placental growth and metabolism (Hay, 1995).

Fetal hyperinsulinaemia, which can be induced by maternal diabetes, increases nutrient availability, increases lipid content (Stevens et al., 1990) and stimulates insulin

growth factors in the fetus (Fowden, 1992). This can cause abnormalities in fetal growth. Ovine research has determined hyperinsulinemia increases the amino acid and nitrogen utilization rates of the fetus independent of glucose (Thureen et al., 2000).

Disturbances, that alter development and growth of either the placenta or fetus, result in intrauterine growth retardation (IUGR). Influence of maternal nutrition is a contributing factor to IUGR and can cause such metabolic issues as insulin resistance, β cell dysfunction, glucose intolerance, and obesity in the developing offspring (Barker and Clark, 1997). In humans, twin pregnancies increase the risk of at least one of the infants developing IUGR, and the IUGR infant will have a higher risk of developing type 2 diabetes mellitus in later stages of life than the twin with normal intrauterine growth (Philips et al., 1994). Early IUGR research looked specifically at the effects of maternal under nutrition on the intrauterine environment, but recent studies suggest both maternal under and over nutrition can induce IUGR (Wu et al., 2004).

In ovine research, maternal nutrient restriction during late pregnancy results in a reduction of placental and fetal growth. Maternal over nutrition decreases placental weight, which directly decreases fetal growth and increases fetal mortality (Wallace et al., 1996; Wallace et al., 2002). In human research, maternal over nutrition results in fetal growth restriction, increased risk of neonatal mortality, and increased obesity during childhood and later stages of life (Castro and Avina, 2002). Minimal research in the horse has looked specifically at IUGR. Winsco et al. (2011) indicated mare dietary manipulation does not result in premature delivery of low birth weight foals or a decrease in placental mass as observed in other species. While indications of IUGR

were not observed in placenta or fetal weight, the effects of fetal programming were observed in the analysis of foal metabolism.

Nitric oxide and placental angiogenesis. Minimal knowledge exists about the specific mechanism of IUGR, but it has been associated with impaired NO production and decreased polyamine synthesis in the placenta. Nitric oxide is a vasodilator, which plays an important role in the regulation of uteroplacental blood flow and nutrient transfer. Polyamines regulate cell proliferation and differentiation in placental vasculature providing an optimal growth environment for the fetus (Bird et al., 2003; Wu et al., 2004).

The placenta functions as the digestive system, respiratory system, and kidney for the developing offspring (Sherwood, 2010). Therefore, placental angiogenesis and blood flow impact the transplacental exchange of nutrients and oxygen from mother to fetus, directly impacting development and growth (Reynolds and Redmer, 2001). Research in sheep has shown maternal placental vasculature develops steadily throughout gestation, while fetal placental vasculature develops rapidly during the last third of gestation in order to provide sufficient nutrients as the fetus increases in size (Stegeman, 1974). Congruently, NO synthesis has been shown to increase during stages of greatest fetal development (Kwon et al., 2003). Since NO and polyamines are both products of L-arginine (ARG) degradation (Figure 1), ARG could potentially be an important molecule in fetal development (Wu et al., 2006).

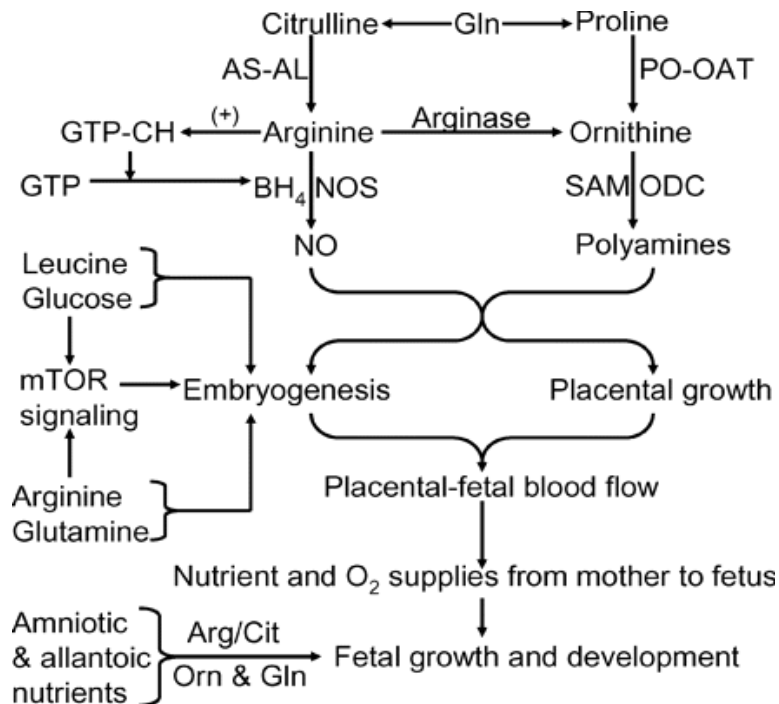


Figure 1. Roles of arginine, NO, and polyamines in fetal growth (Wu et al., 2006)

Arginine Supplementation

Arginine: synthesis and degradation. L-arginine is classified as a basic, non-essential, amino acid in adult humans, but has been shown to be conditionally essential for many young, growing, and pregnant animals (Milner et al., 1974; Gross et al., 1991; Rogers et al., 1970). Endogenously, ARG can be synthesized in the liver via the urea cycle, but is rapidly broken down by the high activity of arginase.

Enterocytes, absorptive epithelial cells in the small intestine, can synthesis L-citrulline (CIT; Figure 2), which is a precursor of ARG. In addition, glutamine, glutamate, and proline in the intestinal-renal axis contribute to the synthesis of both CIT and ARG through the pyrroline-5-carboxylate (P5C) synthetase and N-acetylglutamate

of ARG into cells occurs through System y^+ , which is a high affinity sodium independent transporter of basic amino acids (Flynn et al., 2002). Once inside the cell, ARG catabolism occurs through multiple pathways and is degraded by multiple enzymes to include arginase (Arg), nitric-oxide synthase (NOS), arginine:glycine amidinotransferase, and ARG decarboxylase (Figure 2).

Production of NO occurs as ARG is metabolized to CIT by NOS. This occurs through one of the three NOS isoforms: endothelial (e)NOS, neuronal (n)NOS, or inducible (i)NOS. Because the ARG degradation pathways are complex and involve multiple systems, ARG can have a significant impact on overall body health (Wu and Morris 1998; Hefler et al., 2001; Flynn et al., 2002; Wu et al., 2007; Wu et al., 2009).

Arginine and obesity. Using Zucker diabetic fatty (ZDF) rats genetically altered to exhibit hyperglycemia, dyslipidemia, insulin resistance, and endothelial dysfunction, Fu et al. (2005) found ARG supplementation reduced fat mass, body weight, and epididymal adipose tissue. A 25% decrease in fat mass was observed after a 10-wk treatment of ARG supplementation in the genetically obese rodents. Serum samples revealed elevated NO production in supplemented animals, and adipose tissue had increased expression of ARG products and enzymes. They determined ARG supplementation increased oxidation of both glucose and fatty acid in adipose tissue.

Knockout mice deficient in eNOS and exhibiting inhibited NO production had higher body fat composition, reduced energy expenditure, increased weight gain, and increased insulin resistance compared to wild type mice with normal NO production. It was also found peroxisome proliferator activated receptor γ (PPAR γ) coactivator-1 α (PGC-1 α) is downregulated in the eNOS knockout mice (Nisoli et al., 2003). The

PPAR γ PGC-1 α is a regulator of oxidative phosphorylation, mitochondrial biogenesis, and glucose/fatty acid metabolism. It is activated by NO potentially produced during ARG degradation. Under fasting conditions, the stimulation of PGC-1 α has been shown to increase oxidation of energy substrates to produce glucose (Lehman et al., 2000; Liang and Ward, 2006). In addition, PGC-1 α has increased GLUT 4 activity and increased insulin sensitivity in animals with decreased insulin response (Michael et al., 2001; Yoon et al., 2001).

Arginine effects on insulin. As previously discussed, β -cell function and insulin secretion in adult animals is controlled by circulating metabolites, such as glucose. Additional research reveals amino acids (AA), particularly ARG, can metabolically stimulate the release of glucagon and secretion of pancreatic insulin in humans and in other species (Fajans et al., 1967; Gerich et al., 1974; Fowden, 1980). The pathway in which ARG stimulates the release of insulin differs from glucose as ARG stimulates the release by direct β -cell membrane depolarization (Gresores et al., 1997; Thams and Capito, 2000), and can therefore, initiate a more rapid insulin release than the secondary pathway of glucose.

Pancreatic sensitivity to ARG has also been documented in the pancreas of the developing human fetus (Milner et al., 1974). Research in sheep has shown fetal insulin release actually responded more rapid to ARG than to glucose. Fowden (1980) was able to compare insulin response to glucose and to ARG by utilizing an adrenaline infusion, which reduces basal insulin secretion and abolishes glucose mediated insulin release from pancreatic β -cells. While many of these studies directly infused the fetus with

ARG, the actual maternal-fetal transfer of ARG is slow and requires a significant concentration gradient (Gresores et al., 1997).

Arginine effects on fetal development. In addition to the ARG induced fetal insulin response, ARG supplementation and NO production can impact growth and development of a fetus. Porcine ARG supplementation tends to increase live birth number and live birth weight of piglets as compared to animals without ARG supplementation (Mateo et al., 2007).

Hefler et al. (2001) observed IUGR in eNOS-knockout mice. Fetal mice were observed to demonstrate reduced fetal growth, reduced survival rate, and increased developmental abnormalities resulting from a decrease in NO production. In humans, sampling of infants considered small for gestational age revealed a decrease in NO production as compared to infants of appropriate size (Hata et al., 1998). It was also found premature humans exhibit ARG deficiency (Becker et al. 2000). Furthermore, reduced placental blood flow due to impaired NO production was observed in over nourished sheep (Wallace et al., 2003). Administration of ARG in IUGR sheep increased fetal growth (Lassala, 2008) and increased birth weight of IUGR humans (Xiao and Li, 2005). While the role of arginine in NO production and IGUR has been demonstrated in several species, minimal research has focused on equine ARG supplementation.

Conclusion

The medical implications of obesity and diet leading to metabolic disorders across species compounded with the effects of fetal programming have opened a wide range of potential experimental designs. The common equine management practice of

feeding high starch grains has led to an interest in the role maternal nutrition plays on mare metabolism as well as metabolic development of the foal. Potential ways to mitigate negative effects such as arginine supplementation are of interest. Therefore, the objectives of the current study were to determine the influence of maternal nutritional plane and ARG supplementation on mares and foals. Mare performance, metabolism, foaling parameters, colostrum quality, passive immunity, and reproductive hormones were evaluated, in addition to foal metabolism and growth. The following document focuses only on the objectives related mare and foal insulin and glucose dynamics. It is hypothesized that ARG supplementation will mitigate the negative effects of increased maternal nutrition on mare and foal insulin and glucose dynamics.

CHAPTER II

MATERIALS AND METHODS

All procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee.

Animals and Treatments

Thirty-two pregnant Quarter horse mares (468 to 668 kg BW; 3 to 19 yr), on loan from a single farm, were pasture housed at the Texas A&M University Horse Center, and used in a randomized complete block design. Mares were blocked by expected foaling date and randomly assigned to 1 of 4 dietary treatments within block.

Treatments were arranged as a 2 x 2 factorial with 2 planes of nutrition and 2 levels of L-arginine supplementation. Mares received either 0.5% BW (Mod; as fed) or 1% BW (High; as fed) per day of a commercial concentrate (Vitality Mare and Foal, Nutrena Feeds, Minneapolis, MN). L-arginine (Ajinomoto AminoScience LLC, Raleigh, NC) was fed at 0 or 0.21 g/kgBW/d (Arg) with L-alanine (Ajinomoto AminoScience LLC, Raleigh, NC) used to maintain an isonitrogenous diet (Con).

The NRC (2007) suggests that the daily DE requirement for a 500 kg mare during the 11th month gestation is 21.4 Mcal. The Mod diets provided an average of 28.2 Mcal DE/d which is approximately 120% of NRC DE recommendations while High diets provided an average of 34.6 Mcal DE/d which is 150% of NRC DE recommendations (NRC, 2007). DE intake was calculated from grain consumption and estimated forage intake.

Dietary treatments began 110 days prior to expected foaling date. Animals were fed individually in 3 x 3 m stalls twice a day at 0615 and 1615. In pastures, animals had

access to ad libitum coastal bermudagrass (*Cynodon dactylon*) hay and water. Voluntary forage intake was determined using a dual marker system of ADIA and titanium dioxide as part of a companion study. Grain intake was recorded daily. Table 1 illustrates the nutrient composition of the diet components. After parturition, all mares returned to pasture where they were group fed grain mix at approximately 1% of BW (as fed) twice daily. The composition of the lactation grain mix was similar to that used before parturition (Table 1).

Table 1. Energy and nutrient composition of concentrate and hay (DM basis) fed to mares

Item	Concentrate ¹	Hay ²
CP, %	17.76	8.72
Fat, %	6.96	1.78
ADF, %	8.30	39.70
Starch, %	35.30	3.80
TND, %	80.55	54.91
Ca, %	0.94	0.44
P, %	0.83	0.24
DE, Mcal/kg	1.61	1.10

¹ Commercial concentrate fed at 0.5% and 1.0% BW (as fed; Vitality Mare and Foal, Nutrena Feeds, Minneapolis, MN)

² Hay consisted of coastal bermudagrass (*Cynodon dactylon*)

Mare Performance Measurements

Mare performance was evaluated on d 0 and every 14 d thereafter until parturition with measurements consisting of BW, BCS, and rump fat (RF). Body weight was determined using a GSE 350 electronic scale (Bastrop Scale Co., Inc, Austin, TX) and BCS was determined by 3 individual observers on a scale of 1 to 9 (1 is emaciated and 9 is morbidly obese) as described by Henneke et al. (1983). Rump fat was determined via ultrasonic images on the left hip at a point 5 cm dorsal of halfway

between the first coccygeal vertebra and the ischium (Westervelt et al., 1976), using a SSD-500V ultrasound instrument (Aloka Co., LTD, Mitaka-shi, Tokyo, Japan).

Frequent Sampling IV Glucose Tolerance Test (FSIGT)

A modified frequent sampling intravenous glucose tolerance test (FSIGT) was performed on mares at 11 months gestation (average for the block) and on foals at 5 and 30 d of age using methods previously described (Hoffman et al., 2003; Winsco et al., 2011). Prior to the procedure, mares or mares and foals were moved from pasture to individual 3 × 3 m stalls. The morning feed was withheld. Throughout the FSIGT mares had ad libitum access to hay and water, and foals were allowed to nurse. Nursing times, as well as durations, were recorded. Prior to the FSIGT, animals were weighed using a GSE 350 electronic scale (Bastrop Scale Co. Inc., Austin, TX). The jugular catheter site was prepared by clipping the coat to a sanitary length (blade size 50), and sterilized using Chloradine scrub 4% in addition to isopropyl alcohol 70%. Lidocaine was used as a local anesthetic and injected subcutaneously at the site of catheter insertion. Mare catheters were 14 G × 5.5 inch Abbocath-T Catheter (Abbot Hospitals, North Chicago, IL) and foal catheters were 16 G × 3.5 inch Angiocath Catheter (BD Medical, Medical Surgical Systems, Franklin Lakes, NJ). Catheters were placed and a 30 inch extension set (Hospira, Inc., Lake Forest, IL) was attached to the catheter and secured with Elasticon. Due to the diurnal variation in equine glucose values, the FSIGT was initiated at approximately the same time each morning. Animals were allowed to acclimate for a minimum of 1 hr after catheters were placed and prior to sampling.

After a baseline plasma sample was collected, 0.3 g/kg BW of a glucose bolus composed of 50% Dextrose (AGRIpharm, Agripharm Products, Westlake, TX) was

administered. Blood samples were then collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min. At minute 20, a 30 mU/kg BW insulin bolus of Humulin (Eli Lilly and Company, Indianapolis, IN) was administered. Since equine insulin is not readily available, human insulin is commonly used in equine research as it differs by only two amino acids (Kim et al., 2003). Blood samples collection was continued at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min. Samples were placed into 10 ml evacuated tubes containing sodium heparin (143 USP units; Becton Dickinson, Franklin Lakes, NJ), immediately placed on ice and centrifuged within 20 min of collection (2,700 x g for 20 min). Sample plasma was then collected, placed in microtubes, and frozen at -20⁰C for later analysis.

Glucose and Insulin Analysis

Plasma samples were analyzed for glucose and insulin concentrations. Glucose concentrations were determined using a colorimetric assay (Sigma Diagnostics; Glucose Procedure No. 510, Charleston, WV). A glucose enzyme solution was prepared adding 1 capsule PGO enzyme to 100 mL distilled water in an amber bottle. Each capsule of PGO enzyme contains 500 units of glucose oxidase, 100 purpurogalin units of peroxidase and buffer salts. After inverting the water and enzyme, 1.6 mL of color reagent, o-dianisidine dihydrochloride (reconstituted with 20 mL water), was added. The enzyme mixture, PGO enzyme and o-dianisidine dihydrochloride, were stored used within 30 d. All tubes were prepared in duplicate. A blank was prepared using 0.5 mL water. Standards 1-5 were made using stock β -glucose (1mg/mL). Standard 1 was prepared to a 20-fold concentration (0.05 mg/mL), standard 2 a 10-fold concentration (0.10 mg/mL), standard 3 a 6.67-fold concentration (0.15 mg/mL), standard 4 a 5-fold

concentration (0.20 mg/mL), and standard 5 a 4-fold concentration (0.25 mg/mL). A 10-fold dilution was created for the baseline sample in addition samples from min 4 to 180. Since glucose concentrations were greatest at sample min 1, 2, and 3, a 20-fold dilution was created using reverse osmosis water. Diluted samples were vortex, 0.25 mL was transferred to a separate tube, and 2.5 mL enzyme mixture was added. All tubes were incubated at room temperature (18 to 26°C) for 45 min and then read within 30 min. For analysis, dilutions were placed in cuvetts and read by a DU 730 Vis Spectrophotometer (Beckman Coulter, Inc., Brea, CA) at a wavelength of 450 nm.

Insulin concentrations were determined using a commercial radioimmunoassay (RIA) kit (Coat-A-Count Insulin, Siemens Healthcare Diagnostics, Tarrytown, NY). Polypropylene tubes were labeled in duplicate as T (total counts) and NSB (nonspecific binding). Antibody-coated tubes were labeled A-G in duplicate. Approximately 200 μ L of zero calibrator A was added into the NSB and A tubes while 200 μ L of each remaining calibrator was added to control tubes. For mares, 200 μ L of plasma was added in duplicate to coated tubes. A 5-fold dilution was used in mare samples min 22 to 27. For foals, 200 μ L of plasma from samples 22 to 30 min was used while 2-fold concentration was used for min 0 to 19 and 35 to 180. Within 40 min of placing the first sample in coated tubes, 1.0 mL of I-125 insulin was added to every tube and vortex. Tubes were left to incubate for 18 to 24 hr. Using a foam decanting rack, tubes (except the T tube) were drained and allowed to dry for 2 to 3 min. Tubes were placed in the Packard Cobrat II Gamma Counter (Packard Company, Downers Grove, IL) for determination of insulin concentration.

Statistical Analysis

Area under the curve was determined for plasma glucose and insulin according to the trapezoidal rule using PROC EXPAND of SAS (SAS Inst., Inc., Cary, NC).

All data were analyzed using PROC MIXED of SAS (SAS Inst., Inc., Cary, NC) with the model containing effects for plane of nutrition (Nutr), amino acid supplementation (AA), and their interactions. Additionally, with foal data the effect of age was included in the model. Main effects were considered significant when $P \leq 0.05$ and $P \leq 0.10$ considered a trend towards significance.

CHAPTER III

RESULTS AND DISCUSSION

Mare Measurements

Mare measurements were not influenced by diet ($P \geq 0.69$). Results specify Mod and High least square means. Body weight ranged from 539.4 to 570.5 kg \pm 18.0, mare RF ranged from 0.30 to 0.31 mm \pm 0.007. Mare BCS ranged from 6.2 to 6.6 \pm 0.32 despite providing 120% (Mod) or 150% (High) of NRC (2007) DE requirements. However, it is important to note that all mares, regardless of treatment, were above the ideal BCS of 6 (Henneke et al., 1983). A more detailed evaluation of mare performance will be published in a companion paper.

Mare Glucose and Insulin

Mare peak glucose (PG) and insulin (PI) concentrations, and the area under the curve for glucose (AUC_g) and insulin (AUC_i) are shown in Table 2. Mare glucose and insulin concentrations in response to the FSIGT are shown in Figure 3. Mare AUC_g , PG, and PI concentrations were not influenced by plane of nutritional, AA supplementation, or the interaction between nutrition and AA ($P \geq 0.55$). Mare AUC_i tended to be influenced by the interaction between nutrition and ARG supplementation ($P \leq 0.06$). As expected, HighCon mares had greater ($P \leq 0.05$) AUC_i values compared to those offered ModCon. Additionally, HighArg mares had lower AUC_i values compared to those offered HighCon ($P \leq 0.05$).

Table 2. Effect of plane of nutrition and arginine¹ (ARG) supplementation on peak glucose (PG), peak insulin (PI), glucose area under the curve (AUC_g), and insulin area under the curve (AUC_i)² performed during FSIGT³ in the last third of equine gestation

	Treatment ⁴				SEM	P-value ⁵		
	ModCon (n=7)	ModArg (n=6)	HighCon (n=7)	HighArg (n=7)		Nutr	AA	Nutr x AA
PG, mg/mL	375	379	339	364	18	0.18	0.40	0.55
PI, mU/L	837	733	860	786	153	0.80	0.54	0.92
AUC _g	224	229	220	233	17	0.99	0.55	0.81
AUC _i	9,353	11,193	14,085	9,352	1,79 2	0.41	0.40	0.06

¹Ajinomoto (Ajinomoto AminoScience LLC, Raleigh, NC)

²Values are in least square means

³Frequent sampling I.V. glucose tolerance test (FSIGT)

⁴Grain was fed at 0.5% of BW (as fed) or 1.0% of BW (as fed) and ARG was supplemented at 0 (Ala) or 0.21 g/kg/day (Arg) resulting in 4 treatments ModCon = 0.5% BW (as fed) + Ala, ModArg = 0.5% BW (as fed) + Arg, HighCon = 1.0% BW (as fed) + Ala, and HighArg = 1.0% BW (as fed) + Arg

⁵Nutr = effect of nutrition, AA = effect of amino acid, and Nutr and AA = interaction between Nutr and AA

Winsco et al. (2011) observed greater mare AUC_g when horses were supplemented with grain as compared to mares fed forage alone. However, in the current study, all mares received grain supplementation, which may have altered the AUC_g values compared to those obtained with mares fed forage alone. Mare PG concentrations were similar to those observed by previous research (Hoffman et al., 2003a; Winsco et al, 2011). Their results concluded that nutritional plane and BCS had little effect on PG concentrations during an FSIGT.

With respect to mare AUC_i, ModCon mares had lower concentrations than HighCon, which supports previous findings that feeding high grain diets during the last third of pregnancy in mares can alter insulin production or sensitivity (Winsco et al., 2011). Hoffman et al. (2003b) found conflicting results as mares in their study fed diets

high in starch and sugar (SS) had lower AUC_i than those fed isocaloric diets high in fat and fiber (FF) in late pregnancy. They concluded that mares fed SS did not produce as much insulin as FF mares, which could be associated with adaptation to grain supplementation as mares were fed in both early and late gestation.

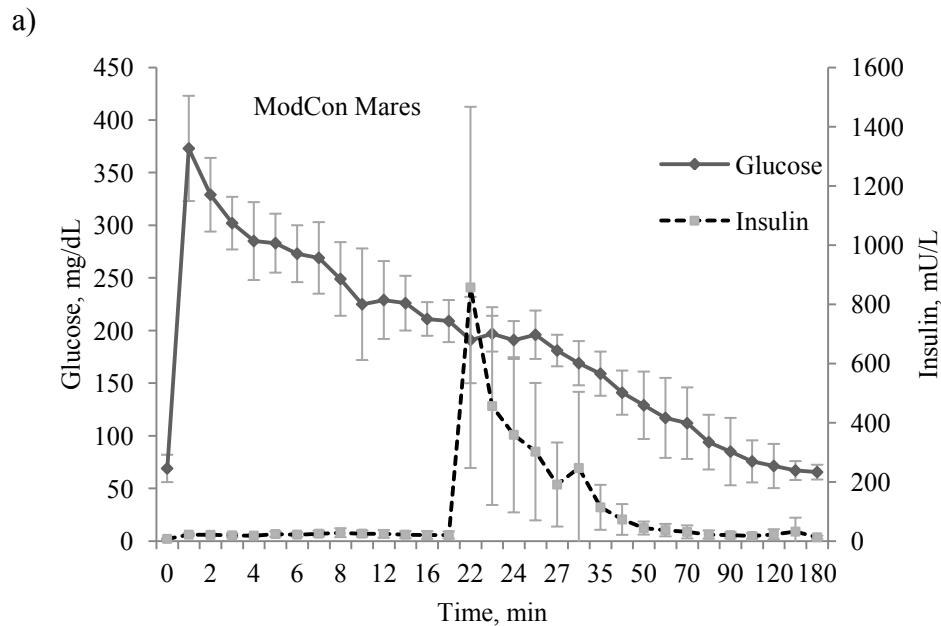
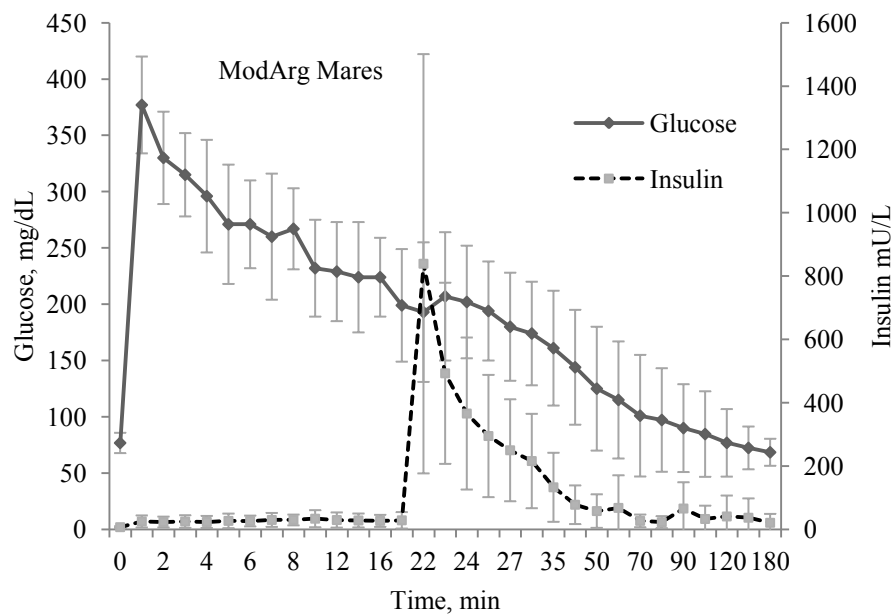


Figure 3. Effects of nutrition and ARG supplementation on mare glucose and insulin during modified FSIGT in the last third of pregnancy. Mare dietary treatments: a) Grain 0.5% BW (as fed) + Alanine (ModCon; n = 7) b) Grain 0.5% BW (as fed) + Arg (ModArg; n = 6) c) Grain 1.0% BW (as fed) + Alanine (HighCon; n = 7) d) Grain 1.0% BW (as fed) + Arg (HighArg; n = 7). Least square means are reported.

b)



c)

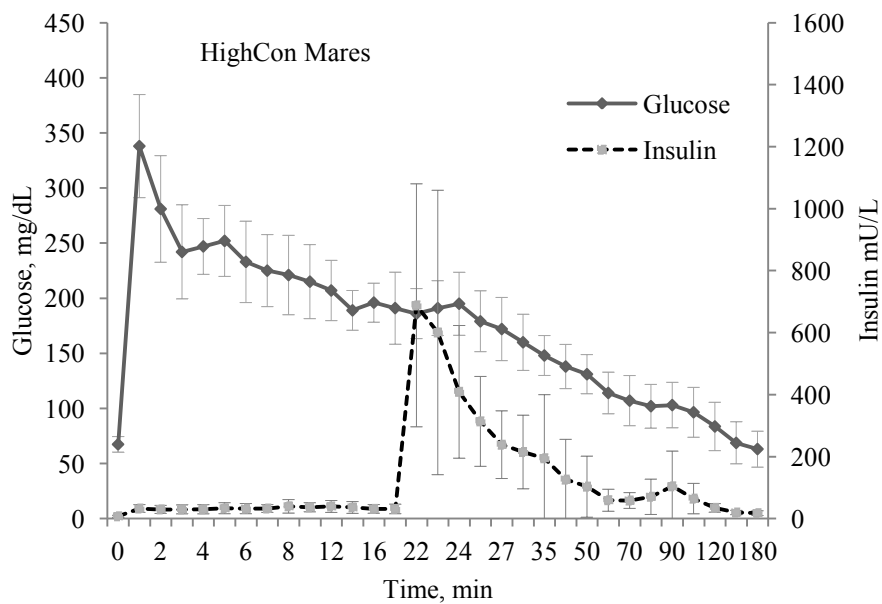


Figure 3 Continued.

d)

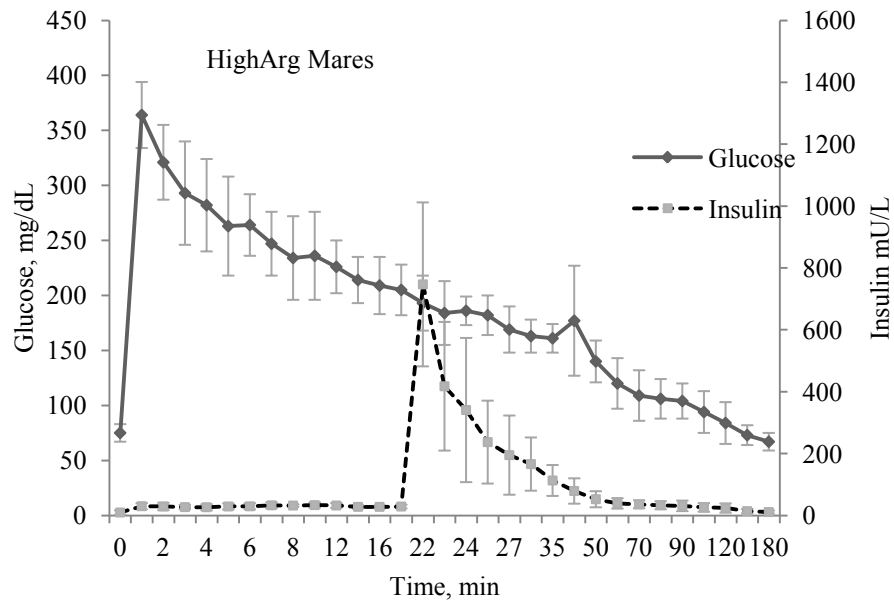


Figure 3 Continued.

In addition, our results concluded HighCon mares had greater AUC_i than HighArg mares ($P \leq 0.05$). This demonstrates the potential for ARG supplementation to mitigate the effects of high starch diets in the horse. Human research has shown ARG supplementation can improve insulin sensitivity in diabetic patients (Piatti et al., 2001). As insulin sensitivity is stimulated, less insulin is needed to initiate regulation of blood glucose. While research with ARG supplementation has determined it can increase pancreatic insulin response in sheep and rats (Fajans et al., 1967), further information regarding ARG supplementation is needed in the horse to determine the approximate mode of action.

Foal Glucose and Insulin

Foal PG, PI, AUC_g , and AUC_i for d 5 and d 30 are shown in Table 3. Foal glucose and insulin dynamics evaluated on d 5 and d 30 are shown in Figures 4 and 5,

respectively. Foal PG (d 5 and d 30 combined) tended to be influenced by maternal AA supplementation ($P \leq 0.09$) with foals from Con mares having greater PG concentration than foals from Arg mares (275 ± 6.5 and 260 ± 5.2 mg/dL, respectively).

Table 3. Effect of plane of nutrition and arginine¹ (ARG) supplementation on peak glucose (PG), peak insulin (PI), glucose area under the curve (AUC_g), and insulin area under the curve (AUC_i)² performed during FSIGT³ on foals at d 5 and d 30 of age.

	Treatment ⁴				SEM	P-value ⁵			
	ModCon (n = 4)	ModArg (n = 8)	HighCon (n = 8)	HighArg (n = 8)		Nutr	AA	Nutr x AA	Age
PG, mg/dl						0.34	0.09	0.67	0.94
d 5	281	264	264	259	12				
d 30	273	268	281	250	17				
PI, mU/L						0.53	0.24	0.62	0.80
d 5	264	258	163	223	68				
d 30	202	267	207	292	78				
AUC _g						0.79	0.38	0.31	<0.01
d 5	274	283	299	285	25				
d 30	260	255	271	233	19				
AUC _i						0.48	0.49	0.22	0.08
d 5	2,466	2,886	2,515	3,313	1,232				
d 30	5,865	2,720	3,165	12,057	5,249				

¹Ajinomoto (Ajinomoto AminoScience LLC, Raleigh, NC)

²Values are least square means

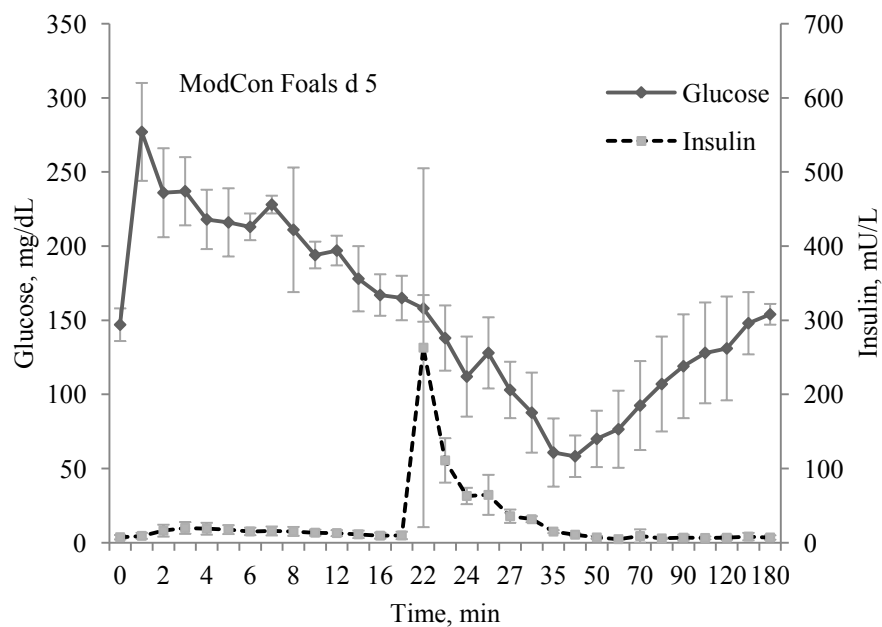
³Frequent sampling I.V. glucose tolerance test (FSIGT)

⁴Grain was fed to mares at 0.5% of BW (as fed) or 1.0% of BW (as fed) and ARG was supplemented at 0 (Ala) or 0.21g/kg/day (Arg) resulting in 4 treatments ModCon = 0.5% BW (as fed) + Ala, ModArg = 0.5% BW (as fed) + Arg, HighCon = 1.0% BW (as fed) + Ala, and HighArg = 1.0% BW (as fed) + Arg

⁵Nutr. = effect of nutrition, AA = effect of amino acid, Nutr. And AA = interaction between nutrition and AA, and Age = effect of age. P-values represent combined (d 5 and d 30) data for all main effects except age

While foal PI was not influenced by nutritional plane, AA supplementation, age, or the interaction between ($P > 0.24$), foal AUC_g was influenced by age, regardless of maternal diet, with d 5 foals having greater AUC_g than d 30 foals ($P \leq 0.003$). In addition, foal AUC_i tended to be influenced by age, as AUC_i was greater in d 30 foals than in d 5 foals ($P \leq 0.08$).

a)



b)

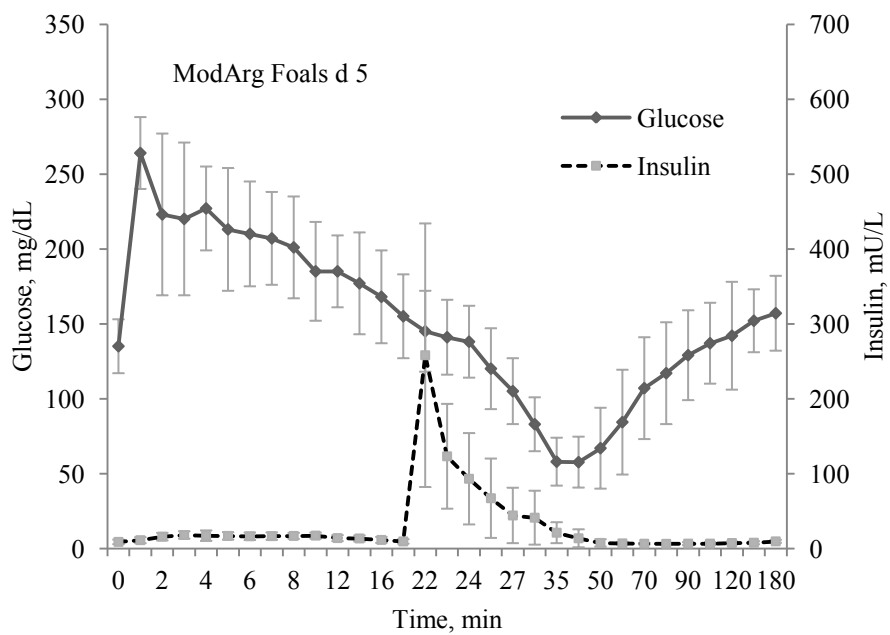
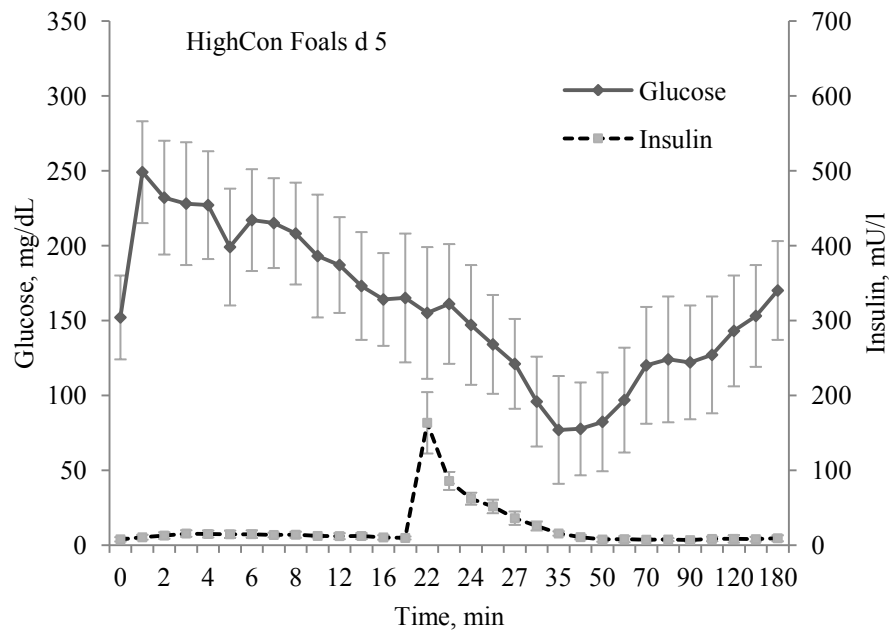


Figure 4. Effects of maternal nutrition and ARG supplementation on foal d 5 glucose and insulin during modified FSIGT. Mare dietary treatments: a) Grain 0.5% BW (as fed) + Alanine (ModCon; n = 7) b) Grain 0.5% BW (as fed) + Arg (ModArg; n = 6) c) Grain 1.0% BW (as fed) + Alanine (HighCon; n = 7) d) Grain 1.0% BW (as fed) + Arg (HighArg; n = 7). Least square means are reported.

c)



d)

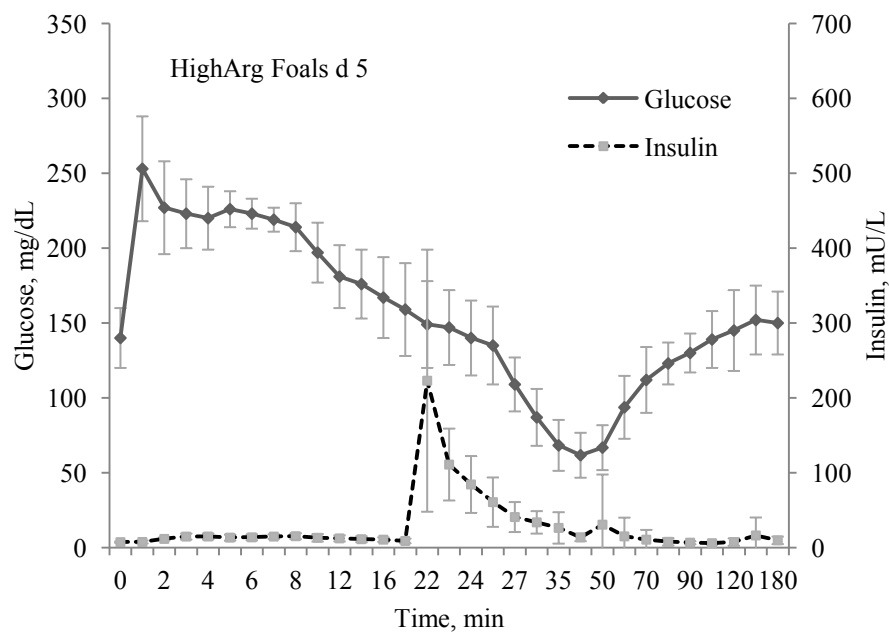


Figure 4 Continued.

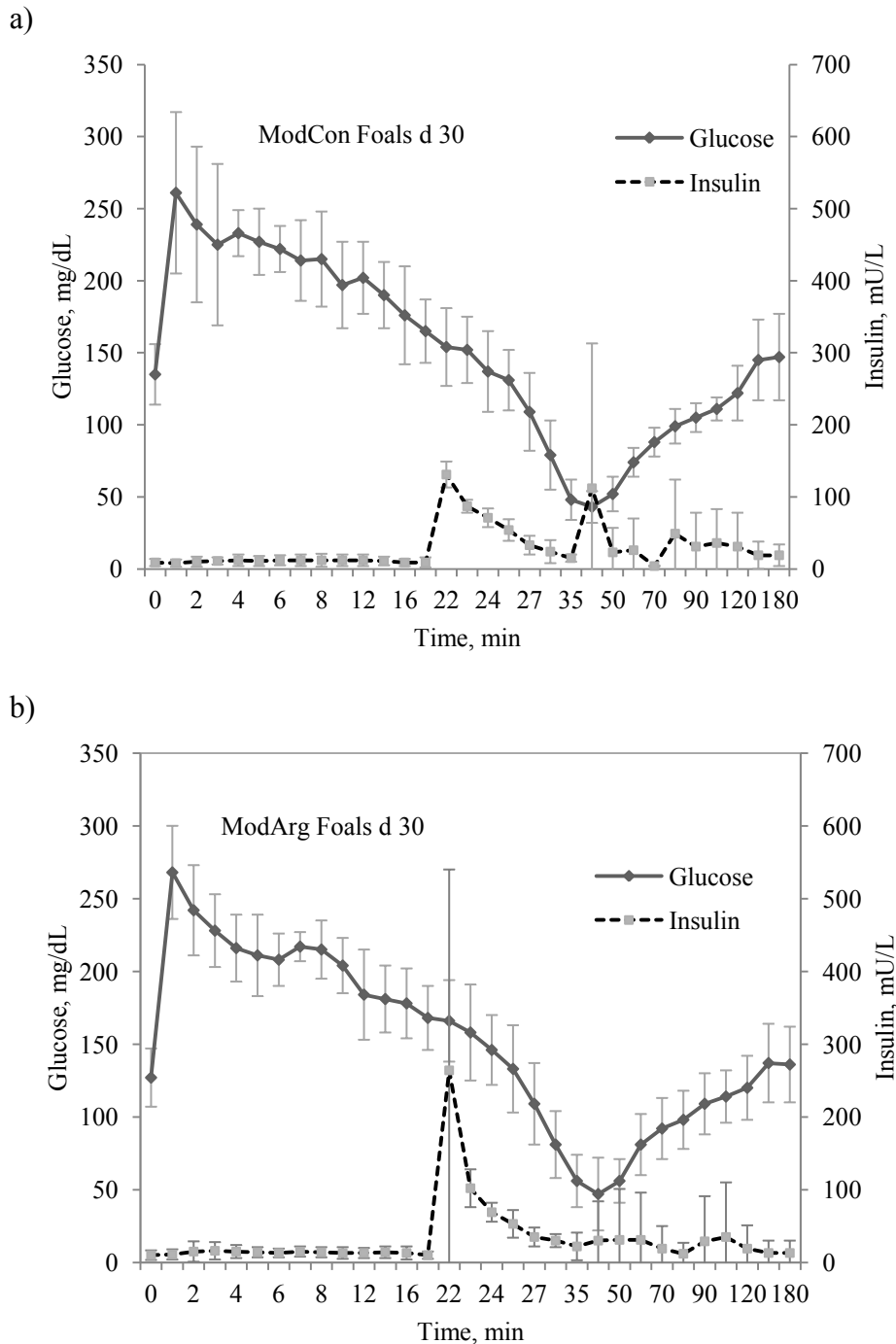
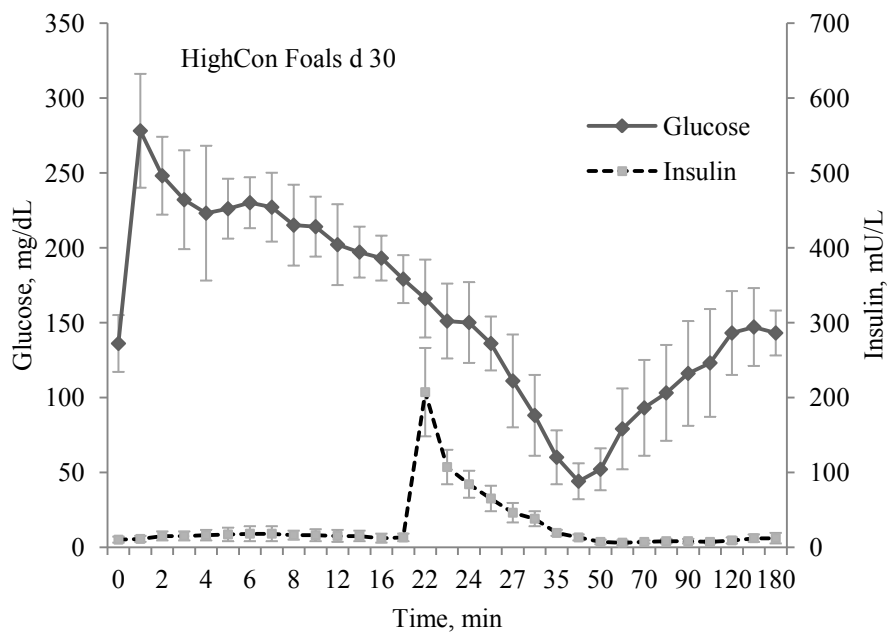


Figure 5. Effects of maternal nutrition and ARG supplementation on foal d 30 glucose and insulin during modified FSIGT. Mare dietary treatments: a) Grain 0.5% BW (as fed) + Alanine (ModCon; n = 7) b) Grain 0.5% BW (as fed) + Arg (ModArg; n = 6) c) Grain 1.0% BW (as fed) + Alanine (HighCon; n = 7) d) Grain 1.0% BW (as fed) + Arg (HighArg; n = 7). Least square means are reported.

c)



d)

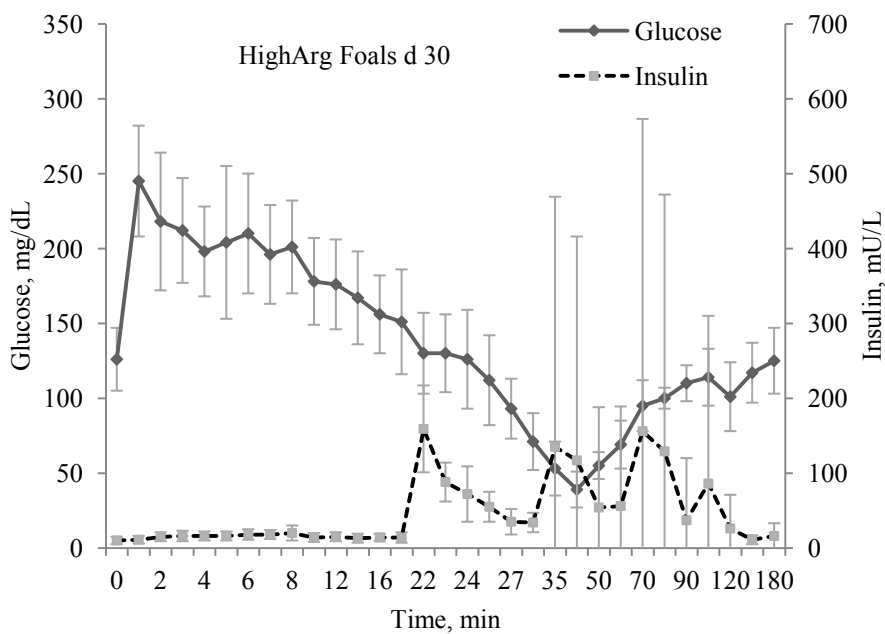


Figure 5 Continued.

In the current study, foal AUC_g decreased and AUC_i increased from 5 to 30 d of age. Previous research has shown the fetal pancreas becomes increasingly responsive to

glucose during late gestation with greater amounts of insulin production in preparation for postnatal glucose homeostasis (Fowden et al., 2005). Once the foal is born, decreased pancreatic activity is observed on the first day of life and progressively increases in the ability to produce insulin for at least 10 d (Holdstock et al., 2004). While these results conflict with previous findings of decreasing insulin concentrations observed in FSIGT performed on foals at 80 and 160 d of age (Winsco et al., 2011), the differences might simply be due to the age of the foals and difference in management across trials. Older foals consume higher percentages of DM, in the form of concentrate and forage, which may alter metabolism and provide variable results compared to younger foals consuming primarily milk. Diet and development of foal metabolism could also be attributed to the differences between foal and mare glucose and insulin concentrations. FSIGT resulted in lower foal concentrations as compared to mares.

Within the data, there were foals with increased insulin concentrations post insulin bolus. In HighArg d 30 foal insulin concentrations the rise in concentrations can be easily seen as additional peaks around min 35, 70 and 120. These additional peaks coincide with a decrease in glucose concentrations. An explanation for these findings remains unclear.

Minimal research in the horse has looked specifically at foal glucose and insulin dynamics, especially relative to maternal nutrition. Additionally, current research differs greatly in experimental design, data collection and analysis, and animal management. Differences in maternal nutrition, foal sampling age, foal sample times and foal sample methods make comparisons difficult. Winsco et al. (2011) found foals from mares supplemented with grain had greater insulin concentrations compared to foals from

mares fed forage only, suggesting maternal grain supplementation decreased offspring insulin sensitivity (SI). George et al. (2009) found foals from mares fed low starch diets had greater glucose clearance and insulin sensitivity as compared to foals from mares fed high starch diets. In the current study, all mares received grain supplementation, and a difference in foal SI relative to nutritional plane was not observed. The significance of this study is the observation of a decrease in peak glucose, which could potentially indicate an increase in SI of foals from mares supplemented with ARG. After the endogenous administration of glucose, foals from ARG mares had lower peak glucose concentrations than foals from Con mares.

Studies in other species, such as the rat, suggest animals exposed to hyperglycemia as a developing fetus, develop impaired glucose tolerance and insulin secretion (Gauguier et al., 1991). In addition, fetal IUGR can alter glucose metabolism during gestation, lead to type 2 diabetes in adult offspring (Simmons et al., 2001) and alter GLUT 4 protein levels (Boloker et al., 2002). Rats from diabetic mothers also demonstrated decreased NO production. Supplementation of ARG has been seen to reduce IUGR in the fetus (Xiao and Li, 2005) and increase NO production in offspring at later stages (Cauanal et al., 2007). While this data is promising, it is difficult to make comparisons across species due to differences in gestational length, fetal growth patterns, and placenta type. Ovine research has shown similar results as ARG supplementation improved fetal growth and development (Wu et al., 2007) and porcine research has shown ARG supplementation increases reproduction and fetal survival rate (Mateo et al., 2007). In other species, the ARG stimulated production of NO appears to be a significant mechanism. Although NO production was not directly measured in the

current study, the increase in NO produced by ARG supplementation could potentially mitigate the effects of over maternal nutrition on mare and fetal metabolism.

Continued research is needed to look at long term effects of maternal nutrition on foal metabolism, and to more thoroughly evaluate the effect of arginine supplementation on the mare and foal. In sheep, ewes fed nutritional planes similar (150% NRC) to the current study resulted in altered mature offspring glucose and insulin metabolism and adipose tissue (Long et al., 2010). In the current study, maternal BW did not significantly increase, but nutritional effect on metabolism was observed which suggest metabolic changes may occur without obesity. Long et al. (2010) observed a 52% increase in BW of obese sheep. Long term effects evaluated offspring of obese sheep. Offspring consumed greater amounts of feed, gained greater amounts of BW and body fat, and demonstrated decreased insulin response and glucose effectiveness during FSIGT as compared to control lambs.

CHAPTER IV

CONCLUSIONS

In conclusion, maternal nutritional plane and ARG supplementation did alter mare and foal metabolism. Results indicated mitigation of increased nutritional plane through ARG supplementation on glucose and insulin dynamics in the reproductive horse. This is the first study to evaluate effects of ARG supplementation in the horse and on fetal programming. Additionally, this study provided valuable information on maternal nutrition and its influence on fetal metabolism, and age related effects on foal metabolism. While further research is needed to determine the direct mechanism and effect of ARG supplementation, results indicate there is an ability of ARG supplementation to increase insulin sensitivity in the horse.

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