MECHANISMS REGULATING PREDISPOSITION OF THE FOAL TO METABOLIC SYNDROME BY MATERNAL OVERNUTRITION

A Dissertation

by

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ABSTRACT

The effects of maternal overnutrition in mares has yet to be fully elucidated; therefore, the objectives of these studies were to determine the effects of maternal overnutrition on neonatal foals at cellular and molecular levels to describe fetal origins of adult disease in the horse. To test these objectives, 16 Quarter Horse mares were used in a completely randomized design and separated by BW, BCS and expected foaling date into one of two dietary treatments consisting of a control (CON; n=8) where diets were formulated to meet dietary requirements during late gestation or overfed (HIGH; n=8) where mares received 40% above CON. Results indicate reduced insulin sensitivity in HIGH mares prior to parturition ($P \leq 0.02$) but was not observed in foals immediately postpartum. Pancreas of HIGH foals had greater number and size of endocrine islets determined by immunohistochemical staining ($P < 0.01$). Relative intensity of immunofluorescent immunoreactive insulin, glucagon and somatostatin within individual islets were not different, however.

Skeletal muscle of HIGH foals expressed larger individual fibers ($P \leq 0.09$) and a shift towards type I slow twitch fibers ($P \leq 0.04$). Changes in expression of genes involved in insulin-stimulated glucose uptake were also observed. Specifically, reduced expression of *glucose transporter 4* and increased expression of *insulin receptor isoform B* and *insulin-like growth factor 1 receptor*. Furthermore, key proteins involved in intracellular insulin signaling were influenced, specifically through increased activation

of extracellular signal-regulated protein kinase 1/2 (ERK1/2) and c-Jun N-terminal kinase (JNK).

Spleen mass was larger in HIGH foals compared to CON (P=0.04), which also resulted in an increase in RBC storage (P<0.01). Lymphocyte localization was not different between dietary treatments; therefore, differences may be with innate immune cells. Collectively, these data are the first to describe the influence of maternal overnutrition on molecular mechanisms related to the predisposition of foals to insulin resistance.

DEDICATION

To: Dr. Josie Coverdale

Mentor, Friend, and Angel

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CONTRIBUTORS AND FUNDING SOURCES

Contributors

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INTRODUCTION

Introduction

Suboptimal conditions during critical periods of fetal development may lead to alterations in offspring phenotype. This is referred to as developmental programming and can be influenced by many environmental factors, such as maternal nutrition, which is known to play a major role. A prominent example of that is the Dutch Hunger Winter during World War II where Germany placed sanctions on the Netherlands, limiting the food supply to the Dutch people. Children born to women pregnant during the winter 1944-1945 experienced a high occurrence of high blood pressure, cardiovascular disease, obesity, and diabetes as adults (Schulz, 2010). More recent evidence has determined the effects to be multi-generational (Veenendaal et al., 2013). As such, the impact of maternal undernutrition has been extensively studied in many species and has been shown to cause intrauterine growth retardation and abnormalities in fetal metabolism that persist into adulthood. This led to the investigation into fetal origins of adult disease.

Although undernutrition is an excellent model for developmental programming in other livestock species, it may not be as applicable with regard to horses. Rather, an overfed model may be more applicable to assess the influence of maternal nutrition on health and performance of the dam and its progeny. This is because many performance horses and broodmares are fed diets high in soluble carbohydrates in an effort to enhance

performance and fetal growth. Specifically, in 2011, the prevalence of obesity in horses was estimated at approximately 50% of the population (Stephenson et al., 2011). The incidence of obesity has been linked to increases in metabolic disorders such as insulin resistance, laminitis, and equine metabolic syndrome, which limit the potential of the equine athlete (Rossdale and Ousey, 2002). To date, the effects of maternal overnutrition during pregnancy on the offspring have yet to be fully elucidated. Studies performed using rat, ovine, and recently equine models have linked overnutrition in the dam to potentially lasting effects on metabolism in offspring (Fowden and Hill 2001; Limesand et al., 2007; Long et al., 2014; Sheen et al., 2018). Research in our lab has begun to identify some of these effects in mares and foals and will be described in further detail in this review (Winsco et al., 2013).

Maternal under- and over-nutrition have influenced glucose metabolism, pancreatic endocrine function, and skeletal muscle response to insulin, adipose tissue metabolism, and alterations in development of many other organ systems. Related to adult-onset metabolic disease, modifications to glucose dynamics and insulin sensitivity in neonates suggest predisposition to adult disease. In humans, the rate of obesity in adult populations has been associated with an increase in obesity rate in children and an increase in type 2 diabetes diagnoses in children and young adults over the last 18 years (Skinner et al., 2018). Research in ovine, bovine, and murine models have documented modifications to glucose and insulin dynamics in response to changes in maternal plane of nutrition in all stages of pregnancy (Limesand et al., 2007; Long et al., 2010; Wilson et al., 2016).

Development and Function of Organs and Tissues

Organ systems develop at different times of gestation, beginning with the central nervous system and ending with maturation of the lungs and respiratory system immediately prior to parturition. Development of organs and functional cells within organs are subject to environmental influences to prepare the fetus for life within that environment. In order to understand changes in organ/tissue development in response to maternal plane of nutrition, normal development of organs and systems must be understood. The pancreas and skeletal muscle are the primary regulators of glucose concentrations, storage, and utilization. Furthermore, the spleen is especially sensitive to maternal plane of nutrition related to size and mass; therefore, this review will focus on the pancreas, skeletal muscle, and spleen development and subsequent influence by maternal plane of nutrition.

Pancreas

Little is known about pancreatic development specific to the horse; however genetic control and expression of transcription factors is likely similar to what has been observed in mice and humans, with differences primarily related to time of gestation (Fowden and Hill, 2001). The pancreas develops in two main phases, primary and secondary, where the pancreatic buds are developed in the primary phase followed by cellular differentiation and activation in the secondary phase. Initiation of pancreatic bud development occurs early in first trimester in conjunction with gastrointestinal development. Cellular differentiation in humans occurs in the second half of the first trimester, and cells are mature and active by the end of first trimester.

The pancreas is derived from the embryonic foregut endoderm and begins with the development of the dorsal bud upon signals from activin, fibroblastic growth factor, and vascular endothelial growth factor (Bastidas-Ponce et al., 2017). Ultimately, the dorsal and ventral buds will fuse to form the adult pancreas. Expression of pancreatic and duodenal homeobox 1 (*Pdx1*) and SRY-Box 9 (*SOX9*) are the primary transcription factors responsible for the stimulation of pancreatic induction with *SOX9* providing a positive-feedback mechanism to prevent cellular default to liver fate (Bastidas-Ponce et al., 2017). Once pancreatic induction has begun, multipotent progenitor cells (MPCs) form under regulation of *Pdx1* and pancreas-specific transcription factor 1a (*Ptf1a*; Burlison et al., 2008). The dorsal bud of the pancreas sits in close proximity to the notochord and aorta; therefore, signaling molecules to induce pancreatic bud formation primarily via suppression of sonic hedgehog (*SHH*) and inclusion of *activin* and fibroblastic growth factor (*FGF*; Bastidas-Ponce et al., 2017). Expression of *FGF*s, *Pdx1*, and *Ptf1a* ultimately prevent the developing pancreas from diverting towards a liver fate in a feed-forward loop (Seymour et al., 2012). Signaling induction of ventral pancreas is still unclear; however, it is known to be derived from the posterior segment of the ventral foregut endoderm (Bastidas-Ponce et al., 2017). The dorsal bud gives rise to the head, neck, body and tail regions of the pancreas where the ventral bud contributes solely to the posterior portion of the head of the pancreas (Uchida et al., 1999).

The specific formation of endocrine cells occurs during the secondary phase of pancreatic development. Endocrine cells are derived from MPCs in the bipotent trunk epithelium (Solar et al., 2009). Endocrine cell fate is determined by the expression of

neurogenin *3* (*Ngn3*; Gradwohl et al., 2000). In mice, the lack of *Ngn3* results in pancreatic development with no endocrine cells resulting in death shortly after birth (Gradwohl et al., 2000). Once committed to an endocrine cell fate, different transcription factors regulate cell specification collectively controlled by *Ngn3*. *Arx* and *Pax4* are dually expressed in endocrine progenitor cells; however, a shift signals specification with high *Arx*, low *Pax4* differentiating α-cells, and low *Arx*, high *Pax4* differentiating β- and δ-cells (Bastidas-Ponce et al. 2017).

Once endocrine cells of the pancreas are mature, the endocrine pancreas functions to regulate fetal blood glucose levels and maintain glucose homeostasis similar to that of a mature animal (Fowden and Hill, 2001). The timing of endocrine maturation varies and has yet to be determined in the horse. In other species, however, this has been investigated with rats reaching endocrine maturation and secreting insulin into fetal plasma at 18 d gestation (term ~21-24 d), sheep at 60 d gestation (term ~147 d), and humans at 84 d gestation (term ~280 d; Fowden and Hill, 2001). Maternal glucose is shunted towards the fetus, increasing fetal blood glucose concentrations. Similar to mature animals, the pancreas responds to elevated blood glucose through the transport of glucose through glucose transporter 2 (GLUT2) on β-cell membranes. On the other hand, when glucose concentrations are low, glucagon is released from pancreatic α-cells, also located within the islets of Langerhans.

The central dogma of glucose-stimulated insulin secretion surrounds the influence of glycolysis paired with ATP production and cellular depolarization (Figure I.1; Meda and Schuit, 2013). Once intracellular, glucokinase converts glucose to

glucose-6-phosphate (G6P) in the first step of glycolysis for ATP production. This first step prevents the return of glucose to circulation and begins the primary sensory mechanism for pancreatic β-cells (Wilson et al., 2017). The metabolism of pyruvate, formed during the last step of glycolysis, enters the mitochondria, generating ATP through the citric acid cycle (TCA) and electron transport chain (Komatsu et al., 2013). The sudden increase in ATP in pancreatic β-cells ultimately stimulates the release of insulin via exocytosis. As a result, ATP-sensitive K^+ channels close leading to membrane depolarization, subsequent Ca^{2+} influx, and insulin exocytosis (Komatsu et al., 2013). Once in circulation, insulin reacts with the insulin receptor on insulin-sensitive tissues to stimulate glucose clearance from circulation.

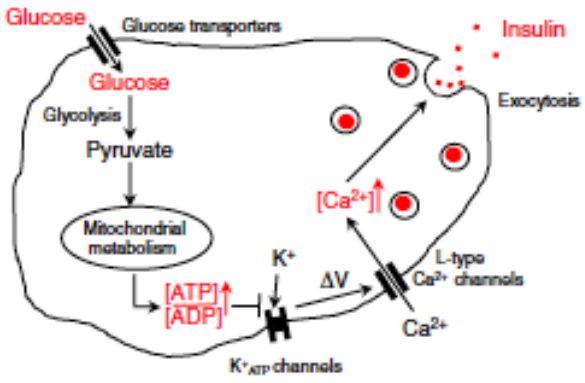

Figure I.1 Regulation of glucose-stimulated insulin secretion from pancreatic β-cells (Meda and Schuit, 2013).

When glucose concentrations in circulation are low, insulin secretion from β-cells is suppressed and α-cells begin secreting glucagon to stimulate the liver to increase gluconeogenesis and glycogenolysis. The specific signals for α-cell stimulation is debated; however, it is suggested that multiple paracrine signals stimulate and/or inhibit the secretion of glucagon to promote glucose homeostasis (Figure I.2 and I.3; Briani et al., 2018). When glucose is high in circulation, insulin is released to stimulate glucose uptake by peripheral tissues, which lowers Ca^{2+} concentration in α-cells, reducing glucagon release. Additionally, somatostatin is released in a pulsatile manner corresponding with insulin and gamma-aminobutyric acid (GABA) release from β-cells. Together, GABA and somatostatin express an inhibitory effect on α-cells to reduce glucagon secretion. Figure I.4 expresses the pulsatile manner in which insulin and somatostatin are secreted in conjunction and the opposing release of glucagon when insulin concentrations are suppressed (Watts et al., 2016).

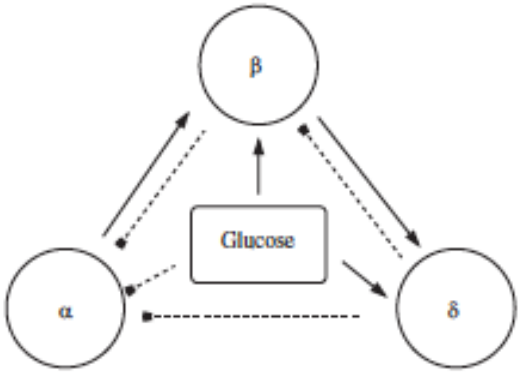

Figure I.2 Paracrine interaction among endocrine cells of the pancreatic islets of Langerhans (Watts et al., 2016).

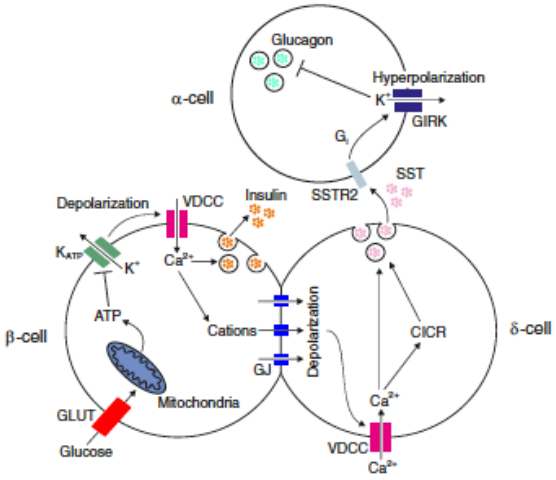

Figure I.3 Inhibition of glucagon via paracrine signaling between β- δ- and α-cells (Briant et al., 2018).

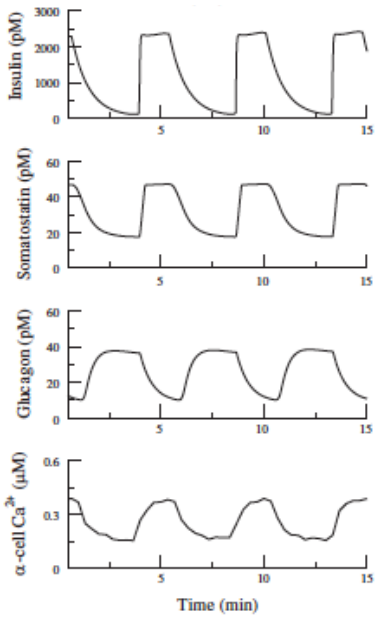

Figure I.4 Effect of increasing insulin on somatostatin and glucagon release from δ-cells and α-cells, respectively (Watts et al., 2016).

Secondary stimulation of insulin release occurs via post-prandial secretion of incretins from enteroendocrine K/L cells including, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1; Nauck and Meier, 2018). When released from enteroendocrine cells, incretins bind to their respective receptors on pancreatic β-cells, increase cAMP and Ca2+ thereby stimulating the release of insulin (Kim and Egan, 2008). It is suggested that incretins are responsible for as much as 50% of β-cell stimulation (Nauck and Meier, 2018). Determination of insulin sensitivity is determined using either frequent sampling i.v. glucose tolerance tests (FSIGTT) or an oral glucose sensitivity test. Using FSIGTT removes the influence of incretins on insulin stimulation and allows for independent determination of glucose-stimulated insulin release. Oral glucose tolerance tests include incretin response, but exact absorption of glucose from the digestive tract is uncertain. Both methods, along with the hyperinsulin euglycemic clamp, are accepted means of determining insulin sensitivity and diagnosing insulin resistance or type 2 diabetes; however, it is important to determine which method is most reliable to test the desired hypothesis.

Skeletal Muscle

Very little research has been performed in the area of prenatal development of equine skeletal muscle. Much of the research has focused on effects of training on skeletal muscle fibers and adaptation; however, it has yet to be determined whether prenatal programming may be limiting the athletic potential of young horses as they enter training. Other livestock species are well ahead in investigations of fetal skeletal muscle, and most conclusions in the horse are derived from information in other species,

which may not be appropriate but provides important information and a basis of understanding.

Similar to pancreatic development, skeletal muscle development occurs in primary and secondary phases. Skeletal muscle originates from somites in the embryonic, paraxial mesoderm (Yan et al., 2013; Nesvadbova and Borilova, 2018). The primary phase of development occurs during the embryonic stage where skeletal muscle fibers, adipocytes, and chondrocytes derive from the same mesenchymal cell population and may be considered competitive processes (Yan et al., 2013). Myoblasts, cells specifically committed to skeletal muscle fate, derive from myotomes with limb muscles originating from the hypaxial myotome (Yokoyama and Asahara, 2011). Commitment involves activation to myogenic progenitor cells via Wingless and Int (Wnt) signaling with expression of *Pax3*, *Pax7*, and myogenic regulatory factors (MRF; Bailey et al., 2001). Following specification to myoblast fate, cells increase in number and fuse to form multinucleated, immature myotubes. The process by which this fusion of myoblasts occurs is not fully understood but signals the end of the embryonic development phase or primary skeletal muscle development.

Secondary myogenesis occurs during the fetal stage of development where the majority of mature muscle fibers differentiate. The number of skeletal muscle fibers formed during secondary myogenesis set the stage for skeletal muscle fiber number in postnatal life and is dependent upon the number of myogenic progenitor cells differentiated during embryonic development. Furthermore, differentiation during secondary development is sensitive to changes in maternal environment, including

maternal plane of nutrition. While skeletal muscle fiber number is fixed, skeletal muscle fiber type and size are programmed during secondary skeletal muscle development.

Skeletal muscle fiber types are classified based on fiber metabolism. Type I skeletal muscle fibers are slow twitch, oxidative fibers, meaning they are slow to react and similarly fatigue, and have a large volume of mitochondria for oxidative metabolism of energy substrates. Type II skeletal muscle fibers are fast-twitch fibers, which are quick to contract, but are also comparatively quick to fatigue. Type II fibers can be further classified as type IIa and type IIx. Type IIa fibers are fast-twitch oxidative fibers where they are similar to type I in their oxidative capacity; however, are quicker to contract. Type IIx fibers, however, are fast-twitch glycolytic with the same capacity for quick contractility, but with fewer mitochondria for limited oxidative capacity. In the horse, type I skeletal muscle fibers are the least abundant followed by type IIx with most abundant being fast-twitch oxidative type IIa fibers.

Energy metabolism is slightly different by skeletal muscle fiber type; however, all skeletal muscle fibers are responsive to insulin stimulation for glucose uptake under normal metabolic conditions. Upon the release of insulin by pancreatic β-cells described in the previous section, insulin reacts with insulin-specific receptors on the surface of skeletal muscle plasma membrane. Upon binding to the receptor, tyrosine kinase is phosphorylated on the intracellular portion of the receptor stimulating downstream activation of key signaling pathways (Figure I.5; Shepherd and Kahn, 1999). Phosphoinositide-3 kinase (PI3K) phosphorylation is the rate-limiting step in stimulating glucose uptake. Following phosphorylation of PI3K, protein kinase B (AKT) is

activated by phosphoinositide-dependent kinase 1 ultimately resulting in the translocation of glucose transporter 4 (GLUT4) from an intracellular vesicle to an active membranous transporter (Alessi et al., 1997). Once intracellular, glucose may be metabolized for adenosine triphosphate (ATP) production or stored for later use as glycogen.

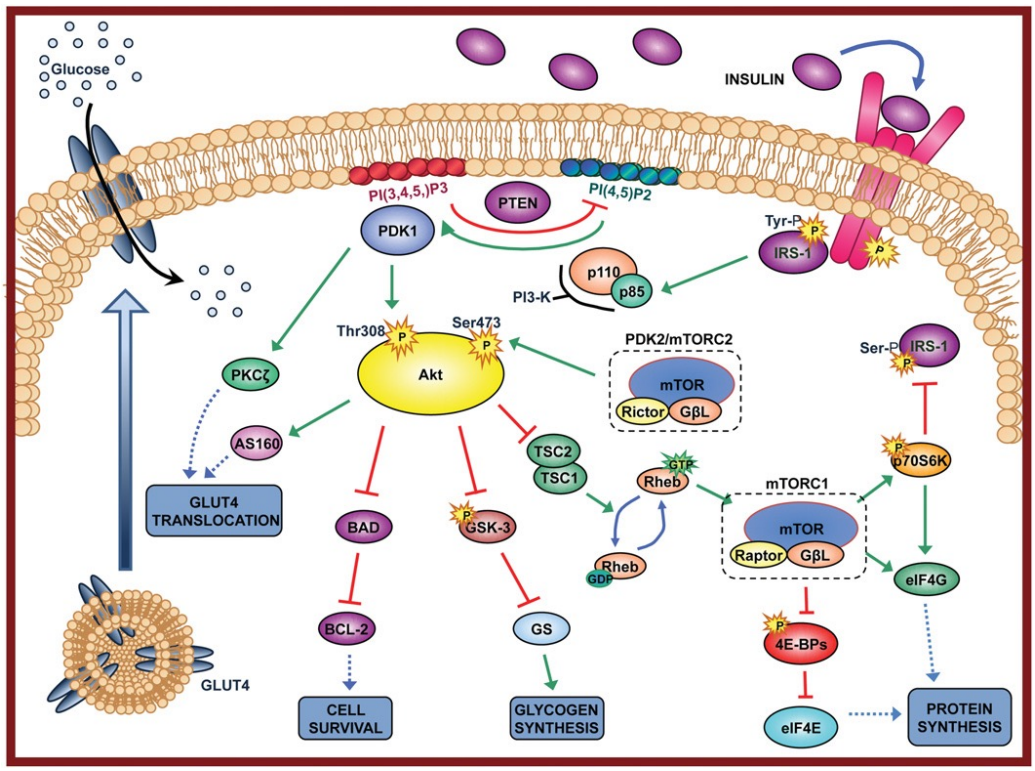

Figure I.5 Insulin-stimulated glucose uptake in skeletal muscle of healthy individuals (Warner and Ozanne, 2010).

Insulin bound to its receptor on skeletal muscle also influences other metabolic pathways involved in cell proliferation resulting in a signaling cascade with extracellular signal-regulated kinase (ERK) phosphorylation to increase transcription thereby promoting cell proliferation through increased protein synthesis (Saltiel and Kahn, 2001; van Ginneken et al., 2006). Furthermore, inflammatory cytokines associated with increased adiposity including tumor necrosis factor-α (TNFα) and free fatty acids (FFAs) activate another intracellular signaling cascade in the c-Jun amino-terminal kinase (JNK) pathway interfering with communication by insulin to stimulate GLUT4 translocation (Guo et al., 2014; Hirosumi et al., 2002).

Insulin-dependent glucose uptake is similar across skeletal muscle fiber types; however, the utilization of glucose or stored glycogen differs under various exercise conditions. Briefly, when ATP levels plummet, glycogenolysis and glycolysis are stimulated in-party by glucagon released from pancreatic α-cells. Type I and type IIa skeletal muscle fibers utilize glucose in the presence of oxygen through glycolysis where resulting pyruvate enters the TCA cycle. Products from the TCA cycle including nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) enter the electron transport chain within mitochondria to produce the greatest number of ATP molecules. Unfortunately, this process may only occur under oxidative conditions or aerobic exercise. Type IIx skeletal muscle fibers lack significant mitochondria; therefore, they lack the ability to oxidize glucose via the TCA cycle or electron transport chains (Zierath and Hawley, 2004). As such, type IIx fibers rely solely on glycolysis for ATP production resulting in lactic acid buildup, lesser ATP

production, and increased rate of skeletal muscle fatigue (Zierath and Hawley, 2004). Although they fatigue quicker and produce lesser ATP per glucose molecule compared to type I and type IIa fibers, type IIx skeletal muscle fibers are imperative for quick contractility during anaerobic exercise.

Spleen

Fetal development of the spleen is not well understood. Much of the work investigating splenic development focuses on immune and lymphatic development or erythropoietic development, independently. The bulk of data compiled is in humans and rats with very little information in livestock species. There is currently no information on splenic development in equine species. The differences between splenic development in humans and rats is vast in relation to timing; however, order of events appear to be similar and therefore may be able to be applicable to livestock species.

The spleen is the largest organ of the lymphatic system serving two main functions: to filter and replace erythrocytes in circulation, and to store white blood cells to mount an immune response in the presence of a foreign invasion. It is made up of two main histological regions; the red and white pulp. Red pulp is predominantly responsible for filtration of erythrocytes while white pulp is responsible for immune cell storage and immune response. Description of development of the spleen is generally divided into the development of immune function and development of red pulp and hematopoiesis.

The spleen develops in the dorsal mesogastrium of the mesoderm in a similar region to the pancreas (Endo et al., 2015). The red pulp matures prior to the white pulp

in the early stage of development during spleen structure establishment in order to prepare the spleen to take over hematopoiesis from the placenta until bone marrow is mature (Endo et al., 2015; Vellguth et al., 1985) Hematopoietic cells are detectable as early as week 7 of gestation in humans with establishment of characteristic lobules and functional vasculature soon to follow (Endo et al., 2015; Vellguth et al., 1985). The white pulp and immune functions of the spleen begin to develop simultaneously with the establishment of lobules and functional vasculature (Vellguth et al., 1985).

In humans, the white pulp develops around 15 weeks of gestation with clusters of intermediate-sized immature lymphocytes collecting around the developing arterioles (Timens et al., 1987). At approximately 22 weeks, T and B lymphocytes begin to segregate and by week 24, white pulp and surrounding marginal zone framework is established (Satoh et al., 2009). In rats, small numbers of immature B and T lymphocytes are not observed until birth with formation of the marginal zone framework occurring approximately a week postpartum. Germinal centers, sites of B cell proliferation and development, are not present until the time of weaning (Holsapple et al., 2003).

Once mature, B and T lymphocytes express specific membranous proteins often used for localization and quantification of respective lymphocytes in histological and protein analyses. Specifically, B lymphocytes express CD20 on the membrane and T lymphocytes similarly express CD3 on the membrane of both cytotoxic and helper T cells, which aid in scientific determination of adaptive immune cell makeup of the spleen. At birth, the spleen no longer performs hematopoiesis; however, stored

erythrocytes are often determined in immunohistological staining through an iron-specific stain, which stains the oxygen-carrying hemoglobin.

Of particular importance to the equine athlete, the mature spleen acts as a reservoir for erythrocytes and filters/replaces old and damaged erythrocytes. Blood from circulation enters the spleen through a series of "cords" and sinuses which are lined with unique endothelial cells and stress fibers (Drenckhahn and Wagner, 1986). When it becomes difficult for erythrocytes to pass through the sinuses due to aging and stiffening of membranes, they are phagocytized by residing macrophages (Mebius and Kraal, 2005). Following erythrophagocytosis, macrophages of the spleen are able to recycle the iron present in hemoglobin of phagocytized erythrocytes and store or release as ferritin (Knutson and Wessling-Resnick, 2003). If released, the ferritin can readily bind to transferrin, an important transporter protein (Knutson and Wessling-Resnick, 2003). Furthermore, the macrophages can use released iron to determine invasion by certain bacteria with a high affinity for iron (Ratledge and Dover, 2000)

In the horse, the spleen is able to contract during maximal exercise, releasing its erythrocyte stores into circulation, increasing hematocrit and oxygen carrying capacity in an effort to delay the onset of fatigue (McKeever et al., 1993). Splenic contraction is induced by an increase in epinephrine in response to exercise and can increase erythrocyte volume by up to 50% (Kurosawa et al., 1998). The spleen in the horse is able to store more erythrocytes than in other species because increased contraction of stress fibers creates a reservoir. At rest, the increased erythrocyte reservoir acts to

reduce stress on the heart by reducing viscosity through reduced PCV (Mebius and Kraal, 2005).

Related to immune function, the spleen plays a role in both innate and adaptive responses. The white pulp plays a role only in adaptive immune responses; however, the marginal zone is able to mount both an innate and adaptive response because of the presence of both macrophage and B-cell populations (Mebius and Kraal, 2005). Macrophages of the marginal zone and the presence of blood-borne pathogens activate B-cells in the marginal zone to initiate an adaptive immune response. The activated B-cells then act as antigen-presenting cells, migrating to the white pulp and activating helper T-cells (Attanavanich and Kearney, 2004). Upon activation of T-cells, they migrate to the edge of the B-cell follicles and are able to exit the spleen via lymphatic vessels to mount an immune response against blood-borne pathogens (Mebius and Kraal, 2005).

Insulin Resistance and Metabolic Disease

Adult-onset metabolic disease is becoming increasingly more common with the incidence of obesity rising. Further, the increase in adult obesity is mirrored by an increase in childhood obesity and an earlier onset of adult metabolic diseases. Often this is related to glucose homeostasis and insulin sensitivity; therefore, the tissues most affected are the pancreas and insulin-sensitive tissues, including skeletal muscle. Metabolic disease is characterized by increased adiposity, obesity, insulin resistance, high blood pressure, and cardiac diseases. Below will detail the influence of insulin resistance and metabolic disease specifically related to the pancreas and skeletal muscle.

Pancreas

Stimulation of the pancreas to release insulin from islet β-cells is imperative for normal glucose regulation. Metabolic dysregulation in the form of insulin resistance or type 2 diabetes mellitus has a significant impact on the pancreas, resulting in hyperinsulinemia followed by β-cell dysfunction. The function of β-cells is closely associated with the physiology and metabolism of insulin-sensitive tissues and are heavily regulated by glucose concentrations in circulation (Cerf, 2013b). Insulin resistance occurs prior to the onset of type 2 diabetes in humans, mice, and livestock species. While horses rarely enter into a state of diabetes, insulin resistance is commonplace and occurs in conjunction with obesity or other metabolic diseases, including equine metabolic syndrome and pituitary pars intermedia dysfunction (Johnson et al., 2012).

The first measurable indication of metabolic dysregulation is hyperinsulinemia at either a basal state or through glucose challenge. When glucose uptake by insulin-sensitive tissues is compromised by obesity, inflammation, or insulin resistance, the pancreas attempts to compensate for the diminished response to insulin by stimulating β-cell replication (Figure I.6; Cerf, 2013b). The increase in β-cell size and number is described as β-cell compensation and results in increased insulin secretion in response to hyperglycemia or post-prandial incretin-stimulation (Tarabra et al., 2012). When β-cell compensation cannot be sustained to address chronic hyperglycemia, β-cell dysfunction occurs as a result of increased apoptosis (Cerf, 2013b).

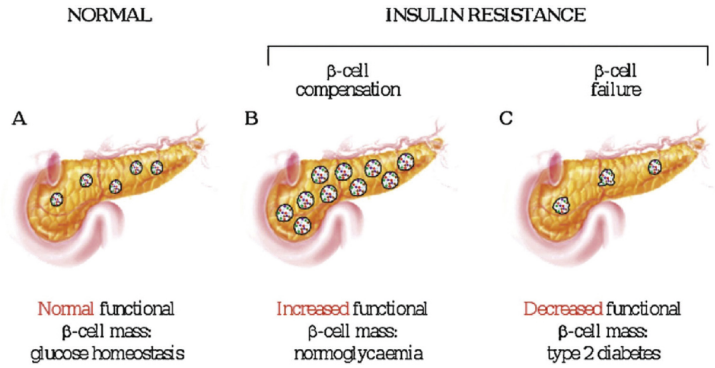

Figure I.6 β-cell compensation in insulin resistant state (El-Kafrawy et al., 2014).

If hyperinsulinemia, described in humans as pre-diabetes, is not appropriately addressed through diet modifications and exercise, apoptosis takes over the ability of β-cells to replicate and compensate, resulting in β-cell loss. Once the population of β-cells declines beyond a point of normal insulin secretion, the patient is diagnosed with type 2 diabetes mellitus (Figure I.7; Rhodes, 2005; Weir and Bonner-Weir, 2004). In early stages of the disease, reversal is possible; however, if not addressed and the disease is allowed to progress, amyloid plaques begin to deposit in pancreatic islets and result in irreversible damage to the pancreas (Weir and Bonner-Weir, 2004). In humans, this occurs approximately 10 years after initial diabetes diagnosis and insulin or incretin therapies are required (Swinnen et al., 2009).

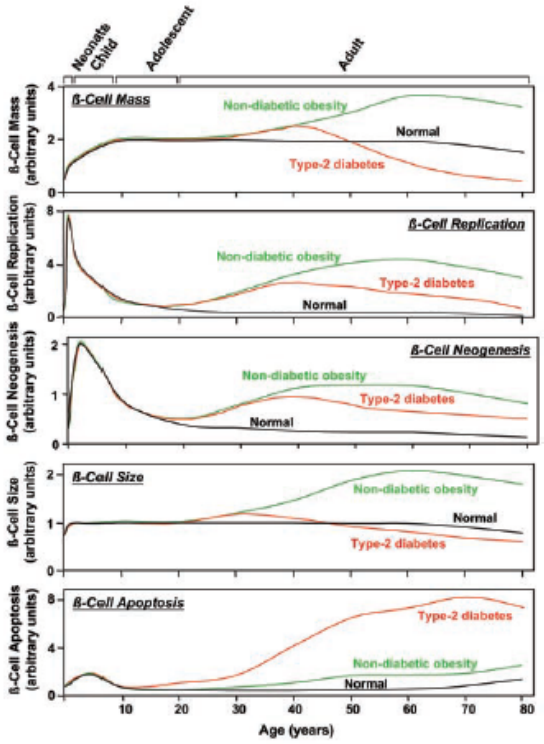

Figure I.7 Postnatal pancreatic β-cell growth in normal, non-diabetic obese, and type 2 diabetic humans (Rhodes, 2005).

As is common with most metabolic diseases, insulin resistance and type 2 diabetes mellitus are multifactorial; therefore, hyperglycemia is not the only culprit in disease progression. Obesity is also associated with increased inflammation and oxidative stress, which play an additional role in pancreatic β-cell damage and peripheral tissue insulin insensitivity. Chronic hyperglycemia and obesity-associated inflammation increase formation of reactive oxygen species resulting in oxidative damage to mitochondria, endoplasmic reticulum, and DNA (Yuzefovych et al., 2013).

Cytokines and adipokines present during the inflammatory response and through excess adipose tissue stimulate a cascade of intracellular signaling leading to cellular

damage through oxidative stress and stimulation of apoptosis. These cytokines include tumor necrosis factor α (TNFα), interferons, interleukins, and prostaglandins. The exact mechanism by which inflammatory cytokines influence pancreatic β-cells is unclear. More extensive research has been performed in insulin-sensitive tissues instead of insulin secreting cells. These data are described further in the following section. It has been suggested that the influence of inflammatory cytokines on insulin release and β-cell health is primarily related to oxidative stress and infiltration of macrophages and other immune cells (Esser et al., 2014). It is believed that the primary cytokine resulting in changes to β-cell metabolism is interleukin-1β (IL-1β; Maedler et al., 2002). Increase in IL-1β presence in pancreatic islets stimulates recruitment of macrophages and other immune cells leading to cellular damage and apoptosis (Esser et al., 2014). These effects stimulate a change in cell metabolism resulting in β-cell compensation and subsequent β-cell decline.

Insulin resistance in the horse is similarly characterized by hyperinsulinemia and regional adiposity. While equine diets are naturally low in fat, diets high in non-structural carbohydrates are common. Excess carbohydrate intake alone can result in reduced insulin sensitivity; however, the conversion of excess carbohydrate to fat via de novo lipogenesis exacerbates the effects of carbohydrates resulting in obesity and related inflammatory effects on insulin sensitivity (Frank et al., 2010).

The similarity in insulin resistance and type 2 diabetes mellitus between horses and humans is striking. Horses exhibit hyperinsulinemia, hyperglycemia, hypertriglyceridemia, and increased systemic markers of inflammation (Frank et al.,

2010). Horses also express a predisposition to laminitis, which may be performance-ending for many horses. While type 2 diabetes mellitus is rare in horses, insulin resistance is common and occurs through similar mechanisms described in humans and murine models. For example, obesity has been associated with an increase in circulating cytokines, increased postprandial insulin secretion, sustained hyperglycemia, and increased leptin concentrations (Carter et al., 2009; Frank et al., 2010; Vick et al., 2007). In horses diagnosed with equine metabolic syndrome, an acute inflammatory insult resulted in sustained insulin release and dysregulation of glucose homeostasis, ultimately confirming insulin resistance and a state of β-cell compensation common in insulin resistance and pre-diabetes in humans and murine models (Tadros et al., 2013).

With glucose and insulin dysregulation documented at the pancreatic level, it is important to then investigate the changes in insulin-target tissues. Skeletal muscle is the primary site of insulin-stimulated glucose uptake, and even slight adjustments in glucose uptake will have significant influence on whole-body glucose homeostasis, energy utilization, and animal performance. Often, horses' performance careers will span across 20 years; therefore, maintenance of glucose homeostasis is important for longevity.

Skeletal Muscle

The sensitivity of skeletal muscle to insulin signaling for glucose uptake is imperative for glucose homeostasis and the maintenance of normal metabolism. In the case of metabolic disease or insulin resistance, skeletal muscle becomes less sensitive to insulin released by the pancreas. As a result, the pancreas releases greater

concentrations of insulin associated with β-cell compensation. Hyperinsulinemia does not result in fewer insulin receptors or affinity of insulin receptor, as may be expected. Instead, resistance occurs within intracellular signaling pathways ultimately leading to reduced translocation of GLUT4 (Cho et al. 2001; Deshmukh, 2015; Guo et al., 2014; Hirosumi et al., 2002).

The most obvious of the pathways to be influenced is the AKT signaling pathway because of its direct relation to translocation of GLUT4 to an active membranous transporter. Knockout models suggest that mice lacking AKT are more likely to develop type 2 diabetes compared to wild type (Cho et al., 2001). Similarly, studies in human patients have determined reduced AKT signaling in skeletal muscle results in the reduced translocation of GLUT4 and sustained levels of hyperglycemia in individuals with type 2 diabetes (Karlsson et al., 2005). Surprisingly, this pathway is not the only pathway impacted in insulin resistance or metabolic disease.

Currently, one of the most heavily influenced pathways is the JNK pathway because of its association with obesity and inflammation. Specifically, excessive adiposity increases the release of FFAs and inflammatory cytokines including TNFα, which decrease intracellular signaling of insulin to stimulate glucose uptake (Figure I.8 ;DeFronzo et al., 2015). When TNFα binds to its TNF receptor and FFAs bind to toll-like receptor 4 (TLR4) on skeletal muscle membrane, there is a resulting increase in phosphorylation of JNK resulting in downstream inhibition of insulin signaling of the PI3K-AKT pathway via serine phosphorylation of insulin receptor substrate 1(IRS-1). Serine phosphorylation of IRS-1 inhibits phosphorylation of AKT thereby down-

regulating the PI3K-AKT pathway and reducing GLUT4 translocation (DeFronzo et al., 2015; Hirosumi et al., 2002). Furthermore, an increase in JNK phosphorylation results in increased activation of inflammatory pathways and cellular response to stress, which contribute to the secondary symptoms of metabolic disease and insulin resistance (Solinas and Becattini, 2017).

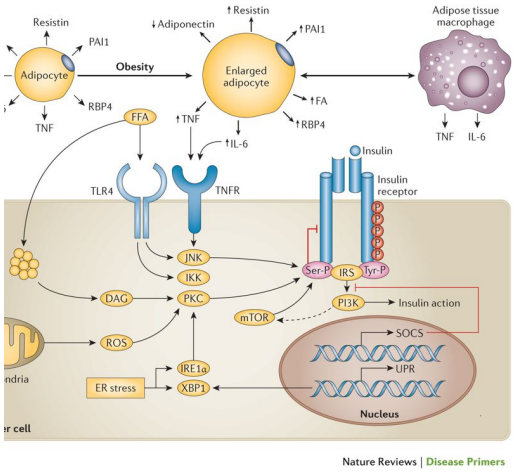

Figure I.8 Influence of adiposity on reduced insulin sensitivity in skeletal muscle (DeFronzo et al., 2015).

Lastly, the cell proliferation pathway involving ERK signaling has similarly been implicated in insulin resistance and metabolic disorders via up-regulation of ERK phosphorylation (Jiao et al., 2013; Ozaki et al., 2016). It is suggested that increased ERK phosphorylation reduces AKT phosphorylation thereby contributing to insulin

resistance by reducing the translocation of GLUT4 (Jiao et al., 2013). Furthermore, elevated phosphorylation of ERK may result in dephosphorylation of forkhead box O1 (FOXO1) causing increased gluconeogenesis when gluconeogenesis should be suppressed (Wu et al., 2010). Because of the multi-factorial effects on various insulin-signaling pathways, ERK has been investigated as a target for the development of treatments against insulin resistance and associated metabolic disorders (Ozaki et al., 2016).

Influence of Maternal Nutrition on Organ Development and Function

Many environmental factors influence offspring phenotype during critical periods of development. The effects became apparent following the Dutch Hunger Winter of 1944-1945 when the German government placed sanctions on the Netherlands, leaving food scarce. Years later, children born to mothers pregnant during the Dutch Hunger Winter had an increased incidence of diabetes, hypertension and cardiovascular disease (Veenendaal et al., 2013). Environmental factors now known to contribute to offspring phenotype and epigenetic modifications include nutrition, air pollution, environmental toxins, and certain drug classes. The prevalence of obesity in humans and domesticated animals makes maternal plane of nutrition and the influence of obesity on offspring an important field of study.

In livestock, both under- and overnutrition during fetal development have been documented to negatively impact offspring development. In periods of drought, undernutrition may be prevalent in some livestock species reared for human consumption. In horses, overnutrition tends to be more common with many horses fed

concentrate diets high in non-structural carbohydrates. With the understanding that maternal plane of nutrition influences offspring phenotype and metabolism, subsequent understanding of mechanisms underlying fetal origins of adult disease are becoming well-documented more than ever before. The field of equine science is just beginning to document these molecular and phenotypic changes in the horse. Because of this, equine scientists must make inferences from other species to begin to answer questions specific to the horse. The abundance of research has been performed using murine and ovine models.

Pancreas

Both under- and over-nutrition during pregnancy have been implicated in offspring pancreatic development and function, predisposing offspring to adult-onset metabolic diseases, including insulin resistance. In livestock species, under-nutrition has been most extensively studied; however, in murine species, overnutrition has been heavily investigated related to human nutrition and health. Insufficiencies in maternal plane of nutrition, whether over- or undernutrition during gestation, alter maternal glucose and insulin dynamics suggesting dysfunction of pancreatic or insulin-sensitive tissues to respond to glucose or insulin, respectively. A study performed in our lab by Winsco et al. (2011) overfed mares during the third trimester of gestation and observed reduced insulin sensitivity in mares 20 d prior to expected parturition in overfed mares compared to control. In offspring, a similar reduction in insulin sensitivity was observed, but was not evident until 6 months postpartum (Winsco et al., 2011).

Similarly, a study in which mares were overfed from mid- to late gestation described a reduction in insulin sensitivity in foals by 19 mos. of age (Robles et al., 2017).

The mechanism by which foals experienced reduced insulin sensitivity by 6 months of age following exposure to high maternal plane of nutrition has yet to be determined in the horse; however, research in other species suggests the pancreas plays a prominent role. The data from these studies are important for developing hypotheses and inferences for equine research. While the effect of over- and undernutrition on pancreatic development appear to oppose one another, insulin resistance and type 2 diabetes mellitus are consistently the end result.

As previously described, overnutrition is more prevalent in the equine industry. Data from nutrient restriction models of developmental programming nevertheless provide valuable information. The thrifty hypothesis developed by Dr. Barker suggests that fetal exposure to maternal undernutrition results in offspring prepared for a nutrient restricted environment; therefore, they are programmed to thrive with lesser nutrient availability (Figure I.9; Hales and Barker, 2001). If these offspring are then exposed to sufficient or excess nutrients postpartum, the consequence is obesity and all associated metabolic disorders (Hales and Barker, 2001). The influence of maternal undernutrition compared to overnutrition on the pancreas are opposite but interestingly, the end result of both circumstances is obesity, type 2 diabetes mellitus, cardiovascular disease, and hypertension (Fowden et al., 2013; Hales and Barker, 2001).

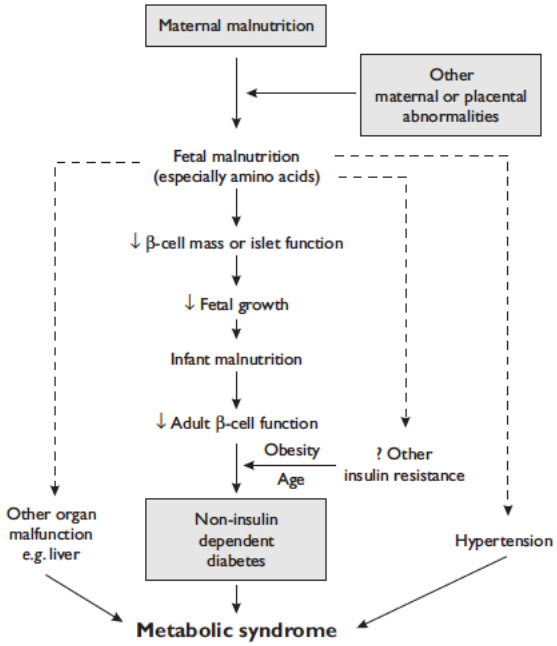

Figure I.9 Influence of maternal undernutrition on the development of metabolic syndrome in offspring (Hales and Barker, 2001).

Maternal undernutrition resulting in intrauterine growth restriction often results in reduced pancreatic mass and endocrine islet area (Petrik et al., 1999). Offspring exposed to low-calorie diets *in-utero* consistently have fewer pancreatic islets with reduced β-cell mass (Boehmer et al., 2017; Garofano et al., 1998; Limesand et al., 2005). As would be expected, reduced β-cell mass results in reduced insulin secretion in offspring and type 2 diabetes mellitus (Limesand et al., 2006). The cause of diminished β-cell mass is believed to be a defect in β-cell replication and altered expression of genes involved in apoptosis, proliferation, and neogenesis (Boehmer et al., 2017; Garofano et al., 1998; Limesand et al., 2005). Offspring exposed to a low protein diet express similarly reduced β-cell numbers at birth, which persists into adulthood and were

attributed to a reduction in *Pdx1* and *IGF-II* expression (Arantes et al., 2002; Petrik et al., 1999). As previously described, *PDX1* is the primary regulatory transcription factor in early pancreatic development to stimulate both endocrine and exocrine differentiation. Sensitivity to IGFs occurs later in gestation; therefore, the timing of maternal plane of nutrition modification affects the organ differently but may result in the same phenotypic expression.

Maternal overnutrition has been less extensively studied, and the mechanism predisposing offspring to adult metabolic disease is not as clearly outlined as that of maternal undernutrition. There is some evidence using ovine and murine models, however. Maternal obesity and overnutrition on the pancreas have revealed variable results. Some studies have observed similar results to what is observed following maternal undernutrition: reduced pancreas size, reduced islet area, and reduced β-cell mass and number (Frantz et al., 2011; Zhang et al., 2011). Other studies, however, have observed increases in islet area and subsequent increases in β-cell number and mass (Ackermann and Gannon, 2007; Ford et al., 2009). The difference is likely related to timing and severity of exposure to maternal overnutrition.

In studies where maternal obesity and overnutrition resulted in similar results to maternal undernutrition, the obesity or overnutrition exposure was extreme and occurred early in gestation (Caton et al., 2009; Zhang et al., 2011). Similarly, in these studies, the rate and length of overnutrition exposure was significant enough to induce intrauterine growth restriction, which explains the similarity in pancreatic effect to nutrient restricted models (Zhang et al., 2011). When imposed for shorter periods of gestation, maternal

overnutrition has resulted in increased β-cell mass, similar to what is observed during β-cell compensation in early insulin resistance prior to diagnosis of type 2 diabetes mellitus (Ford et al., 2009). These data suggest that length and severity of changes in maternal plane of nutrition stimulate pancreatic changes similar to the pathophysiology of insulin resistance and type II diabetes mellitus in mature animals. Specifically, short-term exposure to overnutrition whether *in-utero* or postpartum results in β-cell compensation while long-term exposure results in β-cell loss.

The consistent influence of maternal plane of nutrition on the pancreas demonstrates the potential for downstream impact on insulin-sensitive tissues. With skeletal muscle being the primary tissue for insulin-stimulated glucose uptake, it will be important to investigate the potential effects on glucose and insulin metabolism with skeletal muscle as an important contributor. As with the pancreas, skeletal muscle may be influenced by both under- and overnutrition of the dam.

Skeletal Muscle

The effect of maternal plane of nutrition may influence either embryonic or fetal stages of myogenesis. Embryonic influences of maternal nutrition primarily affect total skeletal muscle fiber number. The number of skeletal muscle fibers present at birth is regulated by the number of available MPCs, which is susceptible to effects of maternal plane of nutrition during the embryonic phase of development (Nesvadbova and Borilova, 2018). Following the embryonic phase of development, skeletal muscle fiber number is effectively fixed. The influence of maternal plane of nutrition during the fetal phase of skeletal muscle tissue development primarily alters skeletal muscle fiber size,

type, adiposity, and collagen. Although skeletal muscle fiber number may not be influenced when maternal plane of nutrition is altered during the fetal phase, these effects during secondary myogenesis may significantly influence the ability of skeletal muscle to adapt postnatally. Furthermore, this may influence the ability of the horse to adapt to training and appropriately metabolize energy substrates for performance.

When maternal nutrition is restricted in the peri-implantation period, the result is reduced skeletal muscle density; however, when the nutritional insult is induced during late gestation the same effect is not observed, supporting the differentiation of skeletal muscle myoblasts during embryonic development (Costello et al., 2008). In a similar study, rats were exposed to a low protein diet during early, mid, late or total gestation and authors observed a similar reduction in muscle fiber number and density of fast and glycolytic fibers of the soleus; however, the gastrocnemius muscle experienced a reduction in oxidative fibers (Mallinson et al., 2007).

Shifts in skeletal muscle fiber type may have a significant impact on muscle quality, performance, and metabolism. Lower muscle fiber density has been associated with increased adiposity and collagen cross-linking when exposed to maternal overnutrition (Yan et al., 2010; Yan et al., 2011). The combination of shift in skeletal muscle fiber types with an increase in adiposity and collagen cross-linking substantially influences skeletal muscle metabolism with the potential to predispose animals to metabolic disorders. Skeletal muscle fiber type responds to maternal plane of nutrition in a manner in which the least essential fiber type appears to be sacrificed; although this has yet to be confirmed (Mallinson et al., 2007).

Changes in skeletal muscle fiber type influences skeletal muscle metabolism with slow twitch and fast-oxidative fibers operating primarily under aerobic conditions, while fast-glycolytic fibers continue metabolism under anaerobic conditions. A shift towards oxidative fibers increases ability to utilize glucose and fat sources of energy in the presence of oxygen. However, under periods of anaerobic metabolism, these fibers are unable to sufficiently operate and reach a level of fatigue more quickly. Paired with changes in metabolism associated with increased adiposity, maternal overnutrition may inhibit the ability of skeletal muscle to perform optimally.

Increased adiposity results in increased paracrine signaling within skeletal muscle. Specifically, adipocytes increase the release of cytokines resulting in reduced intracellular insulin signaling, as previously described (Hirosumi et al., 2002). Furthermore, increased collagen cross-linking has been shown to limit the ability of skeletal muscle to adapt and utilize energy substrates sufficiently (Huang et al., 2012). As such, changes in maternal plane of nutrition during any period of gestation may have a significant impact in skeletal muscle adaptation to exercise and the ability of skeletal muscle to efficiently utilize energy and perform to an animal's genetic potential.

The influence of maternal overnutrition on energy substrate metabolism has been most extensively studied in murine and ovine models. Consistently in both species, maternal overnutrition results in reduced insulin signaling and an increased risk of type 2 diabetes, associated with changes in gene expression and second messenger proteins related to multiple intracellular insulin signaling pathways. Related to the insulin receptor, maternal overnutrition resulted in reduced *insulin receptor* (*INSR*) gene

expression and protein concentration in both sheep and rat offspring (Bayol et al., 2005; Yan et al., 2010). The same study in rats observed a similar decrease in *GLUT4* at weaning, which is symptomatic of insulin resistance (Bayol et al., 2005).

Downstream insulin signaling is more broadly influenced by maternal plane of nutrition than the INSR or GLUT4 transporter. As previously descried, the PI3K-AKT pathway is directly responsible for stimulating GLUT4 translocation. Maternal overnutrition and/or obesity reduced phosphorylation of AKT in lambs, but not total AKT levels, suggesting lesser insulin stimulation of the PI3K-AKT pathway (Yan et al., 2010). A similar response was observed in male rat offspring from overfed dams on a high fat diet (Latouche et al., 2013). Down-regulation of this pathway results in reduced GLUT4 translocation, independent of total GLUT4 available; therefore, glucose clearance from circulation is impaired predisposing offspring to metabolic diseases earlier in life (Waller et al., 2011).

Inflammation associated with increased adiposity affects the JNK signaling pathway and is heavily influenced by maternal overnutrition. Although maternal overnutrition does not influence offspring weight at birth in livestock species, researchers have found a shift in skeletal muscle development towards increased adiposity and reduced skeletal muscle mass (Ford et al., 2009; George et al., 2010; Winsco et al. 2011; Yan et al., 2010). As previously stated, adipocytes and myocytes are derived from the same mesenchymal cell population; therefore, a shift in maternal plane of nutrition during early gestation has a significant impact on the differentiation of mesenchymal stem cell populations (Yan et al., 2013). Interestingly, however, when

maternal plane of nutrition is altered in mid- to late gestation, the JNK pathway is still negatively influenced, suggesting further effects of maternal plane of nutrition following differentiation of mesenchymal stem cells to adipose or muscle cell fates (Bayol et al., 2008; Yan et al., 2010; Zhu et al., 2010).

In sheep, an increase in circulating lipids during mid-gestation resulted in increased phosphorylation of JNK and TLR4 in the placenta, thereby exposing the developing fetal lamb to excess inflammatory mediators (Zhu et al., 2010). Similarly, when exposed to an obesogenic diet during late gestation, resulting offspring had an increase in JNK phosphorylation in semitendinosus skeletal muscle (Yan et al., 2010). As a result, lambs also exhibited reduced phosphorylation of AKT, higher circulating insulin levels, and evidence of reduced insulin sensitivity (Yan et al., 2010). These results have also been observed in murine models exposed to a "junk food" or cafeteria diet during pregnancy and lactation (Bayol et al., 2008).

Similar to the influence of JNK signaling pathway up-regulation, an increase in ERK activation has been observed consistently in offspring of mares on a high plane of nutrition in various periods of gestation. Again, an up-regulation in ERK signaling is believed to decrease phosphorylation of AKT in a similar fashion to JNK, but also to stimulate gluconeogenesis in skeletal muscle and liver. The influence of maternal plane of nutrition on skeletal muscle ERK signaling remains unclear; however, it is suggested that changes in maternal plane of nutrition result in increased MAPK signaling, which is an upstream protein signaling molecule of ERK (Gavete et al., 2005). Furthermore, little research has investigated the effects of maternal overnutrition on the MAPK/ERK

signaling pathway in skeletal muscle; however, maternal obesity has been observed to activate the ERK signaling pathway in adipose, liver, and heart (Fernandez-Twinn et al., 2012). Because liver is also sensitive to insulin, up-regulation of ERK in hepatic tissue also results in reduced insulin sensitivity via a reduction in AKT phosphorylation, similar to observations in skeletal muscle of mature insulin resistant animals (Brozinick et al., 2003).

Spleen

Little research has investigated the influence of maternal plane of nutrition on the spleen and its function in postnatal life. Ultimately, the research in non-human vertebrate spleen function is unclear and limited resulting in confusion and conflicting results. Related to maternal plane of nutrition, much of the data currently documented include only changes in mass (Caton et al., 2009; Vonnahme et al., 2013). Consistently, in response to maternal undernutrition, the spleen is smaller compared to control-fed animals, suggesting potential for reduced function (Vonnahme et al., 2013). However, following maternal overnutrition or obesity, fetal spleen is often heavier than those from control-fed animals (Caton et al., 2009; Vonnahme et al., 2013). Whether maternal overnutrition was implemented during early, mid-, or late gestation, lambs consistently had larger splenic mass than lambs from control ewes (Caton et al., 2009). These data suggest that fetal spleen is sensitive to maternal plane of nutrition, but the influence, if any, this may have on splenic function postnatal is unclear.

With the importance of the spleen in immune function and exercise performance in the horse, any change in splenic function may have a significant impact on

performance or health in adulthood. As previously stated, the prevalence of overfeeding in the equine industry is high; therefore, if foals are born with larger spleens in response to high maternal plane of nutrition, it is reasonable to hypothesize that an increase in splenic weight will increase red blood cell storage capacity. Improved RBC storage capacity has the potential to increase the volume of RBC dumped into circulation following splenic contraction in response to intense exercise, assuming normal stimulation of contraction. In collegiate athletes, splenic mass is quite variable with males having larger spleens than females, but there is no evidence that spleen size influences success in performance as a collegiate athlete (Hosey et al., 2006). Unfortunately, the described study did not interpret potential function of spleen associated with size, they only interpreted spleen size in athletes using ultrasound techniques. While there appeared to be no influence of spleen size on athletic ability, horses are unique in the ability to stimulate splenic contraction during high intensity exercise; therefore, size may play a larger role in the horse than in human athletes. Splenic volume and subsequent contraction are often determined by measuring PCV in exercising horses and may be a reliable measurement in future studies to determine the influence of spleen size on performance and oxygen carrying capacity (de Solis et al., 2012).

Related to immune function, a larger spleen may also increase the capacity for storage of white blood cells, suggesting the potential for improved immunocompetence of both the innate and adaptive immune systems. The ability of the immune system to launch an appropriate immune response to a foreign invader is imperative to maintain

health. If the immune response is insufficient to heal a trauma or combat an infection, the trauma will fail to heal, and an infectious agent has the opportunity to worsen. Conversely, if an immune response is in excess of what is necessary, it could lead to further tissue damage. In avian species, the spleen has been more extensively studied relating spleen size to immunocompetence.

It was first observed in starlings that larger spleens mount a larger cell-mediated immune response following immune challenge with phytohaemagglutinin (Ardia, 2005). Related to nutrition, a protein restriction in rats resulted in smaller spleens, fewer cell volume, and less gene activation (Kenney et al., 1968). More specifically, authors observed a reduction in antibody-forming cells in spleens of rats exposed to a protein-restricted diet (Kenney et al., 1968). Interestingly, Moller and Erritzoe (2000) performed a study in which they compared the spleen mass of birds killed by cats compared to those killed by other means. Authors found a decrease in spleen size of birds killed by predator suggesting predator selection for weakened immune systems (Moller and Erritzoe, 2000). On the other hand, it is possible that an increase in splenic mass not extreme enough to qualify as splenomegaly may have no significant impact on spleen function.

Implications in the Horse

Influence of maternal plane of nutrition on fetal origins of adult disease may have significant implications to the horse. Research specific to equine species is in its infancy but may significantly impact equine performance related to energy metabolism, adaptation to stress, and immunocompetence. Evidence in murine models, humans, and

other livestock species suggests an increased risk of adult-onset metabolic disease, insulin resistance, cardiovascular disease, and hypertension. Specific to the horse, early studies have proven the predisposition of foals to metabolic disorders (Robles et al., 2017; Winsco et al., 2011). The molecular mechanisms by which this predisposition occurs have yet to be elucidated. The ability to determine molecular alterations to describe observed phenotypic changes will provide a starting point in optimizing nutrition and/or development of nutraceutical interventions.

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CHAPTER II

EFFECT OF MATERNAL OVERNUTRITION ON GLUCOSE AND INSULIN DYNAMICS, AND PANCREATIC DEVELOPMENT IN THE FOAL

Introduction

A mounting body of scientific evidence has identified linkages between the uterine environment during pregnancy and postnatal growth and health of offspring (Coverdale et al., 2015). With the prevalence of obesity and metabolic disorders in horses, investigation of the effects of maternal overnutrition is of critical importance for maximizing foal health. While the negative effects have not been as extensively documented in the horse in comparison to other species, recent studies suggest maternal overnutrition during late gestation increases offspring risk for adult metabolic disorders including equine metabolic syndrome, insulin resistance (IR), obesity, and laminitis (Rossdale and Ousey, 2002; Stephenson et al., 2011). A previous study performed by Winsco et al. (2011) determined that maternal overnutrition in late gestation decreases insulin sensitivity in mares 20 d prior to expected foaling date. Resulting offspring were also found to have reduced insulin sensitivity, but this was not evident until 180 d of age (Winsco et al., 2011).

The molecular mechanism by which this predisposition to adult-onset metabolic disease occurs in foals has yet to be investigated. Research in other species has observed differences in pancreatic size, β-cell size, and altered insulin production associated with β-cell compensation or β-cell autophagy depending on timing and duration of altered

maternal plane of nutrition (Dahri et al., 1995; Ford et al., 2009; Zhang et al., 2011). Kou et al. (2014) suggest that an increase in pancreatic islet number may be a major indicator of β-cell mass in humans. Furthermore, an increase in β-cell mass has been associated with β-cell compensation related to insulin resistance and early diabetes in humans, mice, and livestock species (Cerf, 2013). Therefore, the objectives of the present study were three-fold. First, to determine the effects of maternal overnutrition during third trimester on maternal nutrient status and glucose regulation. Second, determine the effects of maternal overnutrition on foaling parameters, foal physical measurements, and foal glucose regulation. Third, to determine the effect of maternal overnutrition on pancreatic histoarchitecture and immunoreactive insulin, glucagon, and somatostatin.

Materials and Methods

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

Horses and Dietary Treatments

Sixteen pregnant Quarter horse mares (initial BW 541 ± 17.3 kg; initial BCS 5.96 ± 0.91) were used in a completely randomized design. Mares were bred to a single stallion to reduce genetic variability. All mares were maintained on pasture at a body condition score (BCS) 6 using the 1 to 9 scale outlined by Henneke et al. (1983). Prior to the onset of the study, mares were stratified by BW, BCS and expected foaling date, and randomly assigned to one of two dietary treatments. All mares were allowed *ad libitum* access to coastal bermudagrass hay (*Cynodon dactylon*) and water for the

duration of the study. Dietary treatments began on gestational d 235 and consisted of a control diet (CON; n = 8) where all nutrients were fed to meet dietary requirements during late gestation, and an overfed dietary treatment (HIGH; n = 8) where mares received an additional 40% above CON (Table II.1). Mares on the CON diet received 0.1% BW commercial forage balancer (Empower Topline Balance, Cargill Animal Nutrition, Elk River, MN). Mares on the HIGH diet received the same as CON plus 0.7% BW commercial mare and foal concentrate (SafeChoice Mare and Foal, Cargill Animal Nutrition, Elk River, MN). Mares were fed twice daily, and adjustments to feed offered were made according to changes in BW every 14 d.

Table II.1 Nutrient analysis of maternal dietary treatments beginning on d 235 gestation.

	Coastal bermudagrass hay	Forage balancer[1]	Mare and foal concentrate[2]
DM, %	90.06	89.14	89.94
CP, %	6.50	34.16	18.26
ADF, %	36.46	7.63	16.84
NDF, %	61.58	16.88	32.17
DE, Mcal/lb	2.35	3.43	1.45
Ca, %	0.29	3.46	1.45
P, %	0.16	1.81	1.07
K, %	1.09	1.82	1.48
Na, %	0.02	0.50	0.49
Cl, %	0.30	0.98	0.98

[1]Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN) fed at 0.1% BW daily (as-fed) to both CON and HIGH dietary treatments.
[2]Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN) fed at 0.7% BW daily (as-fed) fed to HIGH dietary treatment.

Mare Performance and FSIGTT

Mare performance parameters (BW, BCS, and rump fat thickness (RFT)) were recorded and blood samples collected every 14 d via jugular venipuncture until parturition. A final maternal blood sample was collected within 1 h postpartum. Body weights were collected using a digital platform scale, and BCS was determined by 3 individuals. Rump fat measurements were obtained via ultrasound images (Aloka SSD-500V, Aloka, Inc., Tokyo, Japan) on the left hip at a point 5 cm dorsal of halfway between the first coccygeal vertebrae and the ischium (Westervelt et al., 1976).

On gestational d 285 and 315, mares underwent a modified frequent sampling intravenous glucose tolerance test (FSIGTT) using methods previously described (Caumo et al., 2000; Hoffman et al., 2003). On the day of FSIGTT, horses were fasted from concentrate for 7 h and BW was taken using a digital platform scale at 1200. Jugular intravenous (IV) catheters were placed between 1200 and 1300 after aseptic preparation and local analgesia of the overlying skin and secured in place with adhesive bandaging tape. After catheter placement, horses were given 1 h of rest prior to starting the FSIGTT. Horses were allowed *ad libitum* access to water and hay throughout the FSIGTT as complete fasting may affect insulin action in horses (Forhead and Dobson, 1997). Horses were housed in individual stalls for the duration of the FSIGTT.

A baseline plasma sample was harvested immediately prior to starting the FSIGTT, and a glucose bolus of 0.3 g/kg BW (dextrose solution 50%, Producers Cooperative, Bryan, TX) was administered IV (Hoffman et al., 2003). Blood samples were harvested at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min after glucose bolus

administration. At 20 min after initial glucose bolus, an insulin bolus of 30 mU/kg BW porcine insulin zinc suspension (Vetsulin, Intervet Inc., Millsboro, DE; Hoffman et al., 2003a) was administered to mares, and additional blood samples were harvested at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 min after initial glucose injection. Catheter patency was maintained by administering heparinized saline (0.9% sodium chloride containing 10 units of heparin per mL) after harvesting blood. Blood samples were harvested into evacuated tubes containing sodium heparin (158 U.S.P. Unites, Becton-Dickinson, Franklin Lakes, NJ) and immediately placed on ice until centrifugation at 2,700 x *g* for 10 min at room temperature. All samples were centrifuged within 15 minutes of harvesting. Plasma was harvested, and samples were stored at -20°C for subsequent determination of glucose and insulin.

Parturition and Foal Sample Collection

When parturition was imminent, mares were moved into 12 × 12 ft box stalls, were continuously monitored, and parturition was attended. No mares exhibited signs of dystocia. Immediately following parturition, foals were removed from sight, smell, and sound of the mare. Once standing, foals were weighed on a platform scale, and physical measurements were recorded including length, wither height, hip height, and heart girth circumference. Within an hour of birth, a jugular IV catheter was placed using the technique previously described. After a 1-h rest period, foals underwent the same FSIGTT as previously described; however, insulin dosages were adjusted to 10 mU/kg to avoid excessive hypoglycemia (George et al., 2009).

Immediately following foal FSIGTT, foals were humanely euthanized with pentobarbitol (Beuthanasia-D, Merck & Co. Inc., Madison, NJ). All organs were harvested and weighed using a digital scale. Weights for paired organs including adrenals, ovaries, and testis represent the total weight of both. Testicular weight includes the tunica dartos with the epididymis removed. Right kidney volume was determined through water displacement. Tissues were fixed using optimal cutting temperature compound (OCT; Tissue-Plus O.C.T. Compound, Fisher Healthcare™, Pittsburgh, PA), paraformaldehyde, or snap frozen for later analyses.

Leptin, NEFA, Glucose and Insulin Analyses

Maternal plasma samples collected on d 234 and 1 h postpartum along with foal plasma were analyzed for leptin using a commercial multi-species radioimmunoassay validated for the horse using dual parallelism (RIA; catalog no. XL-85K, EMD Millipore, Burlington, MA) and non-esterified fatty acid (NEFA) using a commercial enzymatic colorimetric assay (catalog no. C1057; Wako Chemicals, Richmond, VA). All FSIGTT samples were analyzed for glucose and insulin concentrations using a colorimetric assay and a commercial ELISA kit, respectively (Glucose Assay Kit STA-680; Cell BioLabs, Inc., San Diego, CA; Equine Insulin ELISA, Mercodia, Inc., Uppsala, Sweden).

Histological Analyses of Pancreas Morphology

A hematoxylin and eosin stain (H&E) was used to determine general pancreatic histoarchitecture using methods previously described (Dunlap et al., 2008). Number and size of islets of Langerhans were determined using immunohistochemical localization

for pancreatic α-amylase. Briefly, 5-μm sections of paraffin embedded pancreas were mounted to glass slides. Antigen retrieval was performed using boiling 0.01 M sodium citrate buffer (pH 6.0) for pancreatic α-amylase using a rabbit anti-α-amylase polyclonal antibody (catalog no. sc-46657; Santa Cruz Biotechnology, Inc., Dallas, TX) at 1:100 concentration. Purified nonrelevant rabbit IgG was used as a negative control. Immunoreactive protein was visualized using Vectastain ABC Kit (Catalog no. PK 6101 for rabbit IgG; Vector Laboratories, Burlingame, CA) following manufacturer's instructions and 3,3'-diaminobenzidine tetrahydrochloride (catalog no. D5637; Sigma-Aldrich, St. Louis, MO) as the color substrate. Sections were prepared without counterstaining and a coverslip was fixed using Permount mounting medium (SP15-500; Thermo Fisher Scientific, Waltham, MA). Digital images were captured using Nikon DS-Ri1 camera with NIS-Element AR 4.30.02 software. As endocrine cells lack the presence of α-amylase, the absence of positive staining indicated endocrine cells of the islets of Langerhans. Islet number was determined using the average of 10 images per animal unit in a 20× field of fixed area in Image J version 1.52a (U.S. National Institutes of Health, Bethesda, MD). Islet size was determined by the average area of 10 representative islets per animal unit in a 40× field using the trace tool in Image J.

Quantitative Immunofluorescence

Immunofluorescent localization of immunoreactive insulin, glucagon, and somatostatin in pancreas sections were performed as previously described (Johnson et al., 2001). Briefly, 8-μm cryosections of pancreas were placed on glass slides. Sections were fixed in cold methanol for 10 min at -20°C. OCT was removed from each section

and rinsed in 0.02 M PBS containing 0.3% Tween (PBS/Tween). Sections were blocked with normal goat serum (1:10 dilution) for 1 h at room temperature. Sections were rinsed in PBS/Tween and incubated with the primary antibody overnight at 4°C in a humidified chamber. Primary antibodies include polyclonal goat anti-insulin (catalog no. sc-7389; Santa Cruz Biotechnology), rabbit polyclonal anti-glucagon (catalog no. ab92517; Abcam, Cambridge, MA), and rat polyclonal anti-somatostatin (catalog no. ab30788; Abcam) at 1:100, 1:1000, and 1:100, respectively. Species appropriate nonrelevant IgG were used as negative controls. Sections were probed with rabbit anti-goat IgG Alexa 488, goat anti-rabbit IgG Alexa 594 and goat anti-rat IgG Alexa 488 (Invitrogen, Thermo Fisher Scientific, Pittsburgh, PA) at 1:250 for 1 h at room temp in a humidified chamber, rinsed with PBS/Tween and overlaid with Prolong Gold Antifade (Life Technologies, Carlsbad, CA). Slides were stored at room temp in the dark overnight, and microscopic analyses were performed the following day. Digital fluorescence images were captured with a Nikon DS-Q1Mc camera using NIS-Elements AR 4.30.02 software. Signal for insulin, glucagon, and somatostatin were quantified using Image J software and the standardized procedures previously described (Arques et al., 2012)

Statistical Analysis

Area under the curve (AUC) for glucose and insulin concentrations were determined using trapezoidal summation with individual baseline sample used as the trapezoidal base. All data were analyzed using PROC MIXED of SAS (SAS, Inc., 9.4, Cary, NC). Main effects tested were block, treatment, time, and treatment×day

interaction. Main effects for tissue-specific analyses included only treatment. Foal sex was found to be not significant and was removed from the model. A paired t-test was used to compare CON to HIGH at individual timepoints. Means are reported as LSMeans ± SEM. *P*-values less than or equal to 0.05 were considered statistically significant and less than or equal to 0.10 considered a trend towards significance.

Results

Maternal Physical Measurements and Plasma Analysis

There were no notable refusals of dietary treatments, as all horses readily consumed their respective diets. Maternal change in BW tended to be different between dietary treatments with mares in the overfed group gaining twice that of mares in the CON group during third trimester ($P = 0.07$; Table II.2). Additionally, HIGH mares increased in BCS while mares in the CON group decreased BCS ($P = 0.02$). There was no observed difference in change in RFT measured via ultrasonography ($P = 0.45$). There tended to be a treatment×day interaction ($P = 0.10$) in maternal plasma leptin concentrations where at the start of dietary treatments, there was no difference in maternal leptin; however, at the time of parturition, HIGH mares had twice the circulating concentrations of leptin ($P = 0.02$; Table II.3). There were no differences in NEFA concentrations in maternal plasma prior to the start of dietary treatments or immediately postpartum ($P > 0.10$; Table II.3), however there was an effect of day ($P < 0.01$) characterized by a reduction in circulating NEFA over time in both dietary treatments.

Maternal FSIGTT

There were no treatment×day interactions for maternal FSIGTT performed on d 285 and 315 gestation (Table II.4). There was an influence of time in maternal FSIGTT for peak glucose ($P < 0.01$), baseline insulin ($P = 0.03$), and insulin AUC before administration of insulin ($P < 0.01$) characterized by an increase over time, independent of dietary treatment. Dietary treatment influenced peak glucose ($P = 0.02$) and insulin AUC before administration of insulin ($P = 0.01$) where HIGH mares had higher peak glucose and a larger endogenous release of insulin from maternal pancreas compared to CON.

Table II.2 Effect of maternal overnutrition on changes in mare physical measurements.

	Treatment[1]			*P*-Values
Item	CON	HIGH	SEM	Trt[2]
Change in BW, kg	24.80	45.84	5.51	0.07
Change in BCS	-0.60	0.21	0.14	0.02
Change in RFT[3], cm	-0.03	0.04	0.03	0.45

[1]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).
[2]Trt = effect of dietary treatment.
[3]RFT = Rump fat thickness measured via ultrasound (Westervelt et al., 1976).

Table II.3 Effect of maternal overnutrition on maternal plasma concentrations of leptin and non-esterified fatty acids (NEFA).

		Treatment[1]			*P*-Values		
Item	Day	CON	HIGH	SEM	Trt[2]	Day	Trt*Day
Leptin, ng/mL	235	7.65[a]	7.56[a]	1.52	0.21	0.44	0.10
	Post-Partum	6.43[a]	10.92[b]				
NEFA, mmol/L	235	0.17[a]	0.22[a]	0.03	0.14	< 0.01	0.36
	Post-Partum	0.10[a]	0.15[a]				

[1]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).
[2]Trt = *P*-values of dietary treatment.
[a,b] superscripts indicate treatment*day interaction ($P \leq 0.10$).

Table II.4 Effect of maternal overnutrition on maternal glucose and insulin dynamics using a frequent sampling i.v. glucose tolerance test (FSIGTT) on d 285 and 315 gestation.

			Treatment[1]			*P*-Value		
Item		Time	CON	HIGH	SEM	Trt[2]	Time	Trt*Time
Glucose	Baseline, mg/dL	285	90.61	113.31	13.55	0.52	0.26	0.34
		315	88.02	83.60				
	Peak, mg/dL	285	362.85	415.44	25.23	0.02	< 0.01	0.63
		315	442.73	520.92				
	AUC Before Insulin[3]	285	4,936.16	5,605.44	365.92	0.19	0.16	0.67
		315	5,648.49	5,990.57				
	AUC After Insulin[4]	285	20858.00	18764.00	1,444.39	0.71	0.13	0.31
		315	17,018.00	17,994.00				
	AUC Total	285	25,794.00	24,370.00	1,614.72	0.97	0.31	0.42
		315	22,666.00	23,985.00				
Insulin	Baseline, ng/dL	285	0.04	0.06	0.01	0.25	0.03	0.69
		315	0.07	0.08				
	Peak, ng/dL	285	23.92	23.64	5.35	0.89	0.46	0.85
		315	18.85	20.63				
	AUC Before Insulin	285	3.35	7.05	0.95	0.01	< 0.01	0.47
		315	6.71	8.98				
	AUC After Insulin	285	86.97	107.82	85.15	0.24	0.19	0.34
		315	120.78	312.37				
	AUC Total	285	90.32	114.88	85.16	0.22	0.18	0.34
		315	127.49	321.35				

Table II.4 Continued

[1]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).

[2]Trt = *P*-Values of dietary treatment.

[3]AUC Before Insulin = area under the curve prior to intravenous dosage of insulin during FSIGTT.

[4]AUC After Insulin = area under the curve after intravenous dosage of insulin during FSIGTT.

Foaling Parameters and Foal Physical Measurements

There were no differences in gestation length ($P = 0.68$), placental weight ($P = 0.73$), or colostrum volume ($P = 0.25$; Table II.5). Colostrum quality tended to be greater in CON mares compared to HIGH when measured using a refractometer ($P = 0.10$). Foal physical measurements including BW (CON 41.28 ± 1.69; HIGH 42.69 ± 1.69), hip height (CON 64.06 ± 1.24; HIGH 95.25 ± 1.24), wither height (CON 92.71 ± 1.35; HIGH 93.90 ± 1.35), length (CON 71.44 ± 1.23; HIGH 72.47 ± 1.23) and heart girth circumference (CON 77.23 ± 1.23; 78.11 ± 1.23) were not different between dietary treatments ($P > 0.51$).

Table II.5 Effect of maternal overnutrition on gestation length, placenta weight and colostrum quality.

	Treatment[1]			*P*-Value
Item	CON	HIGH	SEM	Trt[2]
Gestation Length, d	324.40	325.90	2.50	0.68
Placenta Wt., kg	3.98	3.89	0.19	0.73
Colostrum Volume, mL	679.40	991.90	182.90	0.25
Colostrum Quality, Brix %	26.69	20.56	2.64	0.10

[1]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).
[2]Trt = *P*-Values of dietary treatment.

Foal FSIGTT and Pancreas Histology

There was no difference between dietary treatments for foal glucose or insulin baseline, peak, AUC before insulin, AUC after insulin or total AUC ($P \geq 0.22$; Table II.6). Upon harvesting of organs post-euthanasia, there was no difference in pancreas weight ($P = 0.64$; Table II.7); however, the spleen ($P = 0.04$) and testis from male foals (n = 4/trt; $P < 0.01$), weighed more in foals from HIGH mares. Similarly, foal cecum and empty large intestine weights also tended to be larger in foals from HIGH mares compared to CON ($P = 0.06$ and $P = 0.08$, respectively). Pancreatic histoarchitecture (Figure II.1; Table II.8) indicated greater number of islets per area and larger islets of Langerhans in foals from HIGH mares compared to CON ($P < 0.01$). Further analysis of immunoreactive insulin, somatostatin, and glucagon indicates no difference in intensity of staining of each hormone within the endocrine pancreas ($P \geq 0.33$; Figure II.2; Table II.8).

Table II.6 Effect of maternal overnutrition foal glucose and insulin dynamics through a frequent sampling i.v. glucose tolerance (FSIGTT) test at 2 h postpartum.

		Treatment[1]			*P*-Value
Item		CON	HIGH	SEM	Trt[2]
Glucose	Baseline, mg/dL	62.14	63.95	5.48	0.82
	Peak, mg/dL	168.81	170.47	8.88	0.89
	AUC Before Insulin[3]	2,259.95	2,353.51	114.31	0.59
	AUC After Insulin[4]	9,665.50	9,464.41	1,212.67	0.91
	AUC Total	11,467.00	11,788.00	1,422.95	0.88
Insulin	Baseline, ng/dL	0.025	0.027	0.006	0.85
	Peak, ng/dL	4.06	6.14	1.16	0.22
	AUC Before Insulin	2.59	1.82	0.62	0.38
	AUC After Insulin	41.63	38.34	9.73	0.82
	AUC Total	36.88	40.17	8.54	0.79

[1]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).
[2]Trt = *P*-Values of dietary treatment.
[3]AUC Before Insulin = area under the curve prior to intravenous dosage of insulin during FSIGTT.
[4]AUC After Insulin = area under the curve after intravenous dosage of insulin during FSIGTT.

Table II.7 Effect of maternal overnutrition on foal organ weights.

Item	Treatment[1]		SEM	*P*-Value
	CON	HIGH		Trt[2]
Adrenals, g	5.52	6.23	0.38	0.21
Brain, g	312.51	315.64	7.90	0.78
Cerebellum, g	29.53	28.54	0.97	0.47
Intestine, g	1416.80	1419.25	48.74	0.97
Empty Small Intestine, g	621.00	560.57	28.85	0.17
Full Cecum, g	46.03	50.68	2.74	0.23
Empty Cecum, g	41.85	47.62	2.05	0.06
Empty Large Intestine, g	244.92	273.46	10.88	0.08
Heart, g	310.14	335.29	18.83	0.36
Left Ventricle, g	107.91	110.99	6.18	0.73
Right Ventricle, g	95.39	96.15	10.62	0.96
Septum, g	94.66	92.83	7.19	0.86
Left Kidney, g	96.01	94.68	4.62	0.84
[3]Right Kidney Volume, mL	103.75	96.25	8.85	0.56
Liver, g	740.75	736.00	48.36	0.95
Longissimus Dorsi, g	373.50	372.86	17.51	0.97
Lungs, g	542.75	614.00	34.12	0.16
Omental Fat, g	95.16	86.70	6.57	0.36
[4]Ovary, g	32.49	31.87	7.14	0.95
Pancreas, g	16.67	18.21	2.29	0.64
Spleen, g	162.25[a]	200.50[b]	11.99	0.04
[5]Testis, g	20.50[a]	32.93[b]	1.93	< 0.01
Thymus, g	31.28	36.75	3.87	0.33

[1]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).
[2]Trt = *P*-Values of dietary treatment.
[3]Kidney volume was determined using water displacement.
[4]Ovary weight was collected on all female foals (HIGH n = 4; LOW n = 4).
[5]Testis weight was collected on all male foals (HIGH n = 4; LOW n = 4).
[a,b] superscripts indicate differences between dietary treatment ($P < 0.05$).

Table II.8 Effect of maternal overnutrition on foal pancreatic islets of Langerhans and endocrine hormones.

	Treatment[2]			*P*-Value
Item	CON	HIGH	SEM	Trt[3]
Islet Number[4]	4.89	7.40	0.31	< 0.01
Islet Size[5]	8.60	16.15	1.18	< 0.01
Insulin	13.40	16.46	2.25	0.33
Glucagon	11.80	9.92	2.00	0.51
Somatostatin	9.74	11.77	2.33	0.53

[1]Hormones expressed in arbitrary units as relative intensity of immunofluorescent staining.
[2]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).
[3]Trt = *P*-Values of dietary treatment.
[4]Islet number was determined using the average ImageJ calculation on 10 images captured at 10x magnification.
[5]Islet size, mm^2 was determined using the average ImageJ calculation on 10 images captured at 10x magnification.
[a,b] superscripts indicate differences between dietary treatment ($P < 0.05$).

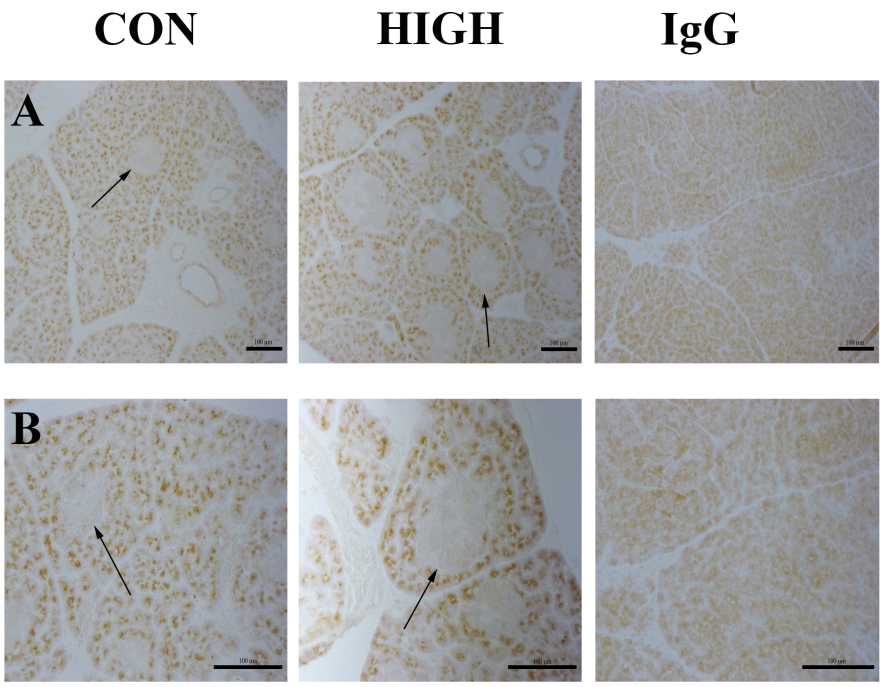

Figure II.1 The effect of maternal overnutrition on foal pancreatic histoarchitecture via immunohistochemical staining for pancreatic α-amylase. Islets of Langerhans are designated by the absence of positive staining. Islets of Langerhans were more abundant (A; 10X) and larger (B; 20X) in foal pancreas from HIGH mares compared to CON. Bar = 100 μm.

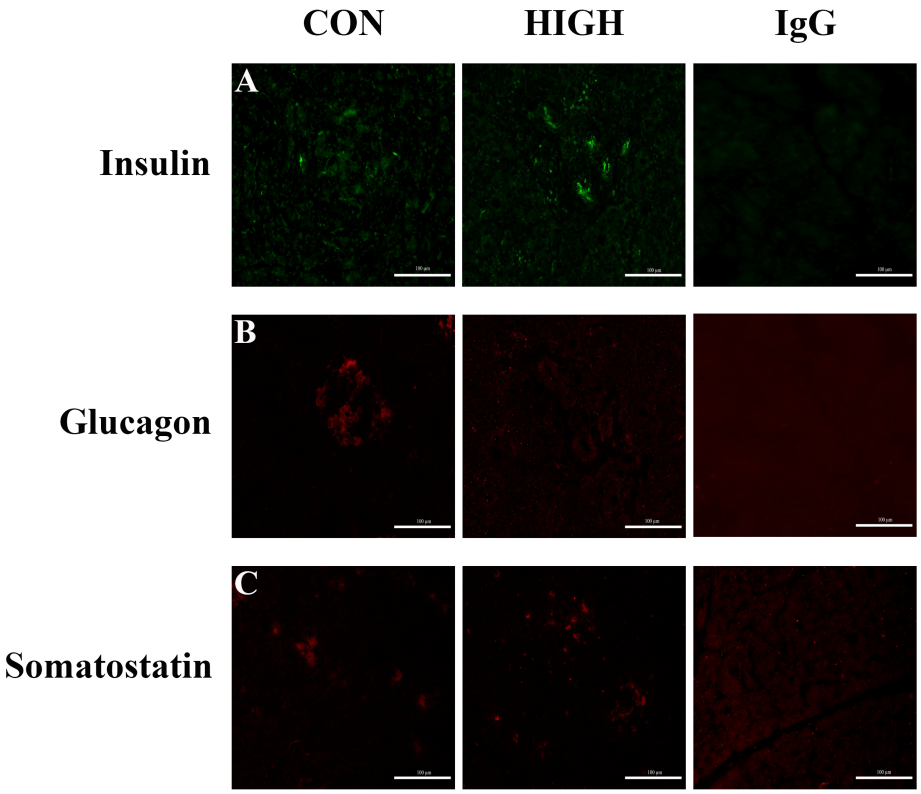

Figure II.2 The effect of maternal overnutrition on immunofluorescent staining of foal pancreas for insulin, glucagon and somatostatin. There was no difference in relative intensity of staining of immunoreactive insulin (A), glucagon (B) or somatostatin (C) using Image J analysis ($P > 0.33$). Bar = 100 μm.

Discussion

The present study examined the effect of maternal overnutrition on maternal nutrient status, glucose and insulin dynamics, foaling parameters, organ weights, and changes in pancreatic histoarchitecture and hormone production. Previous research in our lab has shown changes in offspring glucose and insulin dynamics as early as 180 d postpartum, consistent with a predisposition to adult-onset metabolic disease (Winsco et

al., 2011). Other studies have observed similar metabolic changes in offspring of overfed horses; however, currently, no data exist to suggest the molecular mechanism or histological changes that explain this predisposition of foals to metabolic diseases (George et al., 2010; Robles et al., 2017).

All mares maintained an acceptable BCS throughout all stages of gestation, both during and prior to the onset of dietary treatments. Previous studies have observed a decrease in maternal BW during third trimester when using a hay only diet as the dietary control (Winsco et al., 2011); therefore, in order to avoid this potential state of nutrient restriction, we chose to supplement with the commercial forage balancer to ensure nutrient requirements were met that may otherwise be limited in a hay-only diet during the period of greatest fetal growth and development. The 24.8 kg gained by mares on the CON dietary treatment during third trimester agrees with expected fetal and placental growth during late pregnancy according to the NRC (2007). Mares in the HIGH treatment group, however, gained 45.8 kg during third trimester, supporting the developed overnutrition model.

Determination of NEFA has been used previously to describe a state of nutrient restriction because an increase in circulating NEFAs indicates fatty acid oxidation for energy utilization when dietary energy supply is low (Tygesen et al., 2008). There was no effect of dietary treatment on NEFA concentrations in mares indicating the CON diet was successful in meeting nutrient demands during late gestation. The decrease over time, independent of dietary treatment, indicates that mares were mobilizing fewer fat stores in order to meet energy requirements for maintenance and fetal growth because

both diet sufficiently met energy demands. Furthermore, leptin, a hormone secreted by adipose tissue, is associated with a state of overfeeding and was used in this study to validate the HIGH dietary treatment (Fruhwurth et al., 2018). Leptin was not different between dietary treatments at the onset of the study supporting all mares were similar prior to the start of dietary treatments. By the end of dietary supplementation, however, HIGH mares had twice the circulating concentrations of leptin, suggesting an increase in adiposity in HIGH mares. Furthermore, the increase in leptin in HIGH mares suggests an increase in abdominal fat not observed through the subcutaneous measurement of rump fat thickness via ultrasound in the present study.

Changes in maternal plane of nutrition during late gestation has previously been shown to alter glucose and insulin dynamics independent of gestational-related changes in glucose metabolism (George et al., 2011; Winsco et al., 2011). The present study observed an increase in baseline insulin levels in HIGH mares at d 285 gestation along with an increase in insulin area under the curve prior to administration of an exogenous dose of insulin, indicating an increase in pancreatic response to glucose with no difference in peripheral tissue response to insulin. These data suggest β-cell compensation in response to overfeeding as early as 50 d following the onset of overfeeding. An increase in maternal pancreatic response may have a substantial impact on nutrient partitioning to the fetus, potentially causing the observed fetal pancreatic adaptations that result in adult onset of metabolic disease (Ford et al., 2009; George et al., 2010).

At d 315 gestation, agreeing with previous literature (George et al., 2011), mares exhibited increased peak glucose concentrations and insulin concentrations in an effort to shunt glucose toward the fetus for the final stages of development prior to parturition. George et al. (2011) describe this change in maternal glucose and insulin dynamics in the horse in detail. HIGH mares had higher peak glucose concentrations, suggesting a potential delay in response to endogenously produced insulin in agreement with Winsco et al (2011). These data from both FSIGTTs performed on mares in the present study during third trimester gestation suggest that natural changes, independent of dietary treatment, regulate nutrient supply to the developing fetus during peak fetal growth. Furthermore, maternal plane of nutrition during third trimester reduces insulin sensitivity and pancreatic response to circulating glucose.

Overfeeding during mid-late gestation has not affected offspring size or BW in most livestock species, including the horse, and the same was observed in the present study (Ford et al., 2009; George et al., 2010; Guay et al., 2002; Heidler et al., 2004; Kubiak et al., 1988; Winsco et al., 2011). Maternal overnutrition has, however, shown to alter the size of some organs, particularly using the sheep model (Ford et al., 2009; George et al., 2010). In the present study, empty cecum and large intestine weight in foals from HIGH mares tended to be larger. This has not been previously documented in the horse; however, an increase in cecum and large intestine empty weight may increase fermentative capacity in foals from HIGH mares. Total intestine weight, however, was not different between dietary treatments.

Furthermore, the testis of male foals from HIGH mares were larger compared to CON. This has not been previously documented in the horse; however, a similar study performed by Robles et al. (2017) observed a delay in testicular maturation in response to maternal overnutrition in weaned horses up to two years of age, although no testicular weights were recorded. The specific molecular culprit explaining the delay in testicular maturation has not been described; however, researchers suggest it could be hormonal related (leptin, insulin, and IGF1), or associated with the timing of testicular inguinal migration at approximately d 270 gestation in the horse (Robles et al., 2017).

Lastly, the spleen was larger in foals from HIGH mares compared to CON which has been previously described in other species as the spleen is known to be sensitive to maternal plane of nutrition (Caton et al., 2009). Little research has extended past the documentation of splenic size related to fetal programming; however, the spleen plays a specific role in the athletic horse and this increase in splenic size may indicate changes in splenic function. During heavy exercise, the spleen contracts to release its stored RBC contents into circulation to increase oxygen carrying capacity and delay the onset of fatigue (McKeever et al., 1993). An increase in splenic size may increase capacity for splenic dumping, improving athletic performance. Furthermore, an increase in splenic size may improve immunocompetence and has been previously described in avian species (Moller and Erritzoe, 2000). Further investigation into the effects of splenic size on function in the horse is necessary based on these data.

Specifically related to glucose and insulin dynamics of interest in the present study, the pancreas did not differ in size based on maternal plane of nutrition which

disagrees with studies performed in sheep where an increase in pancreas size was observed in offspring from obese ewes (Ford et al., 2009; George et al., 2010). Species differences or the fact that HIGH mares never achieved a true state of obesity may explain the lack of difference in pancreas size in the present study.

Currently, no studies have observed changes in insulin sensitivity at birth. The earliest observed changes to suggest reduced insulin sensitivity occurred at 160 and 180 d postpartum by George et al. (2011) and Winsco et al. (2011), respectively. A similar study performed using Saddlebred mares overfed from mid-late gestation, observed a reduction in insulin sensitivity in foals at 19 months of age (Robles et al., 2017). In order for these metabolic disturbances to occur so early in life to predispose animals to adult-onset metabolic disease, there is likely a measurable molecular change at birth, programmed *in-utero*, to explain this susceptibility.

Other species have extensively studied molecular changes in the pancreas to explain metabolic diseases such as insulin resistance, diabetes, and metabolic syndrome (Butler et al., 2003; Dahri et al., 1995; Friedman, J. E., 2018; Limesand et al., 2007; Zhang et al., 2011). However, this has not been determined in the horse where metabolic diseases are prevalent. The endocrine pancreas is responsible for maintenance of blood glucose levels by responding to changes in blood glucose concentrations and releasing regulatory hormones including insulin and glucagon to maintain glucose homeostasis. Changes in plane of nutrition or excessive inclusion of a single nutrient can have a negative impact on the ability of the pancreas to respond to fluctuations in blood glucose (Fowden et al., 2001). Insulin resistance is most often observed in

mature, obese horses; however, prevalence has increased over time leading to the postulation of molecular variations *in-utero* (Johnson et al., 2012).

One of the earliest pancreatic molecular change to indicate reduced insulin sensitivity preceding insulin resistance is β-cell compensation, which is characterized by β-cells that are larger in size and number within the endocrine islets (Butler et al., 2003; Weir and Weir, 2004). Displaying signs of β-cell compensation, foals from HIGH mares in the present study had more endocrine islets of Langerhans per area than mares on the CON diet. In humans and mice, this has been observed in patients diagnosed with insulin resistance or pre-diabetes (Weir and Weir, 2004). In a study performed using human pancreas from healthy adults with no history of pancreatitis or diabetes, investigators suggest that pancreatic islet number is correlated with an increase in β-cell mass. It is accepted that an increase in β-cell mass is responsible for the increase in circulating insulin concentrations in humans and mice with insulin resistance and early diabetes (Butler et al., 2003; Cerf et al., 2013; Comstock et al., 2012; Weir and Weir, 2004). These data alone begin to describe the tissue-specific molecular changes *in-utero* that may explain the predisposition of offspring from overfed or obese mares to insulin-related metabolic diseases. Furthermore, larger islets of Langerhans were observed in foals from HIGH mares compared to CON. Kou et al (2013), however, suggests a weaker correlation between islet size and endocrine cell mass.

Lastly, intensity of staining of immuonreactive pancreatic endocrine hormones showed no difference. In animals exposed to maternal overnutrition, pancreatic islets have been shown to produce more insulin relative to animals exposed to a control diet

(Dahri et al., 1995; Ford et al., 2009). In the horse, change in maternal glucose and insulin dynamics has not shown to influence α-cell development or glucagon production at birth, which is in agreement with the present study (Fowden et al., 1999). Somatostatin, however, has not been extensively studied, particularly in the horse, in response to maternal plane of nutrition and this study indicates that maternal overnutrition during third trimester does not influence δ-cell production of somatostatin.

In conclusion, maternal overnutrition during third trimester gestation altered maternal glucose and insulin dynamics 50 d following the onset of dietary treatments. As expected, there was no influence on foal physical measurements or glucose and insulin dynamics, agreeing with previous studies performed in the horse. However, foals exposed to overnutrition during peak fetal growth exhibited an increase in size and number of islets of Langerhans suggesting β-cell compensation. Although there was no difference in staining of immunoreactive insulin, glucagon and somatostatin, the changes in pancreatic histoarchitecture is the first tissue-specific measurement in the horse to explain the predisposition of offspring exposed to maternal overnutrition to adult-onset metabolic disease that was not previously observed until 180 d postpartum.

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CHAPTER III

EFFECT OF MATERNAL OVERNUTRITION ON FOAL SKELETAL MUSCLE DEVELOPMENT AND METABOLISM

Introduction

The ability of skeletal muscle to meet its full genetic potential is imperative for performance horses. Nonetheless, little research has been performed on fetal development of equine skeletal muscle. As research in livestock species has progressed, it has becoming increasingly evident that maternal plane of nutrition plays a significant role in skeletal muscle development and subsequent metabolism which has the potential to limit skeletal muscle adaptation to training in the performance horse. With the prevalence of obesity in the current equine population, the effect of maternal overnutrition is of critical importance.

Skeletal muscle fibers and adipose tissue are derived from the same mesenchymal cell population during fetal development (Yan et al., 2013). Abnormalities in maternal glucose and insulin resulting from overnutrition alters the differentiation of skeletal muscle leading to higher numbers of adipocytes and fewer skeletal muscle fibers at birth in pigs (Cerisuelo et al., 2009). Although fewer skeletal muscle fibers were observed, greater skeletal muscle fiber diameter was recorded in offspring from overfed sows, which reduced the capacity of those skeletal muscle fibers to adapt to exercise demands (Cerisuelo et al., 2009).

Related to skeletal muscle insulin signaling, obesity has been shown to reduce translocation of glucose transporter 4 (GLUT4) through a reduction in insulin sensitivity, related to changes in the insulin receptor (INSR) or activation of second messenger signaling molecules in the insulin signaling pathway (Hirosumi et al., 2002; Simar et al., 2012). We have previously described alterations in maternal glucose and insulin dynamics following maternal overnutrition in third trimester (Winsco et al., 2011). These modifications in maternal metabolism have proven to alter foal metabolism by 180 d postpartum; however, the molecular mechanism by which this occurs is just beginning to be elucidated through analysis of the equine pancreas (Bradbery et al., unpublished; Winsco et al., 2011). The objective of the present study was two-fold. First, determine the effect of maternal overnutrition on skeletal muscle fiber development. Second, determine the effect of maternal overnutrition on skeletal muscle insulin signaling pathways related to adult-onset insulin resistance.

Materials and Methods

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

Horses, Dietary Treatments, and Mare Performance

The nutritional model used in this study is described by Bradbery et al. (unpublished). Briefly, 16 Quarter Horse mares were used in a completely randomized design. All mares were allowed *ad libitum* access to coastal bermudagrass hay (*Cynodon dactylon*) and treatments consisted of a control diet (CON; n = 8), where all nutrients were fed to meet dietary requirements during late gestation, and an overfed

dietary treatment (HIGH; n = 8) where mares received an additional 40% above CON beginning on d 235 gestation.

Parturition and Foal Sample Collection

Immediately following parturition, foals were removed from sight, smell and sound of the mare. At 2 h postpartum, foals underwent a frequent sampling i.v. glucose tolerance tes (FSIGTT) as described by Bradbery et al. (unpublished). At 5 h postpartum, foals were euthanized using pentobarbitol (Beuthanasia-D, Merck & Co. Inc., Madison, NJ) and tissues were harvested and either fixed using optimal cutting temperature compound (OCT; Tissue-Plus O.C.T. Compound, Fisher Healthcare™, Pittsburgh, PA) or snap frozen for subsequent analyses.

Immunofluorescent Histological Analyses of Skeletal Muscle Fiber Type and GLUT4 localization

Immunofluorescent localization of immunoreactive type I and type IIa skeletal muscle fiber types was performed using a modified method described by Tulloch et al (2011). Briefly, 5-µm cryosections were fixed in cold methanol for 10 min at 20°C. Surrounding OCT was removed from each section and rinsed in phosphate-buffered saline (PBS). Immediately after, goat polyclonal anti-collagen V antibody (1:10 dilution; Southern Biotechnology, Birmingham, AL) was applied to each slide and incubated in a humidified chamber for 1 h at room temperature. Slides were washed for 5 min in PBS after which mouse monoclonal antibodies for detection of type I (slow myosin antibody, MAB1628, MilliporeSigma, Dermstadt, Germany) and type IIa (Type 2a antibody, A4.74, Developmental Studies Hybridoma Bank, University of Iowa, IA)

were individually labelled with Alexa fluor-conjugated IgG Fab fragments with emitting 350 and 488 wavelengths, respectively (Zenon Alexafluor 350 mouse IgG labelling kit; Zenon Alexafluor 488 mouse IgG labelling kit, Invitrogen, Thermo Fisher Scientific, Pittsburgh, PA) for 1 h at room temperature in a humidified chamber. Samples were then washed for 5 min in PBS followed by a 15 min incubation in 4% paraformaldehyde. Samples underwent a final wash for 5 min in PBS and were mounted using Prolong Gold Antifade (Life Technologies, Carlsbad, CA). Slides were stored at room temp in the dark overnight, and microscopic analysis was performed the following day. Digital fluorescence images were captured with Nikon DS-Q1Mc camera using NIS-Elements AR 4.30.02 software. Skeletal muscle fiber type was determined using Image J software version 1.52a (U.S. National Institutes of Health, Bethesda, MD). Number of skeletal muscle fibers were determined using 10 images per sample in a fixed area. Skeletal muscle fiber size was determined by the average area of 10 representative fibers per fiber type per animal unit in a 40x field.

Localization of immunoreactive GLUT4 was performed using methods previously described (Johnson et al., 2001). Briefly, 6 μm cryosections of gluteus medius, semitendinosus, and triceps brachii were placed on glass slides and fixed with cold methanol for 10 min at 20°C. OCT was removed from each section and rinsed in 0.02 M PBS containing 0.3% Tween (PBS/Tween). Sections were blocked with normal goat serum (1:10) for 1 h at room temperature. Sections were rinsed in PBS/Tween and incubated with the primary antibody overnight at 4°C in a humidified chamber. Primary antibodies included polyclonal rabbit anti-GLUT4 (1:250; ab33780; Abcam, Cambridge,

MA) and mouse polyclonal rat anti-laminin (1:500; 11576, Abcam), respectively. Species appropriate nonrelevant IgG were used as negative controls. Sections were probed with goat anti-mouse Alexa 488 (Invitrogen, Thermo Fisher Scientific, Pittsburgh, PA) at 1:250 for 1 h at room temperature in a humidified chamber, rinsed with PBS/Tween and overlaid with Prolong Gold Antifade with DAPI (Life Technologies, Carlsbad, CA). Slides were stored at room temperature in the dark overnight, and microscopic analyses were performed the following day. Digital fluorescence images were captured with a Nikon DS-Q1Mc camera using NIS-Elements AR 4.30.02 software.

RNA Isolation and RT-qPCR Analysis

Total cellular RNA was isolated from snap frozen gluteus medius, semitendinosus, and triceps brachii using TRIzol reagent according to manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). Quality and quantity of isolated RNA was determined using an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and the NanoDrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA), respectively. Synthesis of cDNA was performed as previously described from total cellular RNA using random primers (Invitrogen, Carlsbad, CA), oligo-dT primers, and SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA; Stewart et al., 2000). ABI PRISM 7700 with Power SYBR green PCR Master Mix as the detector was used to analyze mRNAs via qPCR according to manufacturer's recommendations and using methods previously described (Satterfield et al., 2008). Genes analyzed included *Insulin Receptor Isoform A* (*INSR-A*), *Insulin Receptor Isoform B* (*INSR-B*), *Insulin-Like*

Growth Factor 1 Receptor (*IGF1R*), *Insulin-Like Growth Factor 2 Receptor* (*IGF2R*), and *Glucose Transporter 4* (*GLUT4*) with *Glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) used as the reference gene (Table III.1). Primers used are described in Table 1. Data were analyzed using 7200HT SDS software (version 2.3, Applied Biosystems, Foster City, CA). The relative quantification of gene expression was determined using the comparative CT method (Satterfield et al., 2008).

Table III.1 Primers utilized for quantitative real-time PCR analyses of foal skeletal muscle.

Gene	Forward/Reverse Primers	Amplicon Size	GenBank accession no.
INSR-A	GTTTTTGTCCCCAGACCGTC GACATCAGCAAGCGCCCT	73	XM_023644610.1
INSR-B	TGGTGCCGAGGACACTAGA TTAGGGAAACCCGGAACCGT	96	XM_023644610.1
GLUT4	TTTGTGGCATTCTTTGAGATTGG CTGAAGAGCTCAGCCACG	65	AF531753
IGF1R	TCCACCGAAGCGTCTTTCTC AGTGTGACCTTGCCACAACA	88	XM_023651179.1
IGF2R	CCCGAGCTGTGCAGTTATACA CAGCACTTGATGACCCACAC	112	XM_023632974.1
GAPDH	CAAGGCTGTGGGCAAGGT GGAAGGCCATGCCAGTGA	59	NM_001163856.1

Western Blot Analyses

Protein was extracted using 100 mg of snap frozen skeletal muscle. Samples were homogenized in protein lysis buffer, centrifuged at max for 15 min at 4°C. Protein concentrations for each sample were determined using the Bradford protein assay (Bio-

Rad, Hercules, CA) with bovine serum albumin as the standard. For western blot analyses, 50 μg protein was separated on 12% SDS-polyacrylamide gels and electrophorically transferred to nitrocellulose membranes. Membranes were blocked using 5% nonfat dry milk in Tris-buffered-saline with 0.1% Tween (TBST) for 1 h at room temperature. Polyclonal antibodies against phosphorylated and total c-Jun N-terminal kinase (JNK; phosphorylated 1:250; total 1:1000; 9251, 9252, Cell Signaling Technologies, Danvers, MA), extracellular signal-regulated kinase (ERK; phosphorylated 1:1000; total 1:1000; 9101,9102, Cell Signaling Technologies), protein kinase B (AKT; phosphorylated 1:250; total 1:1000; 9271, 9272, Cell Signaling Technologies), glucose transporter 4 (GLUT4; 1:500; 33780, Abcam, Cambridge, United Kingdom), and insulin receptor β (IR_{β}; 1:250; sc-81465, Santa Cruz Biotechnology, Dallas, TX) were incubated overnight in 2% milk-TBST at 4°C. Polyclonal glyceraldehyde-3-phosphate (GAPDH; 1:500; 2118, Cell Signaling Technologies) was used as a control protein for gluteus medius samples, and polyclonal tubulin (1:1000; 7291; abcam) was used as a control protein for semitendinosus and triceps brachii. Membrane blots were washed for 30 min at room temperature with TBST followed by incubation with the appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature and a final wash in TBST. Proteins were detected using chemilluminescence (SuperSignal West Pico PLUS, Thermo Fisher Scientific, Waltham, MA) using manufacturer's recommendations. The amount of protein present was quantified by measuring the intensity of bands using a ChemiDoc EQ system and Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA).

Statistical Analysis

All data were analyzed using PROC MIXED of SAS (SAS, Inc., 9.4, Cary, NC). Main effects tested were block and treatment. Foal sex was found to be insignificant and was removed from the model. Means are reported as LSMeans ± SEM. *P*-values less than or equal to 0.05 were considered statistically significant and less than or equal to 0.10 considered a trend towards significance.

Results

The longissimus dorsi (LD) muscle was harvested at the time of euthanasia and used as a reference muscle to determine whole-body skeletal muscle mass. As described by Bradbery et al. (unpublished), there was no difference in foal LD weight of the foal between maternal dietary treatments ($P = 0.97$).

Skeletal Muscle Fiber Type and Size

In gluteus medius, semitendinosus, and triceps brachii muscles, there were greater numbers of type I skeletal muscle fibers ($P \leq 0.04$) in HIGH foals compared to CON (Table III.2; Figure III.1). Type IIx fibers were fewer in gluteus medius ($P = 0.04$) and tended to be fewer in semitendinosus ($P = 0.09$) in HIGH foals. There was no difference in type IIa fiber number for gluteus medius or triceps brachii muscles ($P \geq 0.13$), but there tended to be fewer type IIa fibers ($P = 0.07$) in semitendinosus of HIGH foals compared to CON. All three skeletal muscles had a higher ratio of type I skeletal muscle fibers compared to total type II fibers in HIGH compared to CON ($P = 0.05$).

Table III.2 Effect of maternal overnutrition on skeletal muscle fiber number.

		Treatment[2]			*P*-Value
Muscle	Item	CON	HIGH	SEM	Trt[3]
Gluteus Medius	Type I	3.46[a]	9.02[b]	0.96	< 0.01
	Type IIa	33.03	28.82	1.83	0.13
	Type IIx	36.96[a]	29.34[b]	2.32	0.04
	TypeI:TypeII	0.05[a]	0.16[b]	0.02	< 0.01
Semitendinosus	Type I	1.33[a]	3.98[b]	0.48	< 0.01
	Type IIa	28.01	20.46	2.80	0.07
	Type IIx	32.33	26.21	2.52	0.09
	TypeI:TypeII	0.02[a]	0.09[b]	0.01	< 0.01
Triceps Brachii	Type I	6.48[a]	10.53[b]	1.27	0.04
	Type IIa	25.31	22.41	2.26	0.38
	Type IIx	16.93	10.38	3.10	0.16
	TypeI:TypeII	0.19[a]	0.33[b]	0.04	0.05

[1]Number of skeletal muscle fibers per fixed area using 8 images per muscle per animal.
[2]Dietary treatments are described by Bradbery et al. (unpublished).
[3]Trt = *P*-Values of dietary treatment.
[a,b]superscripts indicate a main effect of treatment ($P \leq 0.05$).

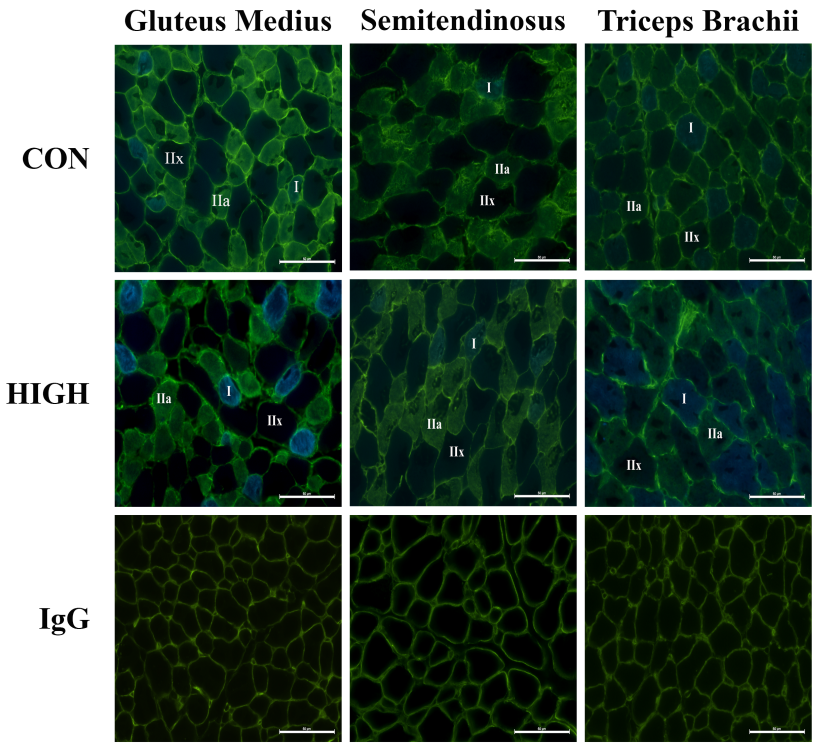


Figure III.1 Immunofluorescent staining of immunoreactive skeletal muscle fiber type in gluteus medius (A), semitendinosus (B), and triceps brachii (C) muscles of foals from control (CON) or overfed (HIGH) mares. Foals from HIGH mares had larger skeletal muscle fibers and an increase in type I slow twitch fibers (blue; I) compared to type II fast twitch fibers (IIa: green; IIx: Black). Bar = 50 μm.

Related to skeletal muscle fiber size (Table III.3), type I fibers were larger in HIGH foals compared to CON for gluteus medius, semitendinosus, and triceps brachii ($P \leq 0.01$). Additionally, type IIa skeletal muscle fibers were larger in HIGH foals compared to CON for all skeletal muscle groups ($P \leq 0.04$). Finally, type IIx skeletal muscle fibers were larger in HIGH foals ($P < 0.01$) in gluteus medius, but there was no difference in type IIx skeletal muscle fiber size for semitendinosus or triceps brachii muscles ($P \geq 0.26$).

Table III.3 Effect of maternal overnutrition on skeletal muscle fiber area.

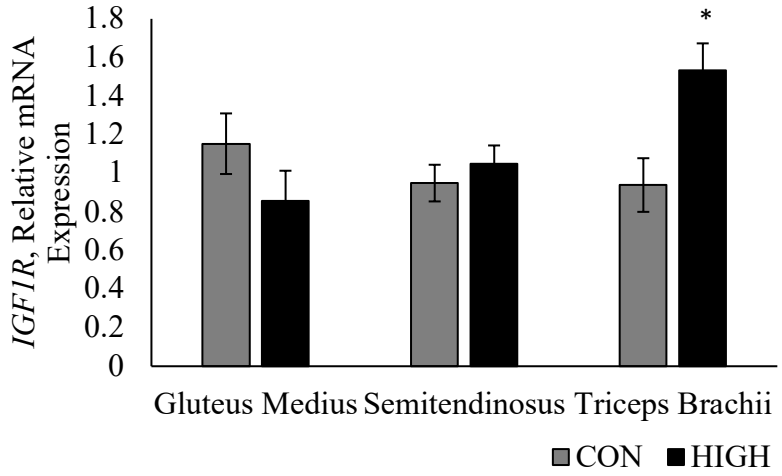
		Treatment[2]			*P*-Value
Muscle	Fiber Type	CON	HIGH	SEM	Trt[3]
Gluteus Medius	Type I	7.75[a]	11.88[b]	1.30	0.01
	Type IIa	8.99[a]	12.25[b]	0.58	< 0.01
	Type IIx	13.68[a]	18.76[b]	0.88	< 0.01
Semitendinosus	Type I	7.02[a]	12.70[b]	0.84	< 0.01
	Type IIa	11.16[a]	143.97[b]	0.72	0.02
	Type IIx	20.60	22.20	2.20	0.61
Triceps Brachii	Type I	17.77[a]	26.20[b]	1.67	< 0.01
	Type IIa	19.78[a]	26.41[b]	2.18	0.04
	Type IIx	25.51	28.83	2.05	0.26

[1]Fiber area give in mm^2.
[2]Dietary treatments are described by Bradbery et al. (unpublished)
[3]Trt = *P*-Values of dietary treatment.
[a,b]superscripts indicate a main effect of treatment ($P < 0.05$).

mRNA Expression

Relative expression of *IGF1R* mRNA (Figure III.2) was higher in triceps brachii of HIGH foals compared to CON ($P < 0.01$) but was not different by dietary treatment in gluteus medius or semitendinosus muscles ($P \geq 0.19$). *IGF2R* mRNA expression tended to be lower in HIGH foals compared to CON ($P = 0.08$) but was not different between dietary treatments for semitendinosus or triceps brachii ($P \geq 0.22$).

A)



B)

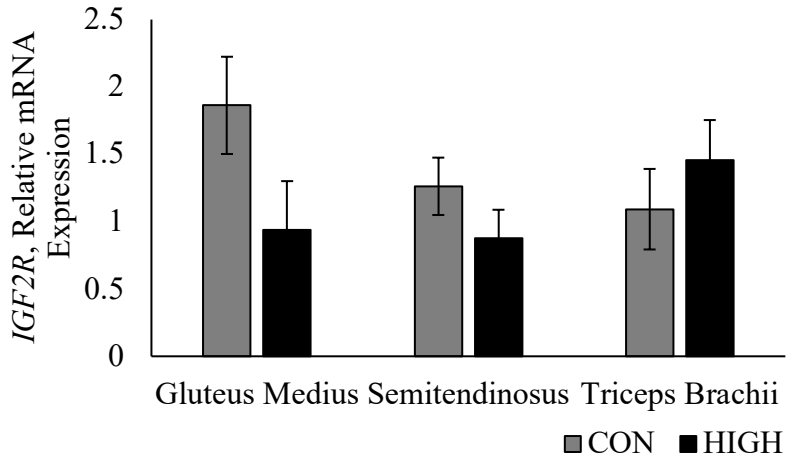
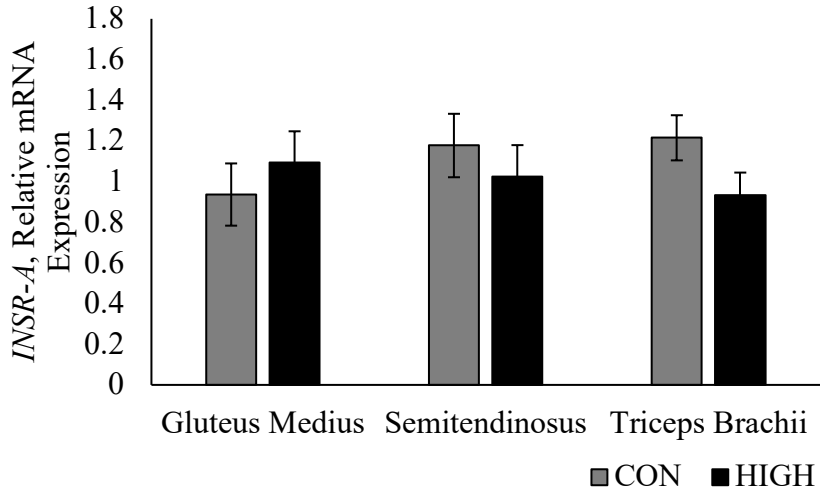


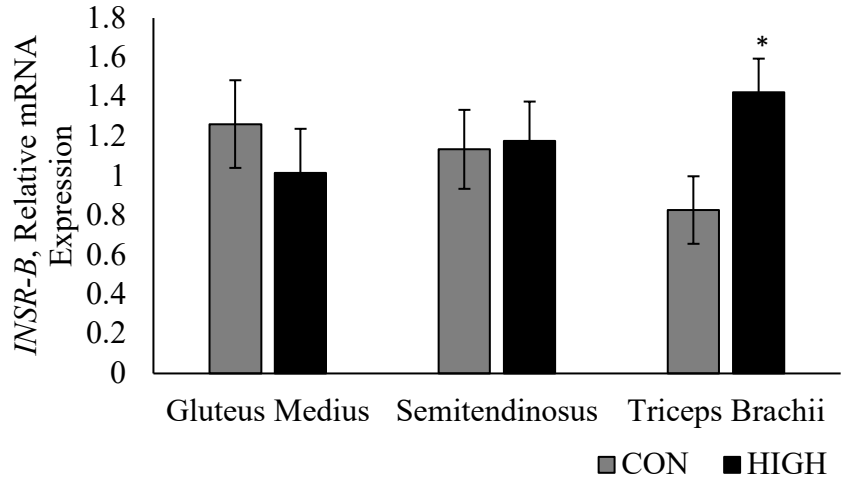
Figure III.2 Relative expression of *IGF1R* (A) and *IGF2R* (B) in gluteus medius, semitendinosus, and triceps brachii of foals from mares overfed during third trimester gestation.

Expression of *INSR-A* mRNA tended to be lower in triceps brachii of HIGH foals ($P = 0.10$), but was not different in gluteus medius or semitendinosus ($P \geq 0.46$; Figure III.3). Finally, *INSR-B* mRNA expression was higher in triceps brachii of HIGH foals ($P = 0.03$) but was not different in gluteus medius or semitendinosus ($P \geq 0.43$). In gluteus medius, *GLUT4* mRNA expression was reduced in HIGH foals ($P = 0.05$) but was not different in semitendinosus or triceps brachii ($P \geq 0.79$; Figure III.3).

A)



B)



C)

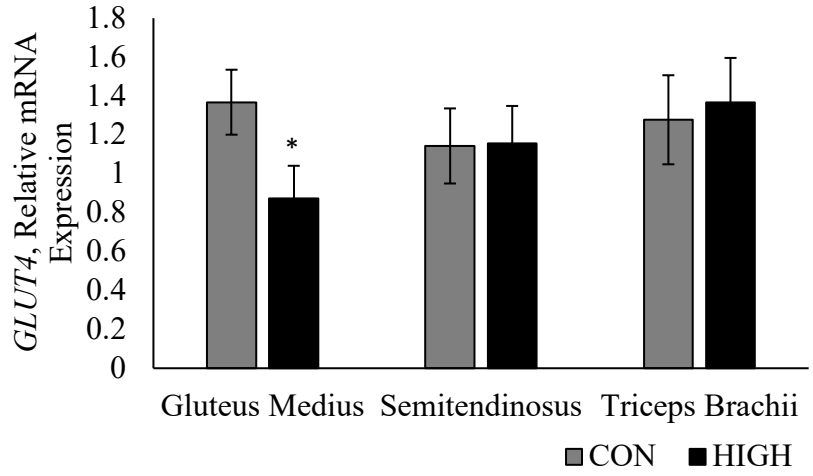
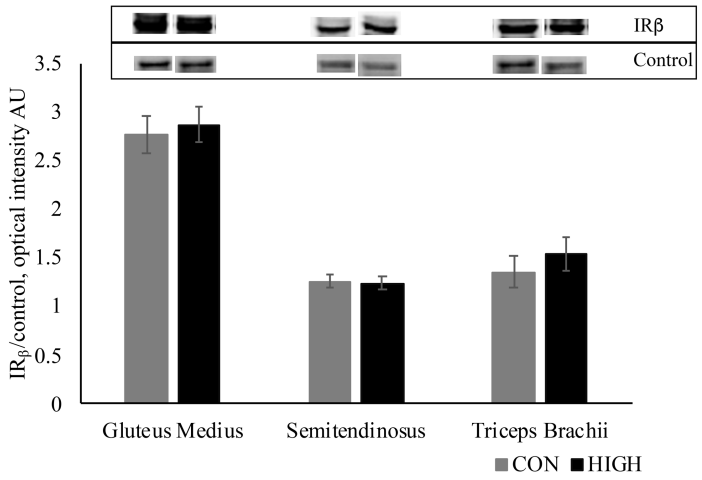


Figure III.3 Relative expression of *INSR-A* (A), *INSR-B* (B), and *GLUT4* (C) in gluteus medius, semitendinosus, and triceps brachii of foals from mares overfed during third trimester gestation.

Key Proteins in Insulin Signaling

There was no difference in protein quantities of IR_β (Figure III.4) between diets in gluteus medius, semitendinosus, or triceps brachii. However, in gluteus medius skeletal muscle samples, ratios of phosphorylated to total ERK1/2 and JNK were elevated in foals from HIGH mares compared to CON ($P < 0.05$; Figure III.5). Additionally, total ERK1/2 tended to be greater in CON mares compared to HIGH ($P < 0.10$; Figure III.6). There was no difference in phosphorylated or total protein levels of AKT irrespective of maternal diet or skeletal muscle type ($P > 0.10$; Figure III.5 and III.6, respectively). There were also no differences in ERK1/2 or JNK in triceps brachii muscles ($P > 0.10$). Lastly, there was no difference in GLUT4 protein levels between diets in any of the three muscle groups tested ($P > 0.10$; Figure 4).

A)



B)

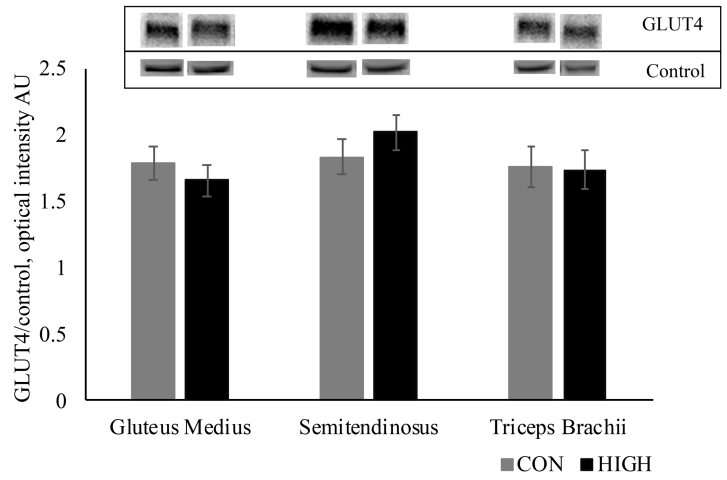
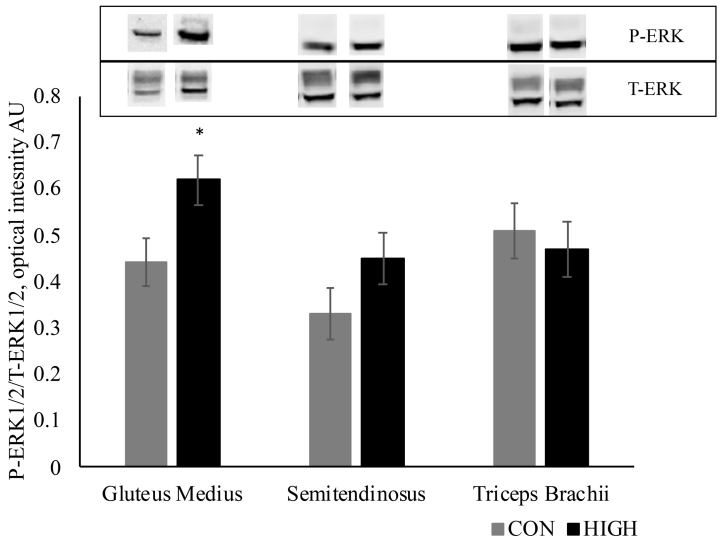
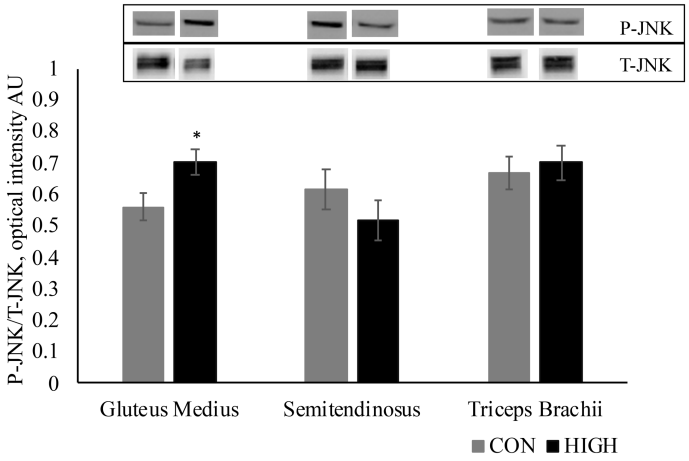


Figure III.4 Total protein concentration of IR_{β} (A) and GLUT4 (B) presented as a ratio of total protein concentration/control protein in gluteus medius, semitendinosus, and triceps brachii of foals from mares overfed during third trimester gesatation. Loading control proteins were tubulin (semitendinosus and triceps brachii) and GAPDH (gluteus medius). There was no influence of treatment on loading control proteins. Data are presented as LSMeans. AU = arbitrary units.

A)



B)



C)

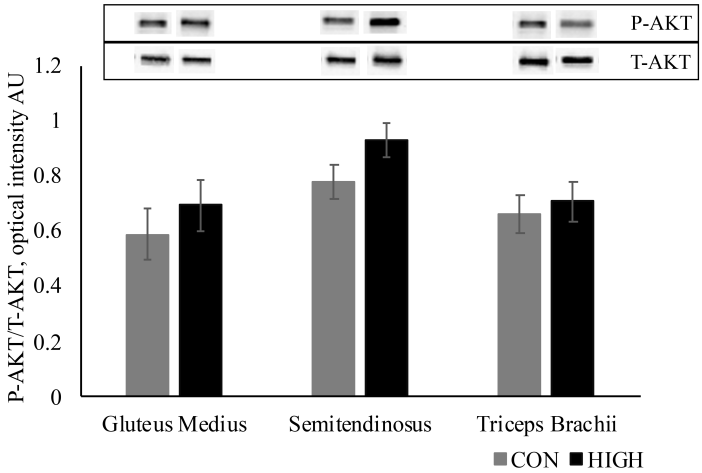
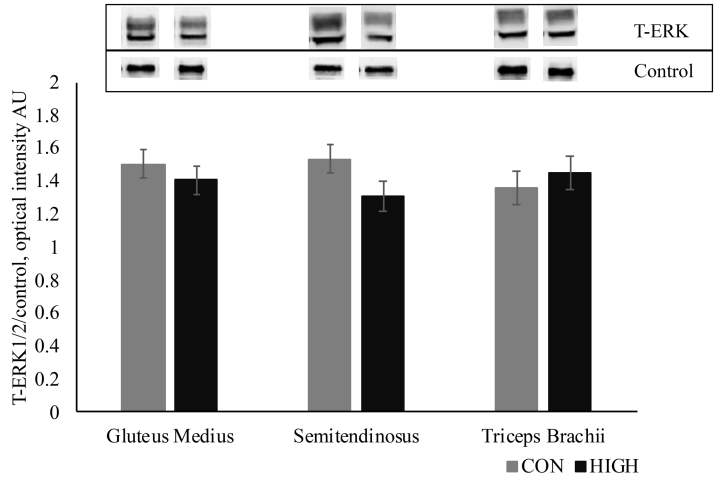
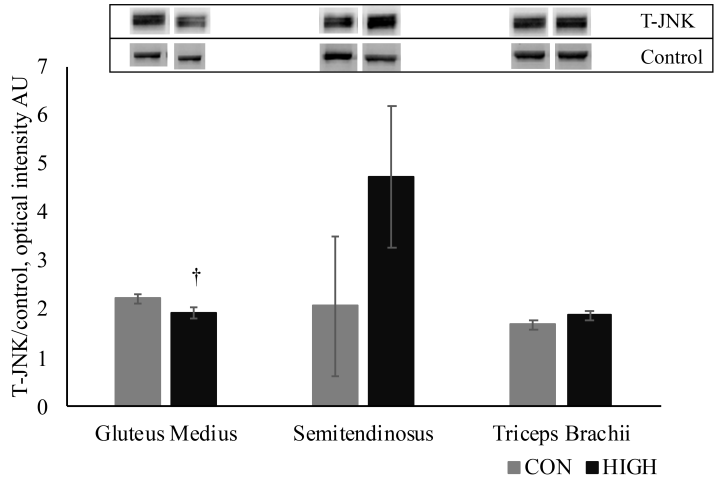


Figure III.5 Protein concentration of phosphorylated ERK1/2 (A), JNK (B), and AKT (C) as a ratio of phosphorylated/total in gluteus medius, semitendinosus, and triceps brachii of foals from mares overfed during third trimester gestation. Data are presented as LSMeans. AU = arbitrary units. Superscripts indicate main effects of treatment ($P < 0.05$).

A)


T-ERK
Control
T-ERK1/2/control, optical intensity AU
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Gluteus Medius
Semitendinosus
Triceps Brachii
CON
HIGH

B)


T-JNK
Control
T-JNK/control, optical intensity AU
7
6
5
4
3
2
1
0
†
Gluteus Medius
Semitendinosus
Triceps Brachii
CON
HIGH

C)

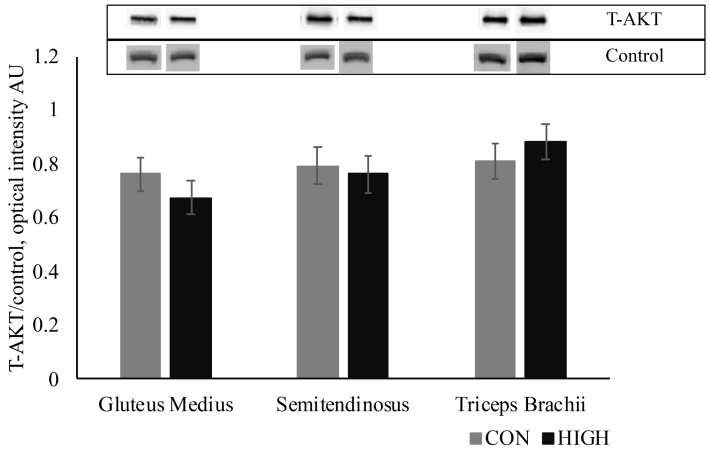


Figure III.6 Total protein concentration of ERK1/2 (A), JNK (B), and AKT (C) presented as a ratio of total protein concentration/control protein in gluteus medius, semitendinosus, and triceps brachii of foals from mares overfed during third trimester gesatation. Loading control proteins were tubulin (semitendinosus and triceps brachii) and GAPDH (gluteus medius). There was no influence of treatment on loading control proteins. Data are presented as LSMeans. AU = arbitrary units. † indicates tendency ($P \leq 0.10$).

GLUT4 Translocation

There was no observed difference in translocation of the GLUT4 transporter in gluteus medius, semitendinosus, or triceps brachii muscles across dietary treatments via immunofluorescent staining of immunoreactive GLUT4 and laminin (Figure III.7).

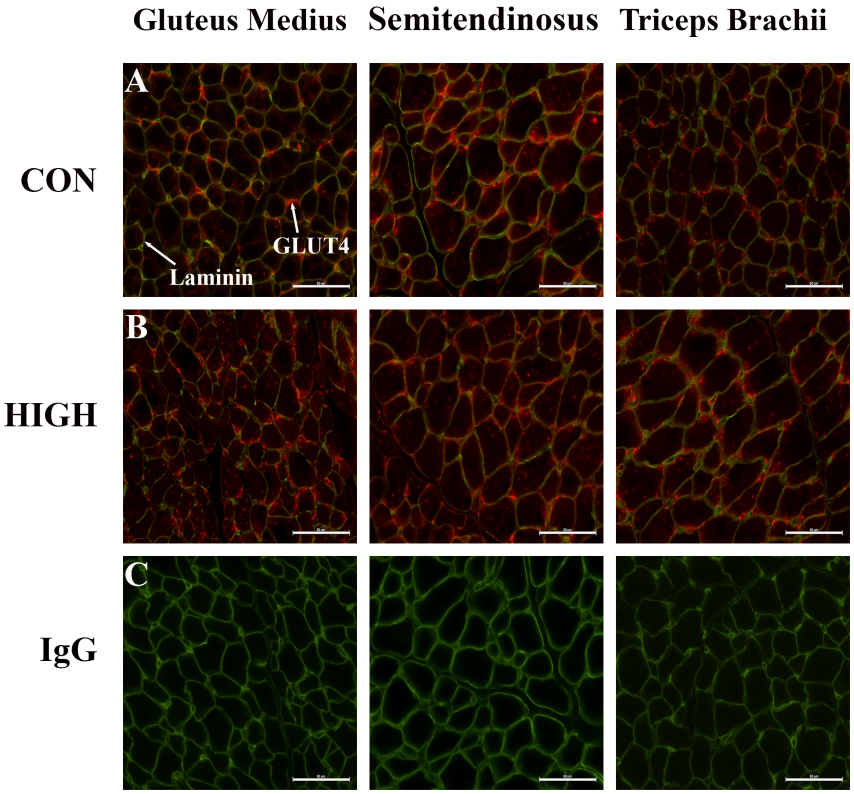


Figure III.7 Immunofluorescent staining of immunoreactive GLUT4 (red) and laminin (green) showed no difference in translocation of GLUT4 from an intramembranous vesicle to an active membrane transporter in foals from CON (A) mares compared to HIGH (B) with IgG used as a control (C). Bar = 50 μm.

Discussion

The present study examined the effect of maternal overnutrition on foal skeletal muscle fiber development and insulin signaling in an effort to determine the molecular mechanism by which foals from overfed mares are predisposed to metabolic disorders (George et al., 2010; Robles et al., 2017; Winsco et al., 2011). Bradbery et al. (unpublished) describe in detail the animals and nutritional model used in the present

study along with foaling parameters, foal physical measurements, and organ weights. The LD was used as a reference muscle to determine skeletal muscle mass, which was not different between dietary treatments. The gluteus medius and semitendinosus muscles were selected based on their nature as hind limb muscles involved in propulsion whereas the triceps brachii was selected as a forelimb muscle playing an important role in elbow extension and the stay apparatus. The present study found that maternal overnutrition during third trimester gestation results in larger skeletal muscle fibers by area and a shift toward type I skeletal muscle fiber type. Furthermore, an increase in phosphorylation of ERK1/2 and JNK in gluteus medius suggests an increased activation of second messenger signaling, which begins to describe the molecular mechanism by which foals from overfed mares are predisposed to adult-onset metabolic disease.

In other species used for meat production, skeletal muscle fibers have been more extensively studied in response to maternal nutrition as it relates to consumer perception. In horses however, where animals are used for athletic performance, skeletal muscle fiber development is of critical importance to meeting genetic potential and may be significantly influenced by maternal plane of nutrition. Most commonly, maternal undernutrition has been shown to consistently reduce skeletal muscle fiber diameter and cross-sectional area in ruminants (Du et al., 2010; Yan et al., 2013). However, Cerisuelo et al. (2009) observed an increase in skeletal muscle fiber size in piglets following maternal overnutrition during secondary fiber development. An increase in skeletal muscle fiber size has previously shown to inhibit the ability of skeletal muscle to adapt to exercise in pigs at weaning (Cerisuelo et al., 2009). The present study observed a

similar increase in skeletal muscle fiber cross-sectional area, which may influence skeletal muscle metabolism and limit the ability of fibers to adapt to early training in young horses.

A shift towards type I slow twitch skeletal muscle fibers has been observed in animals from overfed dams (Cerisuelo et al., 2009; Oliveira et al., 2018). Type I slow twitch skeletal muscle fibers are slow to fatigue, but lack quick reactivity (Maltin, 2008). Conversely, type II skeletal muscle fibers are considered fast-twitch and react in quick bursts, but fatigue at a faster rate. More specifically, type IIa fibers are oxidative with a high concentration of mitochondria for ATP production via the Krebs cycle and the electron transport chain (Maltin, 2008). Type IIb fibers, however, are glycolytic with fewer mitochondria and rely heavily on glycolysis for ATP production. While they are quick to fatigue, they are able to continue to perform under anaerobic conditions (Maltin, 2008). The reduction in type IIx fibers observed in HIGH foals in the present study may be associated with an increase in glucose transport to the fetus during development. It is possible that an increase in fetal glucose and subsequent increase in fetal pancreatic response preferentially shifted skeletal muscle fiber development towards oxidative fiber types I and IIa (Boyle and Friedman, 2011).

Often, related to changes in tissue phenotype, there is a subsequent change in gene expression. The genes of interest in the present study were those related to glucose and insulin signaling with a paired role in regulating skeletal muscle hypertrophy and hyperplasia, as previous research has indicated that maternal overnutrition predisposes offspring to metabolic diseases (Du et al., 2010; Simar et al., 2012; Yan et al., 2013).

This predisposition may occur at the level of the pancreas or a downstream tissue in glucose regulation. With skeletal muscle as the primary tissue for insulin-stimulated glucose uptake where fetal insulin regulates fetal blood glucose by third trimester, any change in gene expression or intracellular insulin signaling will play a major role in the predisposition of offspring to adult-onset metabolic diseases.

Expression of the tyrosine kinase receptors *IGF1R* and *IGF2R* when bound to their ligand are potent regulators of skeletal muscle hypertrophy and hyperplasia (Oksbjerg et al., 2004). Because of their role in muscle growth and development, gene expression of these tyrosine kinase receptors is greatest in young, growing animals, and decreases with age (Oksbjerg et al., 2004). In response to maternal plane of nutrition, a diet containing excess protein resulted in increased expression of *IGF1R* in pigs (Kalbe et al., 2017). Offspring of cattle exposed to nutrient restriction during mid-gestation observed a reduction in *IGF1R* and *IGF2R* in skeletal muscle, indicating that *IGF1R* and *IGF2R* are nutrient sensitive and can have a significant impact on skeletal muscle growth and development *in-utero* (Paradis et al., 2017). In the present study, *IGF1R* was greater in triceps brachii of foals exposed to overnutrition *in-utero* and *IGF2R* tended to be down-regulated in gluteus medius of foals exposed to maternal overnutrition.

The increase in expression of *IGF1R* may explain the increase in cross-sectional area of skeletal muscle fiber in triceps brachii of HIGH foals. There was no difference observed in expression of *IGF1R* in semitendinosus or gluteus medius; therefore, there are likely other factors regulating skeletal muscle fiber size other than expression of *IGF1R*. A tendency towards down-regulation of *IGF2R* in gluteus medius of HIGH

foals is in contrast to previous work which has observed an increase in *IGF2R* expression in offspring exposed to high fat diets and a decrease in expression in offspring exposed to nutrient restriction (Hyatt et al., 2007; King et al., 2013). The lack of effect in gluteus medius and semitendinosus on *IGF1R* and semitendinosus and triceps brachii on *IGF2R* may be related to maternal overnutrition only during late gestation in the present study.

Relative to glucose regulation, expression of the *INSR-A* tended to be lower in triceps brachii of HIGH foals but was not observed in gluteus medius or semitendinosus muscles. Similarly, *INSR-B* was not different between dietary treatments in gluteus medius or semitendinosus, but HIGH foals had greater expression of *INSR-B*. *INSR-A* is primarily responsible for insulin receptor transcription during fetal development while *INSR-B* controls insulin receptor protein transcription postnatally (Belfiore et al., 2017). An abundance of *INSR-A* in postnatal life has been associated with dysregulated cellular proliferation and insulin resistance associated with an increase in pancreatic β-cell hyperplasia (Belfiore et al., 2017). Bradbery et al. (unpublished) observed an increase in size of islets of Langerhans in foals from overfed mares suggesting β-cell compensation; however, there was no difference in *INSR-A* expression in gluteus medius or semitendinosus, and triceps brachii tended to have lower expression of *INSR-A* suggesting the involvement of other factors.

Expression of *GLUT4* transporter mRNA expression was down-regulated in gluteus medius by maternal overnutrition which may greatly contribute to the predisposition of offspring to insulin dysregulation. A similar down-regulation of

GLUT4 mRNA expression was observed in offspring of rats fed a cafeteria diet (Bayol et al., 2005). These data however, disagree with a previous study in adult, insulin-resistant horse where *GLUT4* mRNA expression and total protein concentration were not influenced by obesity or insulin resistance (Waller et al., 2011). The authors instead observed translocation of GLUT4 from an intracellular vesicle to an active membrane transporter was negatively influenced leading to the investigation of second-messenger signaling proteins in the insulin signaling pathway (Waller et al., 2011). The absence of influence on *GLUT4* mRNA expression in the study performed by Waller et al. (2011) may be because the horses studied were adults with insulin resistance unrelated to maternal plane of nutrition. The down-regulation of *GLUT4* mRNA expression in gluteus medius of the present study suggests a potential for reduced GLUT4 protein levels and translocation after weaning when postprandial glucose regulation is enhanced.

Total and phosphorylated concentrations of three intracellular signaling proteins were analyzed along with total protein concentrations of IR_{β} and GLUT4. Furthermore, the use of immunofluorescent staining was used to determine translocation of GLUT4. There was no observed difference in protein concentration of IR_{β} or GLUT4 in the present study. Bradbery et al. (unpublished) performed a FSIGTT on foals exposed to maternal overnutrition and recorded no difference in glucose regulation in response to the FSIGTT; therefore, it is no surprise that the present study observed no difference in IR_{β}, GLUT4 protein concentration, or subsequent translocation using immunofluorescent techniques. However, in gluteus medius, there was an increase in phosphorylation of insulin signaling second messenger proteins, ERK1/2 and JNK. Both proteins have been

consistently implicated in adult human patients with type 2 diabetes and in developmental programming studies (Du et al., 2009; Carlson et al., 2003; Hirosumi et al., 2002).

Specifically, ERK1/2 is associated with the mitogen-activated protein kinase pathway and play a role in cell growth, proliferation, and survival (Guo, 2014). A knockout model of ERK1 induced weight loss and improved insulin sensitivity; therefore, the increased activation of ERK1/2 in gluteus medius of neonates exposed to third trimester overnutrition supports an increased risk of obesity and a predisposition to reduced insulin sensitivity (Carlson et al., 2003; Guo, 2014). Furthermore, increased expression of p-ERK1/2 may explain the increase in size of skeletal muscle fibers in the present study, which could further predispose offspring to abnormal muscle glucose metabolism and limitations to exercise adaptations.

The JNK pathway is closely associated with inflammation and plays a role in obesity-associated insulin resistance caused by an increase in inflammatory cytokines (Hirosumi et al., 2002). The increase in phosphorylation of JNK in gluteus medius of foals from HIGH mares in the present study suggests an increase in inflammatory cytokine signaling. This increase in activation of the JNK pathway may shift development of skeletal muscle towards adipogenesis, downregulating myogenesis (Du et al., 2009). Furthermore, increased phosphorylation of JNK at birth will predispose offspring to obesity-associated insulin resistance in adulthood, making the JNK pathway more sensitive to an increase in inflammatory cytokines associated with increased adiposity (Hirosumi et al., 2002).

The increase in activation of downstream insulin signaling pathways, ERK and JNK, predisposing offspring to metabolic disorders suggests that any excess adiposity during adulthood may exacerbate the influence of adipose tissue macrophages, inflammatory cytokines and free fatty acids resulting in early-onset adult metabolic syndromes (Baskin et al., 2015). Furthermore, the observed increase in type I:type II skeletal muscle fibers in the gluteus medius suggests that maternal overnutrition may drive the gluteus medius to a fiber type phenotype resembling that of a normal triceps brachii muscle with a potential preference for fatty acid energy substrates resulting in reduced glucose utilization during anaerobic exercise.

Downstream insulin signaling was not influenced by dietary treatment in triceps brachii, which may be explained by the observed differences in gene expression previously described. Down-regulation of *IGF2R* combined with the shift in expression from *INSR-A* to *INSR-B* may be compensating and protecting the skeletal muscle cells of the triceps brachii from insulin dysregulation. As a forelimb muscle, the triceps brachii plays a vastly different role than the gluteus medius and semitendinosus of the hind limb which may explain why the differences in gene expression and lack thereof in protein concentration only occurred in the triceps brachii muscle.

In conclusion, maternal overnutrition during third trimester gestation altered skeletal muscle fiber growth and differentiation, expression of genes involved in insulin signaling and glucose uptake, and second messenger signaling protein activation in pathways associated with insulin signaling. Together these data begin to demonstrate tissue-specific changes *in-utero* that will predispose offspring to adult-onset metabolic

diseases, including insulin resistance. Furthermore, larger skeletal muscle fibers paired with a preferential shift in skeletal muscle development towards type I slow twitch fibers may limit the ability of the performance horse to appropriately adapt to training, particularly in disciplines where quick bursts of speed are required, thereby preventing the horse from reaching its genetic potential during performance years.

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CHAPTER IV

EFFECT OF MATERNAL OVERNUTRITION ON SPLEEN DEVELOPMENT IN THE FOAL

Introduction

Maternal overnutrition negatively impacts multiple physiological systems; however, research is just beginning to elucidate these effects in equine species. Multiple species have documented the influence of maternal plane of nutrition on organ mass and consistently describe the sensitivity of splenic mass to maternal plane of nutrition. Exposure to maternal nutrient restriction results in reduced splenic mass whereas maternal overnutrition results in larger splenic mass in sheep and cattle (Caton et al., 2009; Vonnahme et al., 2013). We have previously observed a similar increase in splenic mass in response to the influence of maternal over nutrition on foal development (Bradbery et al., unpublished). While it has been consistently documented that the spleen is sensitive to maternal plane of nutrition, no studies have investigated the influence this may have on the function of the spleen during the offspring's postnatal life.

The spleen serves two major functions, filtration and storage of red blood cells (RBC) and storage of multiple white blood cell (WBC) populations. During the filtration process, aged and damaged blood cells are recognized by abnormal passage rate through interendothelial slits within the spleen (Salehyar and Zhu, 2017). Resident macrophages break down damage cells and replace RBCs and platelets, as necessary.

Furthermore, the extensive population of WBCs allow the spleen to act as a large lymph organ, with the ability to induce an immune response in the event of infection (Mebius and Kraal, 2005). The equine spleen has a unique ability to contract during exercise, releasing its storage of RBCs into circulation at a rate that can double RBCs in circulation (Tablin and Weiss, 1983). The release of RBC stores during exercise increases oxygen carrying capacity (OCC) and packed cell volume to delay the onset of fatigue and encourage continued exercise performance.

The field of developmental programming has yet to describe the functional differences of the spleen in response to maternal plane of nutrition. An affiliated study determined that maternal overnutrition in the mare results in larger spleens in neonatal foals (Bradbery et al., unpublished). The effect of a larger spleen may have positive effects on athletic ability and may lead to differences in immunocompetence. Therefore, the objective of the present study was to determine the influence of maternal plane of nutrition and increased splenic mass on parameters of spleen function.

Materials and Methods

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

Horses, Dietary Treatments, and Mare Performance

The nutritional model used in this study is described by Bradbery et al., (unpublished). Briefly, 16 Quarter Horse mares bred to a single stallion to reduce genetic variability were used in a completely randomized design. Mares were stratified by BW, body condition score (BCS), and expected foaling date and assigned to one of

two dietary treatments beginning on d 235 gestation. All mares received *ad libitum* access to coastal bermudagrass hay (*Cynodon dactylon*) and treatments consisted of a control diet (CON; n = 8) where nutrients were fed to meet dietary requirements during late gestation, and an overfed dietary treatment (HIGH; n = 8) where mares received an additional 40% above CON.

Parturition and Foal Sample Collection

Immediately following parturition, foals were removed from sight, smell and sound of the mare. At 2 h postpartum, foals underwent a FSIGTT as described by Bradbery et al. (unpublished). At 5 h postpartum, foals were euthanized using pentobarbitol (Beuthanasia-D, Merck and Co. Inc., Madison, NJ) and tissues were harvested for analysis and fixed using paraformaldehyde or snap frozen in liquid nitrogen for later analyses.

Wright-Giemsa and Iron Stain

Spleen samples fixed in paraformaldehyde were embedded in paraffin using a commercial laboratory (Veterinary Pathobiology Histology Laboratory, Texas A&M University, College Station, TX). Sections 5 μm in size were mounted to glass slides. A Wright-Giemsa stain was performed on two serial splenic sections, and an iron-specific stain was performed on differing sections using a local, commercial laboratory (Veterinary Pathobiology Histology Laboratory, Texas A&M University). Digital images were captured using Nikon DS-Ri1 camera with NIS-Element AR 4.30.02 software. Percent area positive staining in the average of 10 images section at 20× magnification was used to determine presence of red-type blood cells and iron-

containing cells using Image J version 1.52a software (U.S. National Institutes of Health, Bethesda, MD) and methods previously described (Batchu and Ebong, 2016; Varghese et al., 2014).

Immunohistochemical Analyses of CD3 and CD20

Immunohistochemical localization for splenic CD3 and CD20 were performed to determine localization of T- and B-lymphocytes, respectively. Briefly, 5 μm sections of paraffin embedded spleen were mounted to glass slides. Antigen retrieval was performed using boiling 0.01 M sodium citrate buffer (pH 6.0) for CD3 using a rabbit anti-CD3 polyclonal antibody (catalog no. ab5690; Abcam, Cambridge, MA) and CD20 using rabbit anti-CD20 polyclonal antibody (catalog no. ab27093; Abcam, Cambridge, MA) at 1:100 and 1:300 concentration, respectively. Purified nonrelevant rabbit IgG was used as a negative control. Immunoreactive protein was visualized using Vectastain ABC Kit (Catalog No. PK 6101 for rabbit IgG; Vector Laboratories, Burlingame, CA) following manufacturer's instructions and 3,3'-diaminobenzidine tetrahydrochloride (catalog no. D5637; Sigma-Aldrich, St. Louis, MO) as the color substrate. Sections were prepared without counterstaining and a coverslip was fixed using Permount mounting medium (SP15-500; Thermo Fisher Scientific, Waltham, MA). Digital images were captured using Nikon DS-Ri1 camera with NIS-Element AR 4.30.02 software. Percent area positive staining in the average of 10 images per animal unit at 10× magnification was used to determine presence of CD3 and CD20 positive cells using Image J version 1.52a (U.S. National Institures of Health, Bethesda, MD) and methods previously described (Batchu and Ebong, 2016; Varghese et al., 2014).

Statistical Analyses

All data were analyzed using PROC MIXED of SAS (SAS, Inc., 9.4, Cary, NC). Main effects tested were treatment, sex and treatment*sex interaction. There was no influence of sex on dietary treatments; therefore, it was removed from the model. Means are reported as LSMeans ± SEM. *P*-values less than or equal to 0.05 were considered statistically significant and less than or equal to 0.10 considered a trend towards significance.

Results and Discussion

There was no influence of sex or treatment by sex interaction on any parameter measured, including: RBC, WBC, iron, CD3 and CD20 ($P \geq 0.13$). Splenic weight of foals used in the present study was influenced by treatment ($P = 0.03$); however, spleen weight was not influenced by sex ($P = 0.49$; Female: 187.75 ± 12.62; Male 175.00 ± 12.62). It has been consistently documented that splenic mass is dependent upon sex in humans and many other species (Chow et al., 2015; Corbin et al., 2008; DeLand, 1970). Results of a cohort study of 1,200 healthy humans in the US reveal large spleen length and volume in males compared to females (Chow et al., 2015). In children, however, one study observed no difference based on sex (Megremis et al., 2004).

With little research performed in the spleen, it is possible that splenic size is not influenced by sex until later in life. Several papers equate the difference in spleen size in males to immunosuppressive effects of testosterone and a compensation of the spleen to meet immune demands (Folstad and Karter, 1992). If this theory holds true, it may provide explanation in the lack of sex-related influence on splenic size in neonates in the

present study and children in the study performed by Megremis et al. (2004; Bradbery et al., unpublished). Splenic mass related to sex may only become apparent post-puberty when testosterone levels increase in males.

While there was no influence of sex nor an associated interaction, maternal overnutrition significantly influenced percentage area of RBC, WBC, and the ratio of RBC to WBC ($P < 0.01$; Table IV.1; Figure IV.1). Generally, the spleen is analyzed as red pulp vs white pulp; however, with the understanding that multiple white cells infiltrate the marginal zone for local immune protection, determination of percentage area occupied by RBC or WBC may be more reliable in determining cellular localization, immunocompetence, and RBC storage capacity. The percentage area of RBC was higher in foals from HIGH compared to CON ($P < 0.01$; Table IV.1). Increased storage capacity may contribute to increased athletic ability from a cardiovascular standpoint. The equine spleen contracts during heavy exercise to release stored RBC contents into circulation in an effort to increase oxygen carrying capacity, reduce the load on the cardiovascular system, and delay the onset of fatigue (McKeever et al., 1993).

Table IV.1 Effect of maternal overnutrition on percentage area of red blood cell (RBC), white blood cell (WBC), and iron in the spleen of neonatal foals.

	Treatment[1]			*P*-Values
Item	CON	HIGH	SEM	Trt[2]
WBC	56.54	44.02	2.73	< 0.01
RBC	43.61	55.98	2.77	< 0.01
RBC:WBC	0.81	1.31	0.11	< 0.01
Iron	0.40	0.69	0.09	0.10

[1]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).
[2]Trt = effect of dietary treatment.

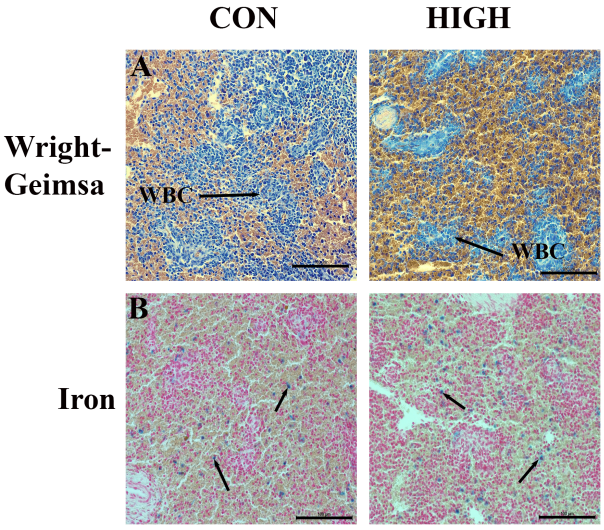

Figure IV.1 Effect of maternal overnutrition on percentage area of RBC and WBC via Wright Geimsa stain (A; 20X; RBC = Blue; WBC = orange) and percentage area of iron via an iron-specific stain (B; 20X; blue stain) in foal spleen samples from HIGH mares compared to CON. Bar = 100 μm.

Hemoglobin is a key protein component of RBCs and contains iron to enable oxygen binding for transport to peripheral tissues. As would be expected, the increase in percentage area RBC resulted in HIGH foals tending to have more iron stores in spleen sections compared to CON ($P = 0.10$; Table IV.1; Figure IV.1). This suggests that spleen from HIGH foals exhibit an increase in oxygen carrying capacity following splenic contraction during exercise compared to CON foals.

Related to the spleen's role as a lymphatic organ, percentage area of WBC understandably was inversely related to percentage area occupied by RBCs. Specifically, foals from CON mares had greater percentage area WBC compared to HIGH ($P < 0.01$; Table IV.1; Figure IV.1). In chickens, splenic size is related to immunocompetence with larger spleens believed to mount a larger immune response (Ardia, 2005). Related to plane of nutrition, a protein restricted diet in mice resulted in reduced antibody-forming cells of the spleen suggesting a diminished immune response (Kenney et al., 1968). In the present study, lesser percentage area of WBC in HIGH foals may indicate that although splenic size is greater, immunocompetence may not be.

The spleen contains white blood cells of both the innate and adaptive immune subsystems (Lewis et al., 2019). Cells of the innate system recognize infection and stimulate a cascade of events ultimately resulting in adaptive immune activation of antigen-specific T- and B-lymphocytes. B- and T- cells are located in different zones of the spleen (Figure IV.2). In the present study, there was no difference in percentage area of B- or T- lymphocytes between dietary treatments. ($P > 0.19$; Table IV.2). These data suggest that while there is a shift in ratio towards RBC storage in HIGH foals, there may

be no difference in antigen-specific immune response of foals exposed to high plane of nutrition *in-utero*.

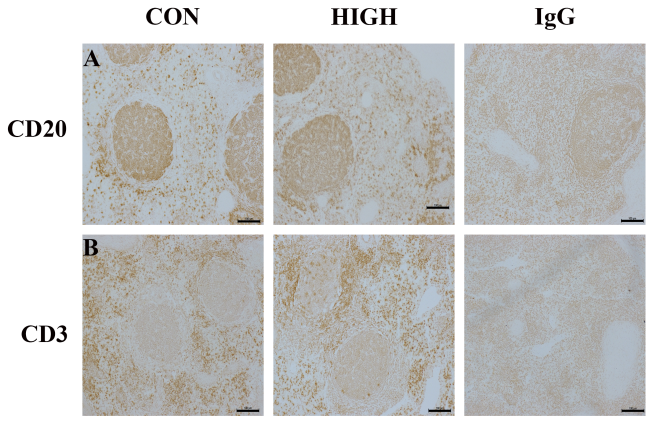

Figure IV.2 Effect of maternal overnutrition on percentage area of B- and T-lymphocytes via immunohistochemical staining for CD20 (A; 10X) and CD3 (B; 10X), respectively, in spleen of foals from HIGH mares compared to CON. Bar = 100 μm.

Table IV.2 Effect of maternal overnutrition on percent area of B- and T- lymphocytes in the spleen of neonatal foals.

	Treatment[1]			*P*-Values
Item[2]	CON	HIGH	SEM	Trt[3]
CD3	12.55	14.75	1.17	0.20
CD20	12.82	14.40	1.47	0.46

[1]Treatments consisted of control (CON; n = 8) where mares received free choice access to coastal bermudagrass hay (*Cynodon dactylon*) and 0.1% BW daily as-fed Nutrena Empower Topline Balance (Cargill Animal Nutrition, Elk River, MN), and overfed (HIGH; n = 8) where mares received the same diet as CON plus 0.7% BW daily as-fed Nutrena SafeChoice Mare and Foal (Cargill Animal Nutrition, Elk River, MN).
[2]CD3 = marker of T-lymphocytes; CD20 = marker of B-lymphocytes
[2]Trt = effect of dietary treatment.

These data are the first to describe the effect of maternal overnutrition on foal splenic development. Maternal overnutrition resulted in increased spleen weight and a shift in favor of RBC over WBC percentage area. With no difference in B- or T-cell area suggesting no change in immunocompetence, maternal overnutrition may improve the capacity of splenic contraction during exercise to increase oxygen carrying capacity and delay the onset of fatigue. These data are purely based on histological analyses and further research is necessary to define immunocompetence in the spleen of foals exposed to a high plane of nutrition *in-utero*.

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CHAPTER V

CONCLUSIONS AND IMPLICATIONS

Previous research in horses suggests reduced insulin sensitivity in foals exposed to maternal overnutrition as early as 6 mo age (Winsco et al., 2011). The purpose of the present study was to further determine the influence of maternal overnutrition on the predisposition of foals to insulin resistance and metabolic disease at the tissue-specific level. The study investigated changes in tissue histoarchitecture, gene expression and activation of second messenger signaling pathways to describe specific molecular changes related to maternal overnutrition. Data from the present studies are the first evidence of molecular changes in offspring of overfed mares to describe the predisposition to adult-onset metabolic diseases.

With the prevalence of obesity in the equine population, increased attention is being paid to metabolic disorders specifically related to glucose regulation and insulin sensitivity. These data suggest a multifactorial molecular influence of maternal plane of nutrition as predisposing factors for abnormal glucose and insulin dynamics. Changes to histoarchitecture, gene expression, and intracellular protein phosphorylation were observed in pancreas and skeletal muscle, the primary organs involved in insulin-stimulated glucose uptake. Specifically, increased number and size of pancreatic endocrine islets paired with down-regulation of *GLUT4* in gluteus medius and up-regulation of key insulin-signaling intermediates suggest altered and compensatory mechanisms involved in glucose regulation promoting a predisposition to adult-onset

metabolic disease. The altered histoarchitecture and molecular mechanisms harbor the potential to prevent optimal energy utilization in performance years, reducing performance ability and longevity of the equine athlete. Failure to perform at a horse's genetic potential with compromised longevity may result in significant economic losses for owners and trainers.

While spleen weight is known to be sensitive to maternal plane of nutrition, little research has investigated the influence this may have on splenic function related to RBC storage capacity or immunocompetence. This study provides the first evidence of nutritional-associated changes in histoarchitecture and cellular localization that may influence splenic function in offspring. The spleen is known to play a significant role during peak performance in the horse through a process of splenic contraction where RBC contents are released into circulation to increase oxygen carrying capacity. The results of the present study indicate an increase in RBC storage as opposed to WBC storage, which may provide an increased capacity for exercise and delay of fatigue. The opposite, however, is a potential down-regulation of immunocompetence. There was no observed influence on percentage area occupied by T- and B-lymphocytes; therefore, the difference may be among the innate immune cells which are responsible for sounding an immediate alarm of a pathogen or injury and stimulate the immune cascade. Any compromise in the innate immune response may limit the ability of the adaptive immune system to mount the appropriate attack, thereby reducing immune function.

In summary, the data collected in this first of its kind equine developmental programming study begin to describe the multitude of effects maternal overnutrition may

have on offspring phenotype, potential performance/longevity, and predisposition to adult-onset disease. Continued collection of data in this field will improve dietary recommendations for pregnant mares and provide potential management techniques in offspring to allow the animal to meet its genetic potential. Furthermore, improvement in longevity and decreased incidence of metabolic diseases will improve the economic contribution of the athletic horse.

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