PLASMA AND MUSCLE AMINO ACID CONCENTRATIONS IN INSULIN RESISTANT COMPARED TO NORMAL HORSES IN THE FED AND FASTED STATE

By

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ABSTRACT

The effects of insulin resistance on protein and amino acid metabolism have not been documented in horses, despite knowledge that insulin stimulates the incorporation of amino acids into proteins and decreases the rate of protein degradation in skeletal muscle. The objective of this research was to investigate protein metabolism in insulin resistant (IR) horses compared to Normal controls. Preceding the study, horses were screened for fasting plasma insulin concentrations with insulin concentrations of 10.1 ± 3.5 mU/L = Normal, and 41.2 ± 15.9 mU/L = IR, and then underwent an oral sugar challenge (OST). Blood samples were collected before and at 60, 75, 90, 120, 180 min after administration of Karo Light Syrup (0.15 mL/kg of BW) for the determination of plasma insulin and glucose. Based on the OST, eight horses (16 $\pm$ 3 yrs), 4 IR and 4 Normal, were studied while receiving Purina Strategy and a mixture of Timothy/Bermudagrass hay fed at 2% BW, a diet which met or exceeded NRC recommendations. After a week of dietary adaption, the morning meal (half the daily ration) was fed on day 7. Blood samples were taken at 0, 1, 3, 4, and 6 h post-prandial for determination of plasma amino acids. On day 8, muscle biopsies were taken and immediately frozen in liquid nitrogen for analysis of free amino acids. Glucose was analyzed using an enzymatic assay, and insulin by radioimmunoassay. Plasma free amino acids and semitendinosus muscle free amino acids were determined using reverse-phase HPLC of phenylisothiocyanate derivatives. Data were analyzed using a mixed model with repeated measures analysis of SAS, with time and group as main effects. The OST data confirmed higher insulin ($P = 0.020$) and a trend for higher glucose ($P = 0.055$) in IR vs Normal horses. There were no differences between IR and Normal for any plasma free amino acids ($P > 0.15$) or semitendinosus muscle free amino acids ($P > 0.17$). Contradictory to this study, hyperinsulinemic clamp procedures in healthy horses and pigs lowered plasma amino acid concentrations, with similar results reported in healthy and diabetic humans. Lack of variation in amino acid concentrations between IR and Normal horses suggests that insulin resistance does not affect amino acid absorption into the plasma pool or incorporation into the muscle in horses.

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CHAPTER 1. INTRODUCTION

As a highly athletic animal, whose body is composed of approximately 50% muscle, maintenance of muscle tissues (i.e. protein and AA) in the horse is of the utmost importance (Gunn, 1987). In a mature individual, protein degradation and protein synthesis are balanced. The pathways that regulate protein and muscle protein synthesis and degradation have been studied. Some factors that alter these pathways still remain elusive.

Insulin is one of the profound regulators of protein and muscle protein synthesis. General insulin effects on protein metabolism are known. Anabolic effects of insulin include increased transportation rate of AA into tissues, increases the rate of muscle protein synthesis, decreased rates of protein degradation in muscle, as well as decreasing the flux of urea formation (Geor et al., 2013). The pathology and effects of insulin dysfunction on muscle have not been directly studied in the horse. Insulin acts on adipose, liver, and muscle tissues. Muscle accounts for 60-70% of the insulin-stimulated glucose disposal in peripheral tissues, as well as postprandial glucose uptake (Smith, 2002). This makes muscle one of the most important sites of insulin action and insulin resistance.

CHAPTER 2. LITERATURE REVIEW

Protein Metabolism

Similar to monogastrics, protein metabolism in the horse begins in the stomach where protein within feedstuffs is denatured by hydrochloric acid and a variety of proteases (Yen et al., 2004). Degraded proteins then enter the small intestine where they are further broken down by proteolytic enzymes (trypsin, chymotrypsin, and carboxypeptidases) secreted by the pancreas into the duodenum. The end products of pancreatic protein digestion are oligopeptides, di- and tripeptide chains, and free AA. Protein degradation is continued by the enterocyte brush border's peptidases (aminopeptidases and endopeptidases), which occurs primarily in the jejunum and ileum. Similar to other monogastrics in the foregut, the hindgut of horses is drastically different anatomically and physiologically. Regardless of their complex gastrointestinal tract, the main product yielding from protein metabolism in the foregut is the same as monogastrics, free AA, which are available for absorption. Monogastrics were found to have lower peptidase activity in the large intestine compared to the small intestine (Bai, 1993). Little research on protein digestion and absorption in the hindgut of horses has been done. Maczulak et al (1995), however, found that bacteria isolated from cecal contents of horses were able to use partially digested protein as a nitrogen source.

Foregut and Hindgut Digestion

Total tract protein digestion in the horses is measured by the amount of nitrogen absorbed through the segments of the gastrointestinal tract (Geor et.al, 2013). Protein is digested in two main locations of the equine gastrointestinal tract, the foregut and

hindgut. Due to the diverse sources of protein being digested in these locations, distinct products are yielded for utilization. Foregut digestion of protein results in AA; whereas, hindgut digestion of protein results in ammonia and microbial protein (Hintz & Cymbaluk, 1994). Figure 2.1 shows the fates of dietary protein (Latham, 2016).

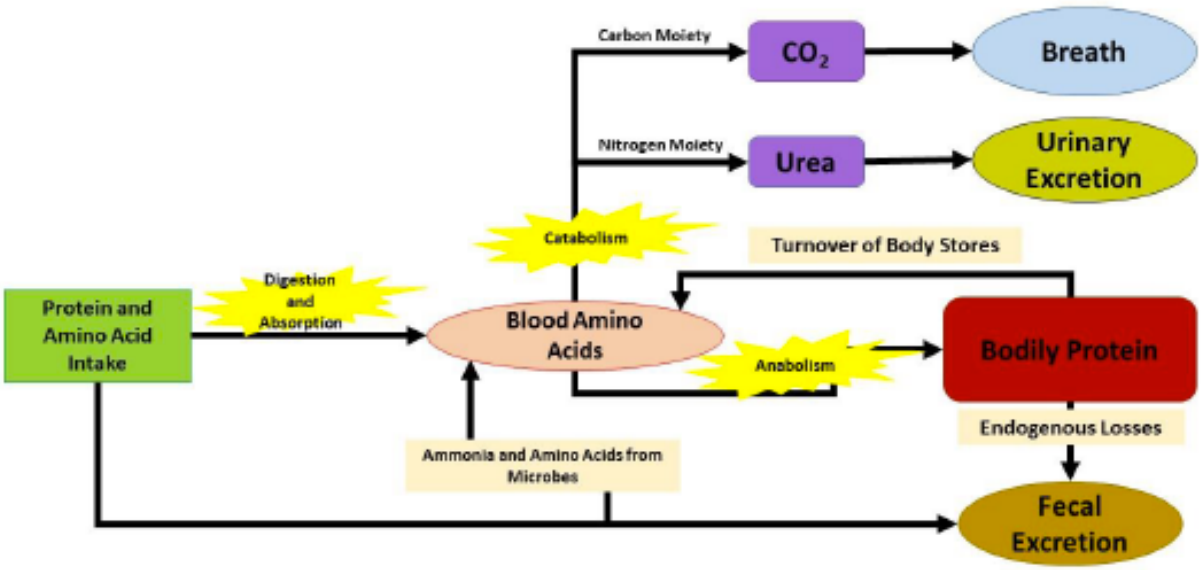


Figure 2.1 Various destinations of dietary protein. Adapted from Latham (2016).

After digestion and absorption, protein and AA can join the AA pool directly, join the AA pool via ammonia and AA from microbial digestion, or get secreted in manure. Once AA are in the pool they can undergo either catabolism or anabolism. AA undergoing anabolism synthesize bodily protein, which can then return to the pool or be excreted. Catabolism of AA from the pool are broken down into two different moieties, carbon and nitrogen. The carbon moiety is excreted as CO2 in the horse's breath, while the nitrogen moiety is exerted as urea in urine (Latham, 2016).

Protein from forage (coastal bermuda grass, low protein alfalfa, and high protein alfalfa) had a prececal digestibility that ranged from 20 to 37% (Gibbs et.al, 1988).

Protein from concentrate (corn, oats, or sorghum) had a prececal digestibility that ranged from 50 to 75% (Gibbs et.al, 1996). The percentage of prececal digestibility of forages and concentrates shows that the foregut plays the primary role in protein digestion and subsequent protein absorption and utilization. Therefore, biological value of proteins ultimately determines the AA profile within a feedstuff (Gibbs et al., 1996). The biological value refers to the nitrogen absorbed from digested protein that is retained for maintenance and growth of an animal (Wu, 2013). Using this method, dietary protein quality can be determined.

In order to determine the digestibility of soybean meal, four mature ponies were fed four quantities of soybean meal, which increased in protein concentration (4.9%, 9.5%, 14.0%, and 16.5%). In ponies, 72% of soybean meal nitrogen was digested in the small intestine; whereas, the hindgut had an overall nitrogen digestibility of 90%. The increase in digestibility in the hindgut was potentially caused by the high volume of protein that was unable to be digested in the foregut and subsequently pushed into the hindgut (Farley et al., 1995). With the diets that contained lower concentrations of protein, small intestine nitrogen digestibility was found to be 75% (Farley et al., 1995). Therefore, if protein is fed at appropriate amounts, the foregut will digest the majority of the protein, which is a positive contributor to the AA pool.

Horses fed grain excreted higher amounts of nitrogen through their urine and horses fed mostly forage diets excreted more nitrogen through their manure (Graham-Theirs & Bowen, 2010). When fed mostly forage diets, horses excreted nitrogen in their manure due to larger intakes of forage in order to meet their caloric requirements (Graham-Theirs & Bowen, 2010). In an earlier study, an increased amount of fermentable fiber increased

microbial protein, therefore leading to an increase in nitrogen content in the manure (Meyer, 1983). An increase in nitrogen in the manure can also occur from protein escaping digestion in the foregut or bypassing digestion completely (Graham-Theirs & Bowen, 2010). Although foregut protein digestion dominates, researchers examining protein quality in the non-ruminant herbivore have found that microbes found in the hindgut of herbivores improve the value of low protein (Kennedy & Hershberger, 1974). However, the protein formed by the microbes is not sufficiently digested and absorbed in the hindgut (Kennedy & Hershberger, 1974). The limited value of protein formed in the hindgut is not able to contribute to the overall absorption of protein unless the protein is broken down and absorbed in the hindgut (Yoshida et al., 1971). Furthering the knowledge of where sources of protein are absorbed and the resulting end products allows for better understanding of how to feed the horse properly. Therefore, feeding protein sources that are easily digested in the foregut are of greater benefit to the horse. Protein that is digested in the hindgut is of little to no value to the horse and thus far, current literature suggests that only the foregut contributes to the AA pool. The protein sources that can be digested in the foregut and contribute to the AA pool become the main objective when feeding protein, which ultimately focuses on protein quality.

Amino Acid Absorption

After digestion, proteins and AA are absorbed by enterocytes that line the small intestine. In addition to the AA absorbed, within the enterocytes peptides are hydrolyzed and the resulting AA are absorbed by AA transporters (Broer, 2008a). Once absorbed, AA enter the blood and are delivered to tissues where they serve as precursors for bioactive molecules, building blocks for *in vivo* proteins synthesis, and as energy

metabolites (Broer, 2008a). Several transport proteins, also known as transport systems, are present in mammalian tissue (Broer, 2008a). PepT1, a peptide transporter, has been identified in the small intestine of several species, including cattle, pigs, sheep, and chickens (Chen et al., 1999). PepT1 has not been found in the intestinal mucosa of the horses. Rabbits showed little to no detection of PepT1 mRNA expression in the cecum, colon, and small intestine (Freeman et al., 1995). *In vitro* incubation of equine jejunal membrane with dipeptide glycyl-L-glutamine showed increase rates of current flow through the membrane, suggesting that PepT1 activity could be present in the small intestine of horses (Cehak et al., 2009). Although Cehak and colleagues (2009) study showed that PepT1 activity could be present in the horse's small intestine, no such study has proven that PepT1 is expressed in the gastrointestinal tract of horses.

AA must be transported across two membranes in order to be absorbed in the small intestine: the apical and basolateral membranes. The apical membrane transports AA into the enterocyte; whereas, the basolateral membrane transports AA both in and out of the enterocyte. These transport systems can either be Na^+-independent or dependent and transport specific AA based on their chemical properties (Broer, 2008a). Woodward et al. (2010) looked to quantify the mechanisms of AA absorption between the small intestine and large intestine of gastrointestinal tract by profiling mRNA abundance of target genes that are responsible for the transport of cationic and neutral AA. Four transport proteins that are Na^+-independent were identified in this study: $b^{0,+}$AT, CAT-1, LAT-2, and LAT-3 (Woodward et al., 2010). Transport protein $b^{0,+}$AT was found to be uniform across the gastrointestinal tract and appears to be unique to the horse (Woodward et al., 2010). Expression of this transport protein in the large intestine has not been found in mice

(Dave et al., 2004) or in pigs (Feng et al., 2008). LAT-2 and LAT-3 were detected in all segments; however, LAT-2 decreased in its expression proximally to distally (Woodward et al., 2010). CAT-1 was the only transport protein found specifically in the jejunum and ileum. $b^{0,+}$AT, LAT-2, and LAT-3 were found in both the small intestine and large intestine of the horses (Woodward et al., 2010). The presence of AA transporters, both cationic and neutral, in the large intestine showed that AA absorption might be plausible in the hindgut (Woodward et al., 2010). The identification of transport proteins along the large intestine is still theoretical at best, until the transporter proteins function can be established. The theory that these transport proteins are functional can be negated by previous studies findings. Reitnour et al. (1970) found no correlation between serum AA concentrations and AA composition of either cecal microbes or fluid, which indicated that the large intestine has little to no influence on whole-body AA status. Bochroder et al. (1994) minimal amounts of lysine, histidine, and arginine were able to cross the apical membrane in an *in vitro* isolated colonic mucosa. Leucine and alanine have been able to cross the cecal mucosa's basolateral membrane; however, no measurable concentrations could be detected in the apical membrane (Freeman et al., 1989, Freeman & Donawick, 1991).

Protein Quality

Protein quality refers to the AA profile and the digestibility of the feedstuff (NRC, 2007). The quality of the AA profile is the specific percentage of AA in feedstuffs that will meet the minimum requirements of the horse. The ratio of AA that is required by the horse would need to be known in order to find protein sources that meet requirements. This brings up the idea of an "ideal protein." A feed or diet that has an essential AA

content that mimics the animal's requirements is said to be an "ideal protein" (Geor et. al., 2013). An ideal protein source has not been found for horses, due to the fact that not all of the AA requirements are known, except for two. Lysine was found to be the most limiting AA in the horse (Ott et al., 1979). When formulating diets, lysine is set to 100% and the other AA are expressed as a percentage of the lysine requirement (NRC, 2007). Threonine has been identified as the second most limiting AA (Graham et al., 1994). Since only these two AA are known, the ideal AA profile is not available for horses. The AA profile may be estimated from the ratios of AA in muscle and milk. Currently, when formulating diets for mature horses the AA composition of a feedstuff is compared to the reference values of muscle (Bryden, 1991), which is logical when formulating diets for mature horses, but not for growing foals. Mare's milk is hypothesized to resemble the AA requirements of growing foals (Davis et al, 1994). Until further research identifies the individual essential AA requirements, milk and muscle amino acids profiles will continue to be the best representation of AA requirements of horses.

Generalized Regulation of Protein Synthesis and Degradation

The largest component of muscle is protein, which makes up 65% of the muscle DM (Badiani et al., 1997). The most influential determinant of muscle mass is the balance between muscle protein synthesis and muscle protein degradation (Urschel et al., 2014). Therefore, it is prudent to understand what stimulates and regulates protein synthesis and breakdown in skeletal muscle. Mammalian target of rapamycin (mTOR) regulates a variety of components of protein synthesis. Although mTOR is a multidomain protein that is related to lipid kinases, its activity is similar to protein serine/threonine kinase

activity (Wang & Proud, 2006). The activation of mTOR signaling is stimulated by AA, insulin, and growth factors (Wang & Proud, 2006).

The mTOR system is composed of mTOR complex 1 (mTORC1) and complex 2 (mTORC2). mTORC1 is composed of five components (Figure 2.2), which are the following: the catalytic subunit (mTOR), regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8), proline-rich AKT substrate 40 kDa (PRAS40), DEP-domain-containing mTOR-interacting protein (Deptor) (Laplante & Sabatini, 2009).

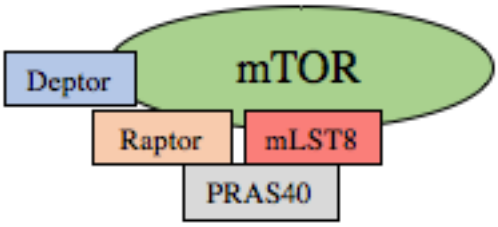


Figure 2.2 Components of mTORC1. Adapted from Laplante & Sabatini (2009).

Similar to mTORC1, mTORC2 is composed of six components (Figure 2.3), which are the following: mTOR, rapamycin-insensitive (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor (Protor-1), mLST8, and Deptor (Laplante & Sabatini, 2009).

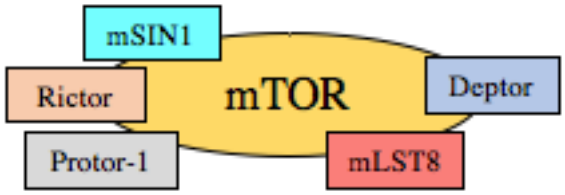


Figure 2.3 Components of mTORC2. Adapted from Laplante & Sabatini (2009).

Although theories have been developed to elucidate the functions of the components of both complexes, they are still largely unknown. Reduced interaction between mTORC1 and PRAS40 and Deptor occurs through direct phosphorylation of PRAS40 and Deptor by the activation of mTORC1. This activation and subsequent phosphorylation activates mTORC1 signaling (Peterson et al., 2009; Wang et al., 2007). Without activation of mTORC1, PRAS40 and Deptor promote the inhibition of mTORC1 (Laplante & Sabatini, 2009). Deptor, similar to mTORC1, is also the inhibitor of mTORC2 (Peterson et al., 2009). mLST8 is vital for mTORC2 function, which was demonstrated by the absence of mLST8 resulted in reduced stability of the complex (Guertin et al., 2006). mLST8 function in mTORC1 contradicts mTORC2. Loss of mLST8 had no effect on the stability of the complex, which leaves its function in mTORC1 still unknown (Guertin et al., 2006). While mTORC2 is undoubtedly an important regulatory complex for cell survival, metabolism, and proliferation, mTORC1 is the main complex that is examined for effects of nutritional factors on protein synthesis and therefore will be examined more closely in this review.

Regulation of mTORC1

There are several simulators of the mTOR complex, including growth factors, energy status, oxygen levels, and AA. Both insulin and insulin-like growth factor (IGF-1) activate mTORC1 through the same cascade. Insulin and IGF-1 bind to the receptor and recruit insulin receptor substrate 1 (IRS1). IRS1 binds and activates phosphoinositidine-3-kinase (PI3K), which stimulates the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP_3). PIP_3 activates phosphoinositidine-dependent kinase-1 (PDK1), which in turn phosphorylates and activates protein kinase B (PKB), also known as Akt. Activated Akt phosphorylates tuberous sclerosis 2 (TSC2) and PRAS40. mTORC1 is inhibited when TSC2 and PRAS40 are dephosphorylated. Forkhead Box Protein O1 (FOXO) is stimulated by decreases in Akt phosphorylation. FOXO stimulates Atrogin-1, which increases protein degradation (Hall, 2008).

Cellular energy and AA can inhibit and stimulate the mTOR pathway. When cellular energy is inadequate (low ATP:ADP ratio), mTOR is inhibited. AMP-activated protein kinase (AMPK) is activated and phosphorylates TSC2, which inhibits Rheb with a net effect of inhibiting mTORC1 (Laplante & Sabatini, 2009). In the absence of other stimulators (i.e. insulin or IGF-1), AA stimulate mTORC1; however, the absence of AA also inhibits of function of the mTOR pathway (Hall, 2008). Leucine and glutamine have both been indicated to produce a strong positive signal the regulates mTORC1 (Laplante & Sabatini, 2009). This mechanism is not completely understood yet. It is known that the activation of mTORC1 by AA is independent of TSC2 (Nobukuni et al., 2005). Rag proteins, a family of four related GTPases, are necessary for the activation of mTORC1

pathway by AA (Kim et al., 2008; Sancak et al., 2008). This occurs when Rag proteins bind to Raptor and relocate mTORC1 to regions that contain its activator Rheb (Sancak et al., 2008). This provides an explanation as to why growth factors (i.e. insulin and IGF-1) cannot stimulate mTORC1 when AA are absent.

mTORC1 Stimulates Protein Synthesis

mTORC1 regulates protein synthesis, lipid synthesis, mitochondrial metabolism and biogenesis, and autophagy. These processes are all important, but due to the scope of this review, mTORC1's regulation of protein synthesis will be the only process examined. mTORC1 positively controls protein synthesis (Laplante & Sabatini, 2009). This positive control is witnessed via the phosphorylation of several proteins (Figure 2.4). Processes that are stimulated via phosphorylation are cap-dependent translocation, translation elongation, mRNA biogenesis, and ribosome biogenesis (Laplante & Sabatini, 2009).

When mTORC1 is phosphorylated it inhibits 4E-binding protein 1 (4EBP1) through phosphorylation, which inhibits eukaryotic initiation factor 4E (eIF4E). eIF4E activates cap-dependent translocation increasing protein synthesis. mTORC1 activates p70 ribosomal S6 kinase 1 (S6K1) via phosphorylation, which in turn activates and inhibits several proteins that contribute to increases in protein synthesis. Activation of S6K1 inhibits programmed cell death 4 (PDCD4) and activates eIF4B through phosphorylation. Both PDCD4 and eIF4B activate eIF4A which also stimulate cap-dependent translocation. In addition, activation of S6K1 inhibits eukaryotic elongation factor 2 kinase (eF2K) via phosphorylation, subsequently stimulating eEF2, which activates translocation elongation. Finally, S6K1 activates both S6K1 aly/REF-like target

(SKAR) and S6, stimulating mRNA biogenesis and ribosome biogenesis, respectively (Laplante & Sabatini, 2009). Through these various proteins, mTORC1 stimulates cell growth by increasing cap-dependent translocation, translation elongation, mRNA biogenesis, and ribosome biogenesis, which leads to an increase in overall protein synthesis.

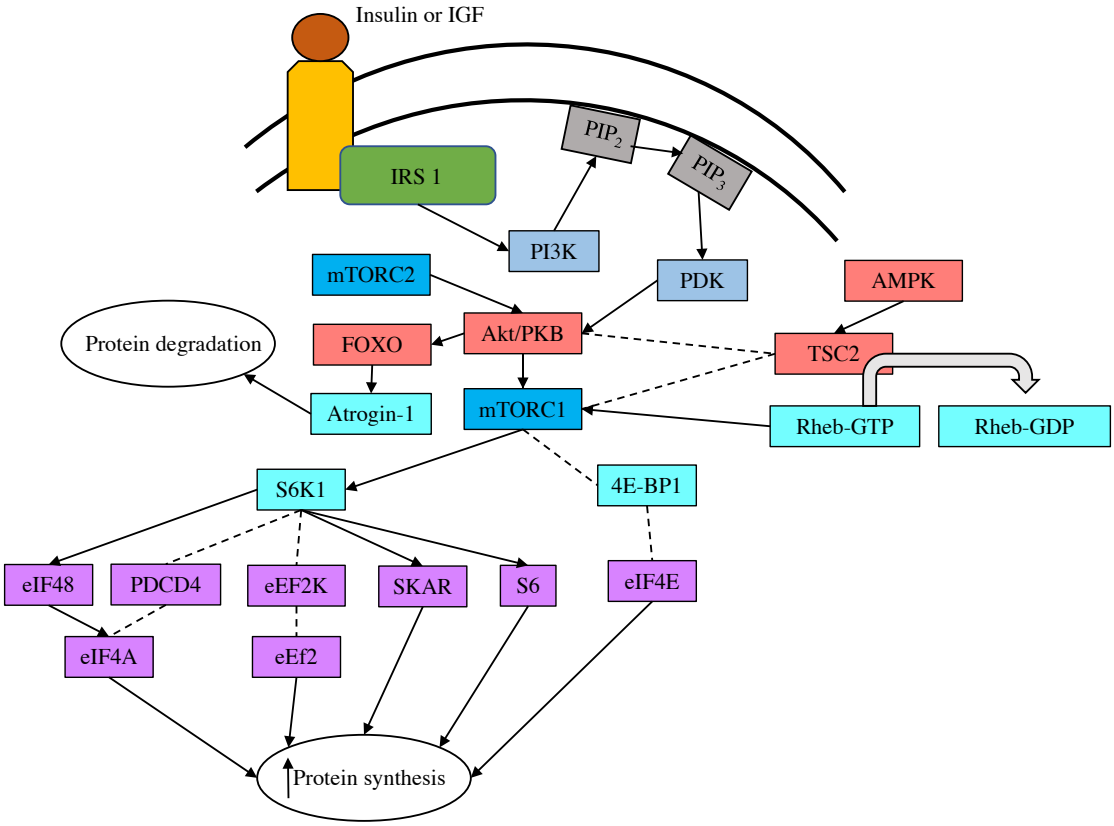


Figure 2.4 Schematic representation of insulin stimulated mTOR activation. Adapted from Laplante and Sabatini (2009). Solid lines represent activation. Dashed lines represent inhibition.

Equine Specific Regulation of Protein Synthesis and Degradation

The mTOR pathway has had little investigation in the horse; however, recent studies aimed to identify how factors such as, exercise, age, feeding, and diet composition regulate muscle protein synthesis. Increases in phosphorylation of 4EBP1 and S6 were found when horses consumed a high-crude protein diet after an 18-hour fast (Urschel et al, 2011). Akt/PKB was not affected by the diet or fasting period. As stated previously, when mTORC1 is phosphorylated it activates 4EBP1 and S6. Urschel et al. (2011) found that S6 and 4EBP1 were 250% and 175% higher, respectively after feeding compared to after the meal was absorbed. This indicates that mTOR was activated directly after the meal was consumed. As a direct result from feeding, AA availability is the primary factor for the increase in mTOR signaling due to greater phosphorylation of 4EBP1 and S6, but not Akt/PKB, after ingestion of the meal and not after the absorption of the meal (Urschel et al., 2011). These results are consistent with Suryawan et al. (2007) and Fujita et al. (2007) who found that providing a mixture of essential AA increase mTOR signaling in piglets and humans. Urschel et al. (2011) findings show an increase in mTOR signaling after the consumption of a meal in the horse, which suggests increased rates of muscle protein synthesis in the mature horse.

Urschel and Wagner (2012) examined the effects of an 18-hour fast followed by a meal on yearlings, 2 year olds, and mature horses in order to measure translation initiation factors, specifically Akt, S6K1, S6, and 4EBP1. Feeding a high-protein diet after an 18-hour fast increased all translation initiation factors in all three groups of horses, suggesting that there is an increase in muscle protein synthesis in all ages of horses after being fed a meal. There was a difference in activation of translation initiation

factors between yearlings and 2-year olds, which suggests developmental differences and responses to insulin and AA (Urschel & Wagner, 2012). Mature horses had a greater response to translation initiation factors from the ingestion of AA and were less sensitive to insulin than the other two groups. Ultimately, this study presented that mTOR signaling is highest postprandial in all 3 age groups. Finding that mature horses had a greater response in translation initiation factors from the ingestion of AA correlates with Graham-Thiers and Kronfeld (2005) who found that AA supplementation lead to aged horses maintaining muscle mass more efficiently than the non-supplemented group. With the availability of AA postprandially, mTOR signaling would be increased thereby increasing muscle protein synthesis, which would result in the increase in muscle mass seen in the AA supplemented group.

Measuring Muscle Protein Turnover in the Horse

Skeletal muscle breakdown can be measured by the metabolites that are excreted in the urine. Granted, there are other methods of quantifying muscle protein breakdown, such as the release of labeled AA from intracellular labeled proteins (*in vitro*), stable isotope tracers (*in vivo*), and leucine oxidation method. Indicator AA used in the *in vitro* labeled method are leucine, valine, tyrosine, phenylalanine, and 3-methylhistidine (3MH) (Wu, 2013).

Some histidine residues undergo posttransitional methylation in actin and myosin proteins, generating 3MH. Since this metabolite is formed through posttransitional events, it cannot be used as a substrate for protein synthesis (Wu, 2013). As stated above, 3MH is found in actin and myosin proteins, which are housed in smooth, skeletal, and cardiac muscles. Therefore, secretion of this metabolite in the urine can be used as index

to quantify muscle protein catabolism. This metabolite does fall short in capturing true muscle protein breakdown. 3MH reacts with β-alanine to form balenine, which is also found in large amounts in skeletal muscle (Wu, 2013). In canines, 3MH undergoes decarboxylation to form 3-methylhistamine. In addition, great quantities of 3MH are excreted in the feces of dogs (Wu, 2013). Diets that consist of meat and bones contain a high quantity of 3MH, but this is not a concern since horses are herbivores. Gallagher (1999) stated that the measurement of 3MH and creatinine are useful to evaluate muscle mass changes between groups of subjects.

Creatinine is a waste product of muscle protein breakdown and is also a useful measurement for kidney function. Creatinine is produced from the breakdown of creatine and phosphocreatine. Creatine, which is formed from the transamination of arginine, glycine, and methionine, is found in the blood and is taken up by muscle and phosphorylated to produce phosphocreatine. Creatine is synthesized in three organs, the liver, kidney, and pancreas. Both substances undergo non-enzymatic and degradation and dehydration to yield creatinine (Wu, 2013). This process most often occurs in muscle, thus making muscle the greatest source of creatinine output.

Insulin

Composed of two, A and B, unbranched peptide chains joined together by two disulfide bonds, insulin targets every organ in the body (Wilcox, 2005). Its most known role is in carbohydrate metabolism, which includes uptake of glucose and glycogen synthesis in skeletal muscle. Insulin also targets other nutrients and produces metabolic alterations, such as: blocking the release of free fatty acids from adipose tissue and the

stimulation of both triglyceride synthesis and the incorporation of AA into various proteins (Wilcox, 2005).

Synthesis

Insulin is synthesized in the beta cells of the pancreas. The 110-AA, preproinsulin, is comprised of a leader sequence, C-peptide, B-chain, and A-chain and begins the synthesis of insulin. The endoplasmic reticulum is the site for the removal of the leader sequence and subsequent folding and disulfide formation of the newly formed proinsulin molecule. It is then transported into the golgi apparatus where it is packaged with zinc into secretory granules. C-peptide is removed from proinsulin by prohormone convertases 2 and 3, which are endopeptidase enzymes. Insulin and C-peptide accumulate in secretory granules in equimolar amounts. When insulin is secreted, all contents of the granule are released, which include proinsulin, C-peptide, and insulin.

Regulation

Secretion of insulin is accomplished by several intracellular signaling pathways (Figure 2.5). Actions of insulin depend solely on the aggregation of various proteins that initiate the signaling cascades. The insulin receptor is composed of α-glycoproteins and β-glycoproteins. The extracellular, α-glycoprotein, contains the insulin binding domain. Whereas, β-glycoproteins span the plasma membrane and contain the tyrosine kinase activity. When insulin binds to its receptor, the α-glycoproteins no longer have inhibitory effects on β-glycoproteins, which allows β-glycoproteins to phosphorylate itself and produce a conformational change. Conformational changes and the subsequent creation of docking sites, can also be accomplished by tyrosine phosphorylation of the insulin

receptor. This allows for other proteins to bind to the receptor, such as insulin receptor substrate (IRS) 1, 2, 3, and 4, which are cytosolic proteins. IRS proteins contain several tyrosine phosphorylation sites and act as a foundation for large signaling complexes, such as PI3K. Phosphatidylinositol-3 kinase (PI3K), a serine/threonine kinase, is stimulated by activated receptors. Receptors are activated by receptor substrates, such as IRS 1, 2, 3, and 4 and activate PI3K-Akt pathways (Boucher, Kleinridders, & Kahn, 2014). Activation of catalytic subunits and subsequently phosphorylation leads to the conversion of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol (3,4,5)-triphosphate (PIP_3). PIP_3 binds to Akt/PKB, which allows PDK to phosphorylate T308. Activation of T308 partially, but sufficiently activates Akt/PKB to activate mTORC1 (Hemmings & Restuccia, 2012). This cascade, as described in previous sections, promotes protein synthesis and cell proliferation. When Akt/PKB is in its fully activated form it stimulates several cellular functions including metabolism, growth, proliferation, survival, transcription, apoptosis, and protein synthesis (Hemmings & Restuccia, 2012).

PI3K and Akt/PKB have negative regulators. These three negative regulators all act on different proteins within the signaling cascade (Figure 2.6). Phosphatase and tensin homolog (PTEN) inhibits the conversion of PIP_2 to PIP_3, thus inhibiting the partial activation of Akt/PKB by PDK. Protein phosphatase 2 (PP2A) and PH-domain leucine-rich-repeat-containing protein phosphatases (PHLPP) inhibit the downstream effects of Akt/PKB (Hemmings & Restuccia, 2012).

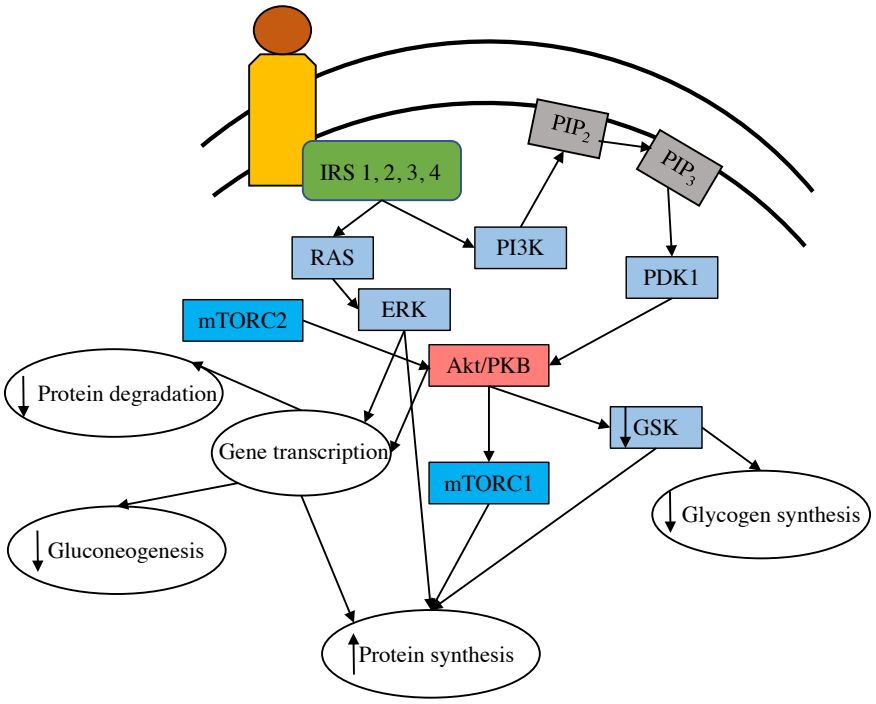


Figure 2.5 PI3K and Akt/PKB signaling cascade. Adapted from Hemmings and Restuccia (2012) and Goodman (2009). Solid lines represent activation. Dashed lines represent inhibition

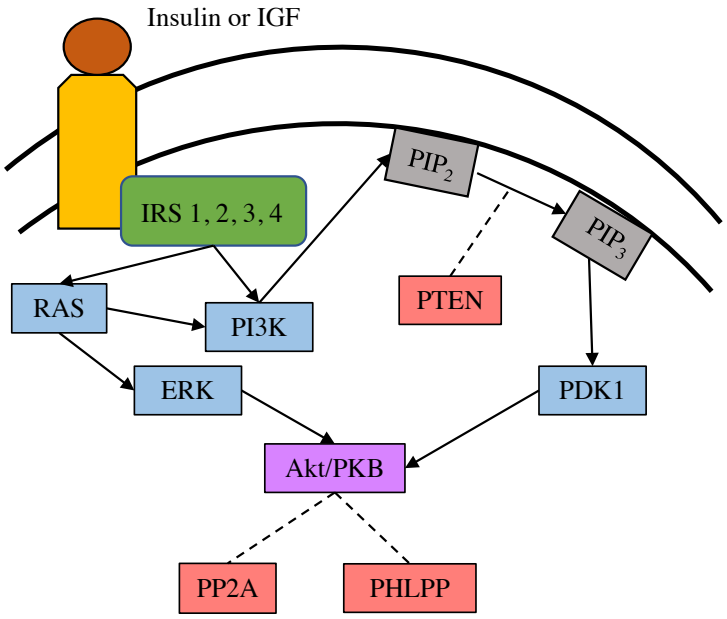


Figure 2.6 Inhibitors of PI3K and Akt/PKB signaling cascades. Adapted from Hemmings and Restuccia (2012). Sold lines represent activation. Dashed lines represent inhibition

Insulin Resistance in the Horse

Defining IR

Similar to insulin action, insulin resistance (IR) specifically targets pathways and tissues. Thus, IR can vary in severity between adipose, liver, and muscle tissue between individuals. IR is defined where a normal or elevated insulin levels produces a weakened biological response (Wilcox, 2005). Alterations at the cellular level are believed to be the most common cause of IR. Wheatcroft et al. (2003) proposed possible sites of alterations: down-regulation, deficiencies or genetic mutations of tyrosine phosphorylation of the insulin receptor, IRS proteins, or PIP_3, or malfunctions in the GLUT4 transporter.

Diagnosing IR in the Horse

Several diagnostic tests exist for diagnosing IR in the horse, including basal glucose and insulin concentrations, oral sugar test (OST), intravenous glucose tolerance test (IVGTT), frequently sampled insulin glucose tolerance test (FSIGT), insulin tolerance test (ITT), and euglycemic hyperinsulinemic clamp procedures (Firshman & Valberg, 2007). Combined glucose-insulin test (CIGT) assess both glucose and insulin response. Horses that have normal insulin action show a return of blood glucose to baseline within 1 hour during the CIGT (Frank, 2009). IR horses take 45 minutes or longer for blood glucose to return in baseline. Insulin's response to the CIGT is assessed in both normal and IR horses at 0 minutes and at 45 minutes later (Frank, 2009). If fasting insulin concentrations are above 20 mU/L, then the horse is suspected to be IR. If insulin concentrations exceed 100 mU/L at or after the 45-minute mark, the insulin response to the CIGT is considered excessive (Frank, 2009).

IVGTT avoids the variability that is seen in the OST (Kronfeld, Treiber, & Geor, 2005). For this test, insulin resistant horses have a higher peak in blood glucose, followed by a >2-hour delay in the return to baseline glucose concentrations. Whereas in normal horses have an immediate rise in blood glucose concentrations followed by a return to baseline within an hour (Firshman & Valberg, 2007). ITT is a direct measure of the blood glucose response to an injection of insulin, thus measuring the tissues response to insulin. This test's outcomes can be affected by age, diet, and stress levels (Firshman & Valberg, 2007).

Basal glucose and insulin concentrations, OST, IVGTT, and ITT pose a disadvantage to measuring insulin action. These diagnostic tests produce uncontrollable fluctuations in insulin secretion, which alter glucose homeostasis (Firshman & Valberg, 2007). Therefore, glucose clamp procedures are used to control this variability, which include the hyperglycemic clamp and the hyperinsulinemic euglycaemic clamp (HEC). Hyperglycemic clamp procedures quantify the sensitivity of the pancreatic β cells to glucose (Firshman & Valberg, 2007). HEC quantifies insulin sensitivity of muscle and adipose tissue by providing steady state insulin concentrations and glucose infusion that maintains euglycemic (Firshman & Valberg, 2007).

The OST used by Schuver et al. (2014) was conducted by orally administering corn syrup using a 60-mL catheter-tipped syringe at a dosage of 0.15 mL/kg of body weight. The manufacturer of the corn syrup would only disclose that this dosage would provide 150 mg of digestible sugars per kilogram of body weight; however, that information could not be verified by the Schuver et al., 2014 study. The corn syrup used contained glucose, maltose, and starch, but not fructose (Schuver et al., 2014). Blood was

collected via venipuncture before and after the administration of the corn syrup at 30, 60, 90, and 120 minutes.

Diagnostic tests described above, with exception to basal glucose and insulin concentrations and the OST, require hospitalization and can be expensive to conduct. An OST, is used to describe blood glucose clearance (Firshman & Valberg, 2007). OST quantifies the amount of glucose the liver can absorb, the endocrine function of the pancreas, and the absorption of glucose from the small intestine (Firshman & Valberg, 2007). Thus, providing an accurate presentation of normal physiological functions of glucose uptake and insulin secretion in the horse undergoing testing. Schuver et al. (2014) compared the IVGTT to an OST. Both IVGTT and OST were conducted in ten equine metabolic syndrome horses and eight healthy adult mares. OST and IVGTT results were positively correlated, which suggests that the OST is a reliable diagnostic test for insulin dysfunction (Schuver et al., 2014). Equine metabolic syndrome (EMS) horses were found to differ from control horses, regarding both glucose and insulin concentrations for both tests, further validating the OST (Figure 2.7 & Figure 2.8; Schuver et al., 2014).

The best method for determining IR in horses is still elusive. Banse and McFarlane (2014) compared the insulin response to dexamethasone test and OST to the HEC. In this study, the OST was performed in the stall and the pasture, showing a strong, positive correlation ($r = 0.76$, $p = 0.02$ = stall; $r = 0.72$, $p = 0.04$ = pasture) in insulin concentration at T0 and T75, indicating that an OST has the same results in both a stall and pasture setting. Banse and McFarlane (2014) found no differences between mares and geldings ($p > .10$). OST was conducted throughout the year and it was found that

September plasma glucose concentrations at T75 were higher than summer plasma glucose concentrations during the at T75 (Banse and McFarlane, 2014). Other differences were not found for plasma glucose or insulin concentrations at T0 or T75 across seasons (Figure 2.9; Banse and McFarlane, 2014).

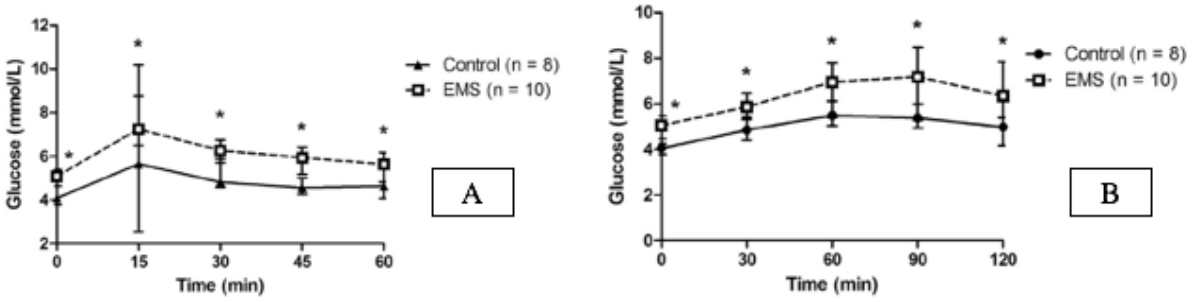


Figure 2.7 Glucose concentrations in the IVGTT and OST. Adapted from Schuver et al. (2014)
(A) Represents glucose concentrations from the IVGTT.
(B) Represents glucose concentrations from the OST.

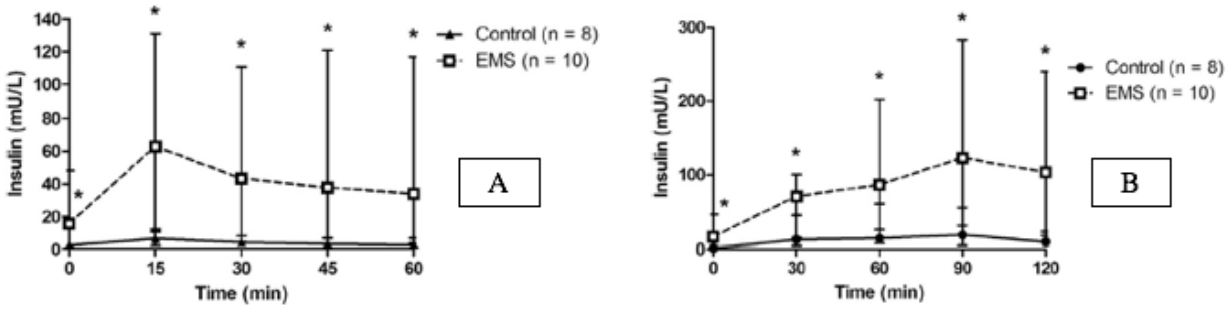


Figure 2.8 Insulin concentrations in the IVGTT and OST. Adapted from Schuver et al. (2014)
(A) Represents insulin concentrations from the IVGTT.
(B) Represents insulin concentrations from the OST.

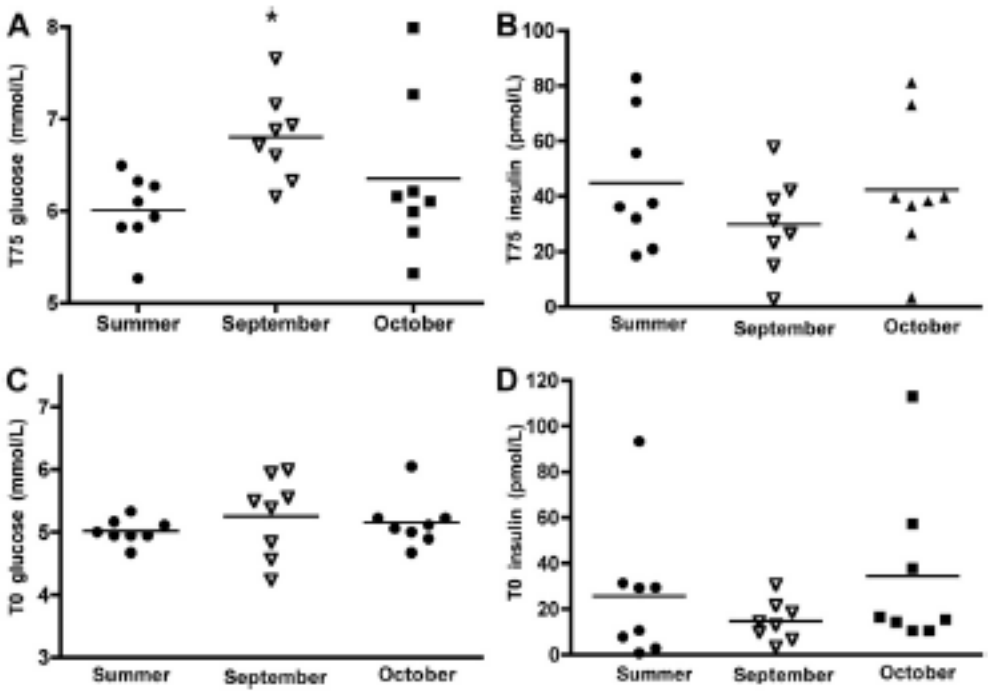


Figure 2.9 OST results for plasma glucose and insulin across seasons. Adapted from Banse and McFarlane (2014).
(A) OST T75 glucose.
(B) OST T75 insulin.
(C) OST T0 glucose.
(D) OST T0 insulin.

Factors Affecting Insulin Concentrations

Insulin is a pleiotropic hormone that mediates several metabolic pathways and organ systems. Pertinent metabolic pathways have been described in previous sections. Insulin targets three main tissues: adipose, liver, and skeletal muscle. This gives rise to explore insulin's effects on more than just carbohydrate metabolism, such as protein synthesis and degradation. Insulin secretion is effected by exercise, diet, insulin resistance, age, and breed. These factors affect the production and secretion of insulin, thus modifying both protein synthesis and muscle protein synthesis (Figure 2.10). Effects of exercise will not be covered in this review; however, diet, IR, age, and breed will be examined.

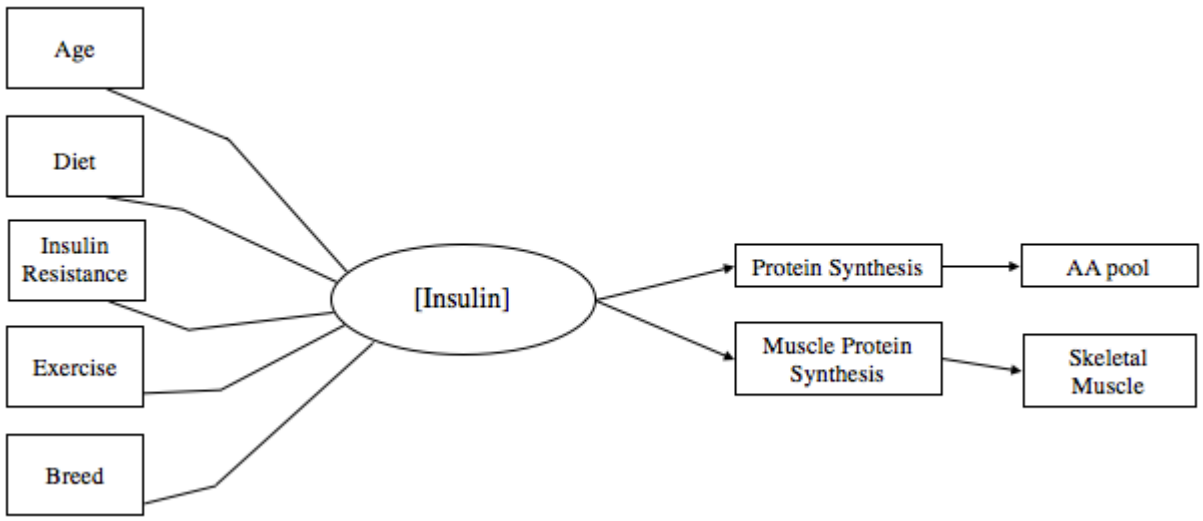


Figure 2.10 Factors affecting insulin sensitivity. Designed by author. All factors (age, diet, IR, exercise, and breed) affect the concentration of plasma insulin secreted, which subsequently effects protein and muscle protein synthesis.

Age

From birth, pancreatic β cells mature continuously until 3 months of age. This was established by Smyth et al. (1993) who found similar levels of pancreatic β cell function in mature horses. In humans, insulin sensitivity decreases with age; whereas, the time it takes to develop IR in horses is dependent on environment, diet, and genetics (Barbieri et al., 2001). Urschel and Wagner (2012) found an increase in activation factors of Akt/PI3K in yearlings compared to two-year olds, suggesting that yearlings are more insulin sensitive than 2-year-olds horses. Urschel and Wagner (2012) also found that mature horses have a greater response to post-prandial AA and were less sensitive to insulin compared to yearlings and 2-year-olds. Age plays a role in the development of insulin dysfunction. If age is accounted for in a research setting, accurate interpretations can be made.

Breed

Results of studies that examine insulin sensitivity between breeds are difficult to compare due to being conducted at different institutions and at different times. Methodology was similar between studies and therefore can be used to compare breed differences in regards to insulin sensitivity. Standarbreds were found to have greater insulin sensitivity than ponies (Jeffcott & Field, 1986). This finding was validated by Rijnen and van der Kolk (2003), which was done via HEC and found that ponies had a lower glucose disposal rate than Warmbloods. Compared to Quarter horses, Belgian drafts had a greater insulin sensitivity, which was quantified based on the amount of glucose infused to attain euglycemia (Firshman et al., 2006). Rate of glucose disposal during a HEC for Standardbreds was greater than both Belgian draft horses and Quarter

horses, suggesting that Standardbreds are more insulin sensitive (Pratt et al., 2006). When measuring insulin concentrations in research settings, accounting for breed differences is crucial, especially for studies that use mixed breeds.

Diet

Insulin sensitivity can occur independently of obesity. Long-lasting, positive energy balance, however, can contribute to insulin dysfunction (Schmidt & Hickey, 2009). A high fiber grain and hay were compared in mature ponies using an OGTT (Murphy et al., 1997). Murphy et al. (1997) found that hay had a higher plasma glucose response than the high fiber grain. Also in mature ponies, Argenzio & Hintz (1972) found that the grain only diet produced lower blood glucose concentrations than that of the hay only diet. Furthermore, Hoffman et al. (2003a, b) found that insulin sensitivity was lowered in horses fed grain and molasses compared to those fed high fat and fiber diets. Feeding non-structural carbohydrates for 6 weeks has also been shown to decrease insulin sensitivity (Pratt et al., 2006).

Schmidt and Hickey (2009) suggested that high-protein diets would be appropriate treatments for horses with metabolic disorders; however, protein and AA have independent effects on insulin's actions. Linn et al. (1996, 2000) reported that in humans, high protein diets negatively impact whole body insulin sensitivity. Type I diabetics showed increased hepatic glucose production (Linn et al., 1996). Similar findings were reported in healthy, non-obese adults, who showed suppressed hepatic glucose output, increased gluconeogenesis, and elevated fasting blood glucose (Linn et al., 2000). Short-term elevations in plasma AA has suppressed insulin-mediated glucose uptake (Tessari et al., 1985; Flakoll et al., 1992; Krebs et al., 2002). As mentioned

previously, Urschel and Wagner (2012) found that consumption of a high protein diet in horses resulted in the increase in activation of Akt/PKB, S6K, S6, and 4EB1. Compared to 2-year-olds, mature horses were found to have a greater response to consuming a high protein diet (i.e. presence of AA) and were less sensitive to insulin's actions (Urschel and Wagner, 2012). When feeding protein to horses with metabolic disorders, protein quality, not quantity, should be of the utmost importance.

Effects of Insulin and IR on Protein and Muscle Protein Synthesis

As stated previously, insulin acts on adipose, liver, and muscle tissues. Muscle accounts for 60-70% of the insulin-stimulated glucose disposal in peripheral tissues, as well as postprandial glucose uptake (Smith, 2002). This makes muscle one of the most important sites of insulin action and insulin resistance. Insulin increases glucose uptake in muscles by stimulating the relocation of the glucose transporter, GLUT4, from the intracellular pool to the cell membrane (Dimitiadias et al., 2011). In a mature individual (i.e. non-growing), protein degradation and protein synthesis are balanced (Figure 2.11). In healthy subjects, insulin promotes glycogen synthesis in the fed state, which allows for energy to be released anaerobically via glycolysis. In horses, increased plasma concentrations of insulin have shown to induce protein and muscle protein synthesis in mature horses (Urschel et al., 2014). Insulin deficiency results in massive breakdown of muscle protein, which is accomplished through insulin stimulated release of AA, which are then used for gluconeogenesis (Goodman, 2009).

Anabolic effects of insulin include increased transportation rate of AA into tissues, increased rate of muscle protein synthesis and other tissues, decreased rate of protein degradation in muscle, as well as decreased flux of urea formation (Geor et al.,

2013). Insulin functions in not only muscle, but other tissues as well, are clearly anabolic. Ultimately, insulin decreases the release of AA from muscle, while simultaneously incorporating AA into muscle. To maintain this anabolic state, sufficient plasma AA need to be present in the body (Dimitiadias et al., 2011). If dietary AA are not supplied in sufficient quantities (e.g. low protein-high carbohydrate diet), insulin will inhibit gluconeogenesis, therefore decreasing the use of AA in the liver, which prevents the decrease in plasma AA concentrations (Dimitiadias et al., 2011).

Validating muscle's vital role in insulin action and insulin resistance, increases in vasodilation and capillary recruitment results in increased blood flow to muscle, which is stimulated by insulin (Dimitiadias et al., 2011). The increase in blood flow in muscle mediated by insulin, as well as insulin stimulated glucose uptake, make muscle a vital determinant in insulin sensitivity (Dimitiadias et al., 2011).

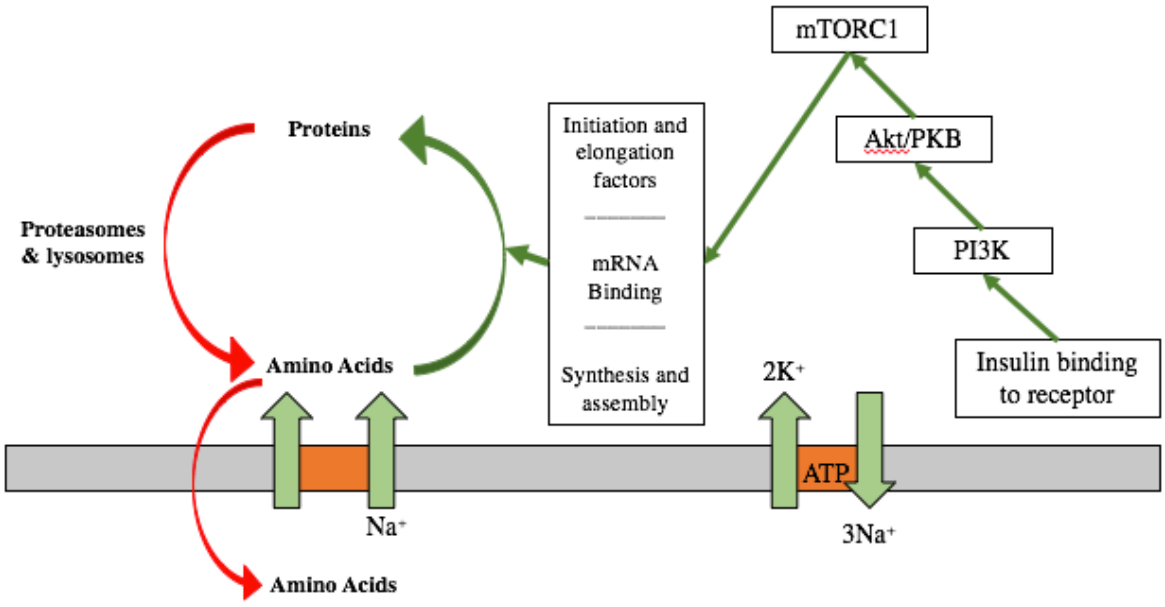


Figure 2.11 Insulin's effect on protein turnover. Adapted from Goodman (2009). Actions stimulated by insulin are represented by green arrows. Actions inhibited by insulin are represented by red arrows.

Insulin Stimulates Protein and Muscle Protein Synthesis

Insulin stimulates muscle protein synthesis. Wray-Cahen et al. (1998) used a hyperinsulinemic-euglycaemic-AA clamp on 7- and 26-day old piglets to examine insulin's actions on muscle protein synthesis. Insulin was infused to mimic plasma insulin concentrations seen in the fasted, fed, and refed states. Whole body AA disposal was calculated from the necessary amount of infused AA to maintain plasma essential AA near fasting levels. As insulin infusion increased, whole body AA disposal increased. Muscle protein synthesis stimulated by insulin was greater in 7-day old pigs compared to 26-day old pigs. This study suggests that muscle protein synthesis is more responsive to insulin than disposal of whole body AA (Wray-Cahen et al., 1998).

A major difficulty in studying *in vivo* effects of insulin on protein synthesis and degradation is maintaining plasma AA concentrations at baseline, which is vital when trying to compensate for the lowering effect of insulin (Chevalier et al., 2003). Protein turnover measured during hyperinsulinemia and hypoaminoacidemia have shown either stagnant or decreased rates of whole body protein synthesis (Tessari et al., 1982; Castellino et al., 1987; Petrides et al., 1991). Decreased or stagnant rates of whole body protein synthesis could be due to reduced availability of essential plasma AA (Chevalier et al., 2003). Thus, researchers attempted to control this unwanted effect by infusing a mixture of AA, as well as using a HEC. The combination of these procedures, most often produces hyperaminoacidemia, thus increasing protein synthesis (Castellion et al., 1987; Petrides et al., 1991). Therefore, methods used to study insulins effects on protein and muscle protein synthesis are complicated at best.

Branched-chain AA (BCAA) have been documented to be the most sensitive to insulin, thus important for determining protein metabolism (Felig, Marliss, & Ohman, 1970; Luzi, Castellino, & DeFronzo, 1996). Chevalier et al. (2004) determined the roles of insulin and AA in protein synthesis and degradation by controlling baseline concentrations of BCAA by varying infusion rates of AA solution during a HEC. Reasons behind this methodology, according to the authors, were that by clamping the BCAA, the other AA found in the solution infused, that are known regulators of protein metabolism, potentially might be clamped as well (Chevalier et al., 2004). Using this procedure, Chevalier et al. (2004) found that protein synthesis increased with insulin and protein breakdown decreased. Thus, through suppression of whole-body protein degradation, in vivo protein anabolism was induced when controlling postabsorpative plasma AA concentrations in conjunction with high plasma insulin concentrations (Chevalier et al., 2004).

Varying levels of insulin were infused using an isoglycemic, hyperinsulinemic clamp procedure in horses to determine the effects on plasma AA concentrations (Urschel et al., 2014). Four different rates of insulin infusion rates stimulated mTOR signaling in skeletal muscle. The final 30 minutes of the clamp procedures revealed that plasma insulin concentrations were 12, 83, 327, and 1001 mU/L for the four infusion rates. A decrease in the concentration of BCAA was seen in the gluteus medius, which was dose-dependent. In this study, as insulin increased, circulating plasma AA decreased. Marked decreases in plasma AA were seen, as well as increases in the activation of mTOR signaling factors, indicating that circulating insulin produces changes in protein and muscle protein synthesis (Urschel et al., 2014). This study concurs with a previous study

mentioned, Wray-Cahen et al. (1998), that showed the rate of AA infusion required to maintain normal plasma AA concentrations increased with increases doses of insulin infusion.

Insulin Resistance Effects on Protein and Muscle Protein Synthesis

Insulin resistance impairs glucose disposal. Insulin is a known anabolic hormone, which stimulates protein synthesis and suppresses protein degradation. Halvastsiotis et al. (2002) measured leucine flux (marker for protein degradation) in diabetic and non-diabetic humans to determine if insulin's normal action of suppressing protein degradation is altered. Diabetic patients had higher insulin levels than non-diabetic patients; however, AA concentrations were similar between both groups ($p < 0.01$). Suppression of whole-body protein synthesis in response to short-term insulin is normal in people with Type 2 diabetes (Halvastsiotis et al., 2002).

Tai et al. (2010) used 263 non-obese men and distributed them into lower and upper tertiles of IR (Tai et al., 2010). IR was diagnosed using homeostatic model assessment (HOMA), which assess β-cell function and IR from basal glucose and insulin concentrations. Tai et al. (2010) found that individuals with high HOMA had higher concentrations of alanine, proline, valine, isoleucine, leucine, phenylalanine, tyrosine, glutamate, glutamine, and ornithine compared to patients that had low HOMA. When adjusted for age and BMI all the amino acids listed remained higher, except for phenylalanine. Overall, alterations in plasma AA concentrations were found to be associated with IR in this sample of human patients (Tai et al., 2010).

Conclusion

Insulin has been shown to stimulate whole-body protein synthesis in horses. Insulin resistance is becoming more prevalent in horses, as it is associated with EMS, PPID, and glycogen storage diseases and may affect protein and muscle protein status. To the author's knowledge, there are currently no studies that have used IR horses to examine the effects of insulin dysfunction on protein and muscle protein metabolism.

CHAPTER 3. RATIONALE & OBJECTIVES

Rationale

Little is known about the effects of IR on protein metabolism in the horse. IR is most often a symptom of EMS, equine glycogen storage disorders, pars pituitary intermedia disorder (PPID), and cancer. IR can also be seen in obese horses, but can be independent of obesity.

Arguably the most important circulating hormone in the body, insulin, functions in multiple tissues, including adipose, liver, and muscle. Insulin secretion is primarily triggered by the presence of glucose. Muscle accounts for 60-70% of the insulin-stimulated glucose disposal in peripheral tissues, as well as postprandial glucose uptake (Smith, 2002). Thus, skeletal muscle tissue is one of the largest and most prominent sites of insulin action and insulin resistance.

A previous study examined the effects of varying concentrations of insulin on whole-body and muscle protein synthesis (Urschel et al., 2014), using increasing HEC insulin doses to stimulate the mTOR signaling pathway. The clamp procedure revealed that as insulin increased, circulating plasma AA decreased and mTOR signaling factors increased. This indicated that circulating insulin produces changes in protein and muscle protein synthesis in the horse (Urschel et al., 2014).

Urschel et al. (2014) was performed in eight healthy, mature (13.8 $\pm$ 3.4 yrs) horses weighing 604.5 $\pm$ 37.4 kg with BCS ranging from 5 to 7. Therefore, horses exhibiting from IR have not been used to examine insulin's effects on protein metabolism. It could be said that IR was synthetically induced in the Urschel et al. (2014)

study. In order to capture the actual physiological effects of IR on protein metabolism, IR horses would need to be used.

Objectives

The aim of this study was to investigate IR's effect on protein metabolism in normal horses compared to horses diagnosed with IR. Urschel et al. (2014) found that as plasma insulin increased, plasma AA decreased. Thus, the hypothesis of the current study was that IR horses would have lower plasma and muscle amino acids. Muscle mass and skeletal muscle synthesis were also hypothesized to be lower in IR horses, which was quantified by urinary creatinine and 3MH.

CHAPTER 4. MATERIALS & METHODS

Animals, Housing, and Diets

Eight mature horses from Emory and Henry College in Bristol, Virginia were used. Horses weighed an average of 617 $\pm$ 84 kg, with an average of 5.6 $\pm$ 1.1 BCS. Three mares and five geldings were used for this study with an average age of 16 $\pm$ 3 years. Breeds included one Hanoverian, one Oldenburg, one Warmblood, one Welsh pony, one Holsteiner, one Dutch Warmblood, one QH Warmblood, and one Selle Francais. Prior to the start of the study, horses were housed in stalls at Emory and Henry College and received ad libitum access to pasture grass during turnout for approximately 12 hours a day. Horses underwent initial adaption to stalls and treatment diets one week before the initiation of experimental treatments and sample collection. During the trial, horses were housed in stalls except for their allotted overnight turnout (~12 hours), where they had ad libitum access to grass. Horses were fed and housed individually during the adaption and collection period in 3 x 3 m stalls. BW were recorded using an electronic scale (Model AL660-LA, Cambridge Scaleworks, Honey Brook, PA) on days 0 and 6 of the experimental period and feed quantities offered were adjusted accordingly. The protocol was approved by the Institutional Animal Care and Use Committee at Emory and Henry College, where the horses were owned and housed. All research followed guidelines stated in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010).

Experimental Treatment and Diets

One month prior to the beginning of the study, horses were screened for IR by taking fasted baseline blood samples to measure plasma glucose and insulin concentrations. Horses were fasted overnight for 12 hours with access to ad libitum water. At 0800 h the next morning, basal blood samples were drawn via venipuncture to determine plasma insulin and glucose concentrations. The American Association of Equine Practitioners (AAEP) diagnoses insulin resistance with fasting insulin concentrations > 30 mU/L. (Frank, 2013). Horses with fasting insulin concentrations of <20 mU/L are considered to have normal insulin concentrations. Normal fasting glucose concentrations are <100 mg/dL. Based on the screening concentrations, horses identified as IR had fasting insulin (41.2 $\pm$ 15.9 mU/L; Figure 5.1A) that met the AAEP criteria and was greater ($p = 0.026$) than Normal horses (10.1 $\pm$ 3.5 mU/L; Figure 5.1B). Fasting glucose concentrations were within normal ranges (100.8 $\pm$ 1.6 mg/dL vs 93.8 $\pm$ 7.3 mg/dL), and not different ($p = 0.29$) between IR and Normal horses, respectively.

The experimental treatments included an oral sugar test (OST), which has been validated by Schuver et al. (2014), and a controlled and balanced hay and grain diet. After the last blood collection of the OST, horses began the adaption phase of the trial. Trial diets were administered for one week prior to collection. Trial diets simulated horses consuming the same proportion of nutrients, especially protein (Table 2.2 & 2.3). All horses (both IR and non-IR) received the trial diet, which was composed of Purina Strategy (Purina Animal Nutrition LLC.) and a Timothy/Bermuda grass hay mixture fed at 2% of BW/day. The trial diet was designed to meet or slightly exceed requirements for mature horses at maintenance (NRC, 2007). Horses were fed half of their daily intake at

7:00 am and the remainder was fed at 3:00 pm. Feed refusals from the previous day's meals were collected prior to offering the next day's meal and recorded. Unlike the adaption phase, horses only received half of their daily ration before collection.

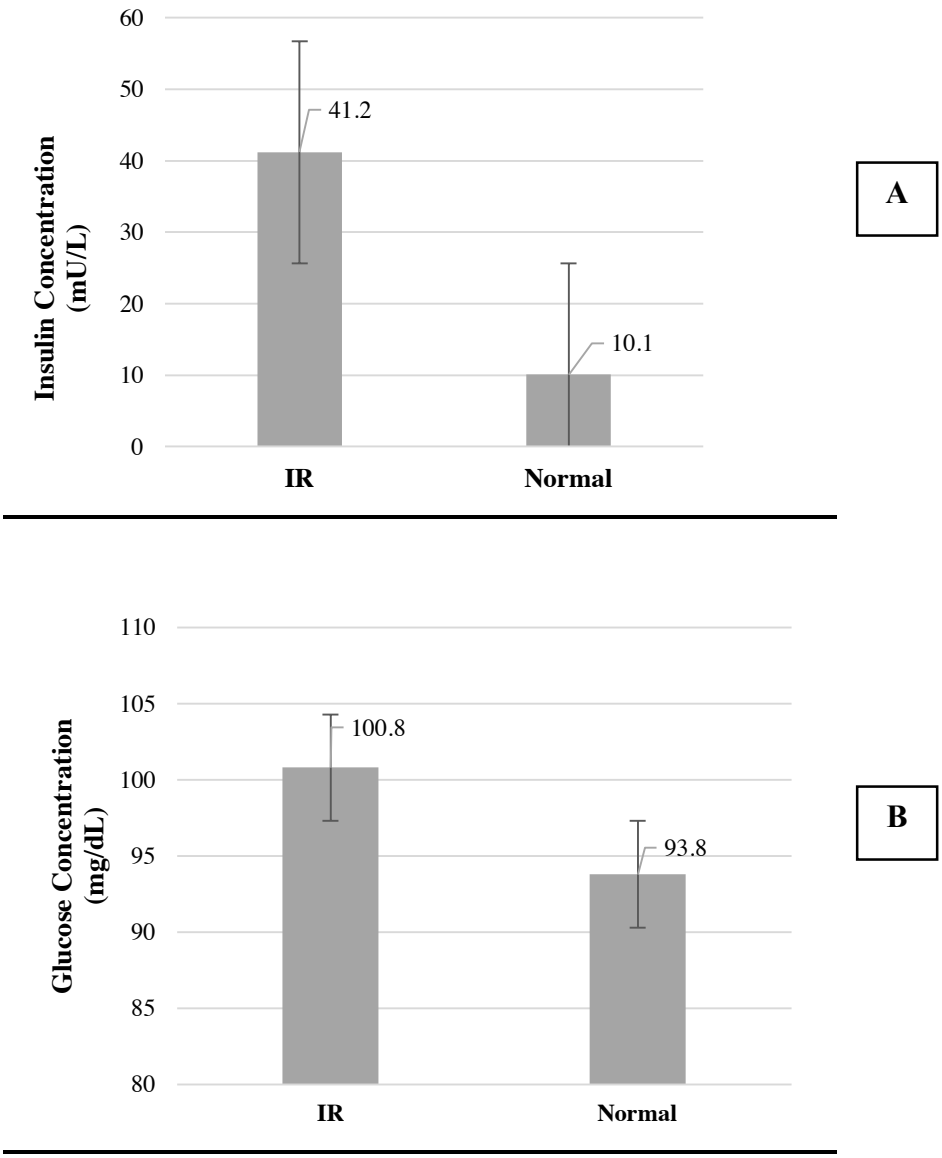


Figure 4.1 Screening plasma insulin and glucose concentrations.
(A) Plasma insulin concentrations ($p = 0.026$).
(B) Plasma glucose concentrations ($p = 0.29$).

Table 4.1 Diets[1] of allotted proportions of mixed Timothy/Bermuda grass hay and grain (Purina Strategy) fed to each horse over the course of the study.

	Horse							
	1	2	3	4	5	6	7	8
Hay (kg)	10.1	12.6	11.1	7.6	10.1	9.6	9.6	9.6
Grain (kg)	2.5	3.2	2.7	0	2.6	2.5	2.5	2.5

[1]Daily intakes were calculated based on daily intakes of 2% of BW/d.

Table 4.2 Purina Strategy analysis

Component	DM
Crude Protein	17.8%
Available Protein	17.0%
ADICP	0.78%
Soluble Protein % CP	32%
NDICP	3.05%
ADF	15.8%
aNDF	33.9%
Calcium	1.35%
Phosphorus	0.81%
Magnesium	0.34%
Potassium	1.35%
Sodium	0.30%
Iron	958 ppm
Zinc	445 ppm
Copper	112 ppm
Manganese	284.5 ppm
Sulfur	0.31%
Horse DE	1.37 Mcal/Lb

Moisture – 9.8%, DM – 90.2%

Table 4.3 Timothy/Bermuda grass hay mix analysis

Component	DM
Crude Protein	8.55%
Available Protein	7.6%
ADICP	0.95%
Soluble Protein CP	39.5%
Degradable Protein CP	64.5%
NDICP	2.7%
ADF	42.35%
aNDF	64.4%
Lignin	5.65%
NFC	18.8%
Starch	1.15%
WSC	8.15%
ESC	5.95%
Crude Fat	2.55%
Total Fatty Acids	0.89%
Ash	5.78%
Calcium	0.52%
Phosphorus	0.22%
Magnesium	0.19%
Potassium	1.67%
Sulfur	0.12%
Chloride Ion	0.44%
Lysine	0.20%
Methionine	0.11%
DE	0.95 Mcal/Lb

Moisture 8.85%, DM – 91.2%

Experimental Design

All horses (both IR and Normal) consumed the same experimental diet. The experimental diet was fed 1 week prior to a two-day collection. Day 0, horses underwent the OST and started the adaption phase (day 0-6) prior to collection. Day 7, baseline and post prandial (1, 3, 4, and 6 hours) blood samples were taken for the determination of plasma amino acids, insulin, and glucose concentrations. Day 8, muscle biopsies were taken after allowing horses to consume half the daily ration.

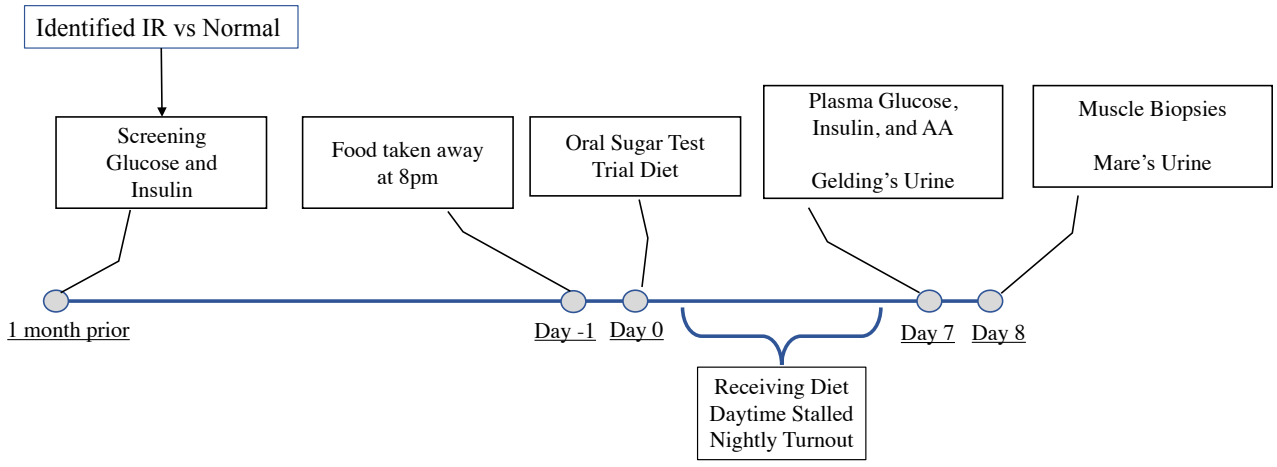


Figure 4.2 Experimental design.

Sample Collection

Oral Sugar Test

The day before the OST, horses had their remaining hay removed at 8:00 pm and were fasted overnight with ad libitum access to water. On day 0, starting at 8:00 am, horses were dosed with Karo Light Corn Syrup (ACH Food Companies) at a dosage of 0.15 mL/kg of body weight (Schuver et al., 2014). The Karo syrup was administered orally through a 60 mL catheter-tipped syringe. Blood samples were collected via venipuncture before and at 60, 75, 90, 120, 180 minutes after the administration of the Karo syrup. After the last blood collection, horses began their treatment diets.

Pre- and Post-Prandial Blood Samples

On day 6 at 8:00 pm, the remaining hay was taken away and horses were fasted overnight with ad libitum access to water. On day 7 at 8:00 am, baseline blood samples (T0) were collected (10-mL Vacutainer tubes containing sodium heparin, Becton Dickinson, Franklin Lakes, NJ). Horses then received half of their daily ration, which included hay and grain and blood collection followed an hour after offering the meal. Post-prandial blood samples were taken at 1, 3, 4, and 6 hours via venipuncture. Plasma was separated by centrifugation at 3,400 x g at 22°C for 15 min, and then frozen for later analysis of amino acids, insulin, and glucose.

Muscle Biopsies

On day 8, horses received half of their daily ration, which included both hay and grain at 7:30 am. One hour later, horses were tranquilized, tails wrapped, and secured out of place for the collection of the muscle biopsy from the semitendinosus muscle. An area approximately four inches below the point of the buttock was shaved and sterilized with

betadine. Lidocaine (10 mL) was injected intramuscularly into the sample site. After the lidocaine settled for 2-3 minutes, an incision approximately and inch in length was made through the skin. A 7mm Acu-Punch skin punch (Acuderm Inc, Fort Lauderale, FL) was inserted into the site and manipulated to remove a piece of muscle. After the tissue was removed, the sample was weighed and the procedure was repeated until approximately 300 mg of muscle tissue was obtained. The tissue was immediately frozen in liquid nitrogen for later analysis of free amino acids. After the biopsy was complete, the incision site was closed with 1-2 stiches and horses were taken back to their stalls to recover from sedation.

Urine

On day 7, after receiving half of their daily ration, which included both hay and grain, geldings were fitted with collection harnesses that allowed for the separate collection of feces and urine in separate compartments (Equisan, South Melbourne, Australia). For this study, only urine was collected from the horses, manure was discarded. Horses were monitored closely and urine was collected as soon as horses urinated. The urine was then transferred from the collection harness to the specimen cup and frozen for later analysis of 3MH.

On day 8, after receiving half of their daily ration, which included both hay and grain, the mare's urine was collected. While anesthetized and after the collection of the muscle biopsy, the mare's vulva was sterilized and a stallion urinary catheter (Jorgensen Labs, Loveland, Colorado) was passed to the bladder. Urine was collected directly into specimen cups and frozen for later analysis of 3M-histdine and creatinine.

Laboratory Analysis

Pre- and Post-Prandial Blood Samples and Muscle Biopsies

Plasma glucose was analyzed in duplicate using Glucose AutoKit (Wako Diagnostics, Richmond, VA). Plasma insulin samples were analyzed in duplicate by radioimmunoassay (BET Laboratories, Lexington, KY). Plasma amino acid and semitendinosus amino acid concentrations were determined via HPLC analysis of the phenylisothiocyanate derivatives, previously described (Urschel et al., 2011).

Urinary 3MH and Creatinine

Urine collected from both geldings and mares were analyzed using an internal standard (ISTD) method. When analyzing 3MH, a dueterated-3MH molecule will be used for analysis, using gas chromatography/mass spectrometry, and quantification as the ISTD (Heartland Assays, Ames, Iowa). This procedure has been validated by Rathmacher et al. (1992) and Rathmacher, Flakoll, and Nissen (1995). Creatinine was analyzed using colorimetric assay by the Jaffe reaction (Heartland Assays, Ames, Iowa).

Statistical Analysis

Data were tested for normality using a Shapiro-Wilk statistic. Glucose, insulin, plasma amino acids, semitendinosus amino acids, and urine were analyzed using a mixed model with repeated measures analysis with time and group as main effects (SAS, ver 9.25, SAS Inst., Cary, NC). Similarly, 3MH and creatinine were analyzed using a mixed model with repeated measure analysis with group as main effects. Pearson's correlation coefficients were used to examine relationships between semitendinosus free amino acids

and basal insulin. Statistical significance was considered at $P \leq 0.05$ and trends were considered at $0.05 < P < 0.10$.

CHAPTER 5. RESULTS

OST – Insulin and Glucose Concentrations

The night before the OST, horses were fasted with access to ad libitum water. Starting at 8am the next morning, blood samples were collected before and at 60, 75, 90, 120, 180 min after administration of Karo Light Syrup (0.15 mL/kg of BW) for the determination of plasma insulin and glucose. Area under the curve (AUC) for glucose concentrations during the OST were not different ($p = 0.28$), but AUC of insulin tended to be different ($p = 0.061$) between Normal and IR horses (Table 5.1). The OST data confirmed higher insulin ($P = 0.020$) and a trend for higher glucose ($p = 0.055$) in IR vs. Normal horses over time. As expected, there was a post-OST change in insulin ($p = 0.03$) and glucose ($p = 0.007$) over time. There was no difference for the interaction of group and time for insulin ($p = 0.25$) or glucose ($p = 0.28$). Insulin peaked in the IR group at 75 and 120 minutes (Figure 5.1). Normal horses peaked insulin at 60 minutes and declined thereafter (Figure 5.1). Glucose peaked in the IR group at 75 minutes and subsequently declined (Figure 5.2). Glucose also peaked at 75 and 120 minutes for Normal horses (Figure 5.2). Glucose concentrations for Normal horses decreased back down to baseline values at 180 minutes.

Table 5.1 AUC for glucose and insulin concentrations during the OST

	Normal	IR	*p*-value
Glucose, $mg/dL^{-1}*min^{-1}$	2154 ± 704	3785 ± 1186	0.28
Insulin, $mU/L^{-1}*min^{-1}$	761 ± 261	4522 ± 1610	0.061

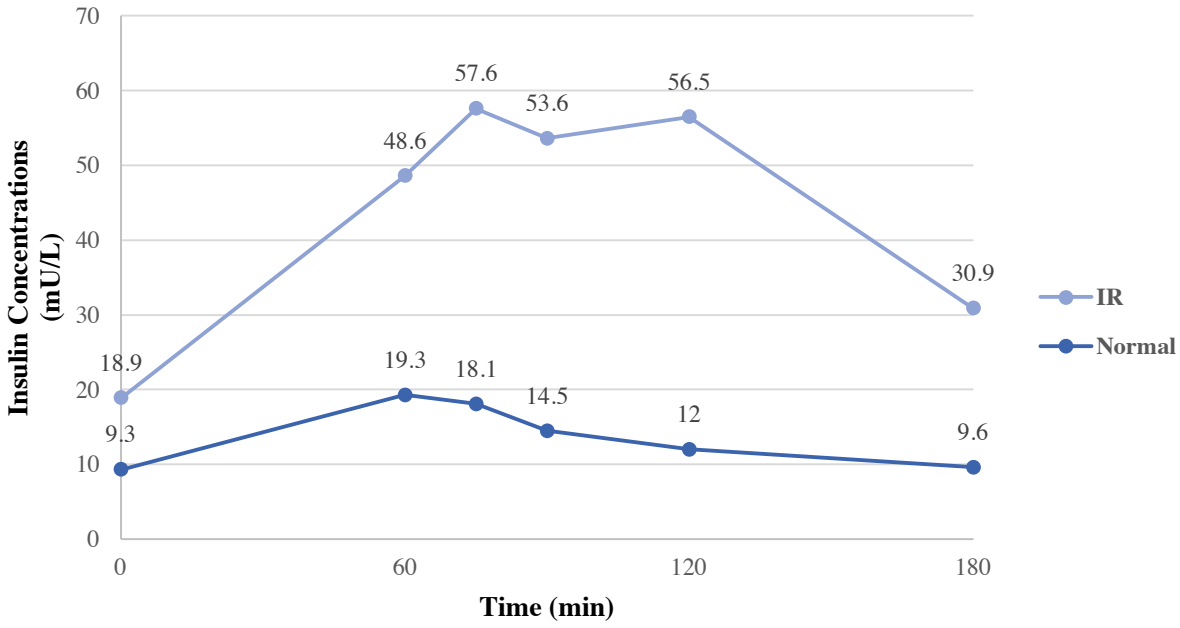


Figure 5.1 Plasma insulin concentrations during OST. ($p = 0.25$)

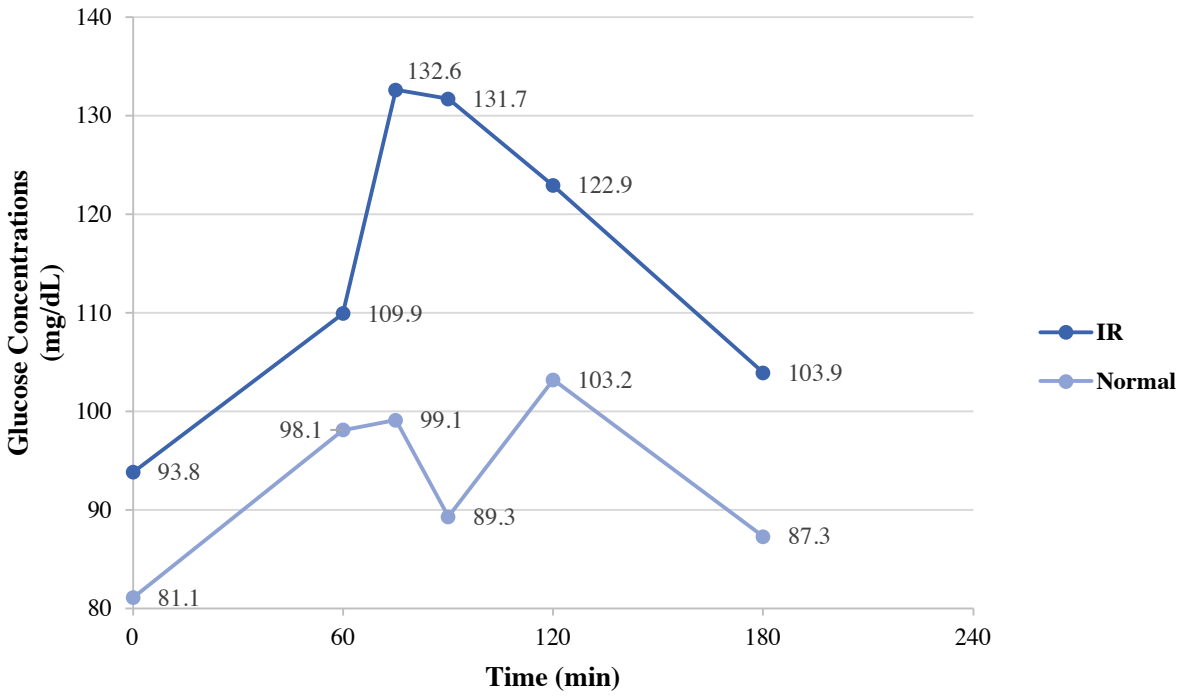


Figure 5.2 Plasma glucose concentrations during OST. (p = 0.28)

Plasma Amino Acids

Plasma free amino acids were not different between Normal and IR horses ($p > 0.15$; Table 5.2). Plasma free amino acids were not affected by the interaction of time and group ($p > 0.37$; Table 5.3). As expected, plasma free amino acids were affected by time ($p < 0.0001$).

Table 5.2 Plasma free amino acids for Normal and IR horses

Amino Acid	Normal (μmol/L)	IR (μmol/L)	SE	*p*-value
Alanine	292.45	432.58	76.55	0.24
Arginine	176.99	184	15.26	0.76
Asparagine	62.02	68.81	8.63	0.6
Aspartate	12.66	11.52	0.65	0.26
Citrulline	141.28	134.91	12.65	0.73
Glutamine	646.44	659.27	59.56	0.88
Glutamate	46.61	51.78	6.79	0.61
Glycine	829.35	768.87	81.61	0.62
Histidine	104.99	110.27	7.22	0.62
Isoleucine	87.75	103.01	10.49	0.34
Leucine	159.16	192.72	24.12	0.36
Lysine	168.54	159.57	19.15	0.75
Methionine	90.77	87.19	9.56	0.8
Ornithine	58.08	69.47	9.65	0.44
Phenylalanine	105.35	106.7	6.91	0.89
Proline	211.26	207.96	22.09	0.92
Serine	368.21	486.53	50.06	0.15
Taurine	82.77	72.57	11.18	0.54
Threonine	146.38	152.48	19.71	0.83
Tryptophan	21.82	20.86	3.52	0.85
Tyrosine	174.21	182.52	21.21	0.79
Valine	299.41	340.61	37.13	0.46

Table 5.3 Time*Group interaction for plasma free amino acids for Normal and IR horses

AA, μmol/L	Group	Time After a Meal, hours					SE	P-value
		0	1	3	4	6		
Alanine	Normal	177.8	370.7	356.1	276.9	280.8	8.3	0.68
	IR	340.8	550.3	512.3	396.6	362.9		
Arginine	Normal	124.9	236.5	206.3	158.8	158.1	20.1	0.90
	IR	139.8	237.4	226.3	165.0	151.4		
Asparagine	Normal	37.7	86.0	80.7	55.0	50.7	11.2	0.96
	IR	46.2	93.6	91.4	62.8	50.1		
Aspartate	Normal	9.1	14.7	14.1	13.1	12.2	1.3	0.78
	IR	9.5	13.2	13.5	11.1	10.4		
Citrulline	Normal	130.3	141.1	153.4	132.3	149.3	14.5	0.51
	IR	127.9	140.1	156.4	124.4	125.8		
Glutamine	Normal	505.3	745.7	743.3	592.0	646.1	71.8	0.54
	IR	538.9	770.9	822.1	594.0	570.5		
Glutamate	Normal	43.0	49.1	45.29	46.13	49.51	7.7	0.38
	IR	44.56	60.34	57.38	46.06	50.57		
Glycine	Normal	832.5	888.5	879.9	724.0	833.7	93.1	0.42
	IR	770.2	866.7	879.9	670.9	656.55		
Histidine	Normal	81.2	114.1	122.2	99.3	108.1	10.3	0.60
	IR	89.6	126.8	135.0	102.7	92.3		
Isoleucine	Normal	71.3	136.2	103.1	68.5	59.6	13.8	0.91
	IR	88.5	141.1	124.3	89.7	71.4		
Leucine	Normal	124.6	251.3	182.8	122.1	115.0	29.9	0.88
	IR	162.6	264.7	227.7	171.3	137.3		
Lysine	Normal	100.7	253.1	215.5	149.2	124.2	25.7	0.96
	IR	107.6	235	210.9	136.8	107.6		

Table 5.3 (cont.)

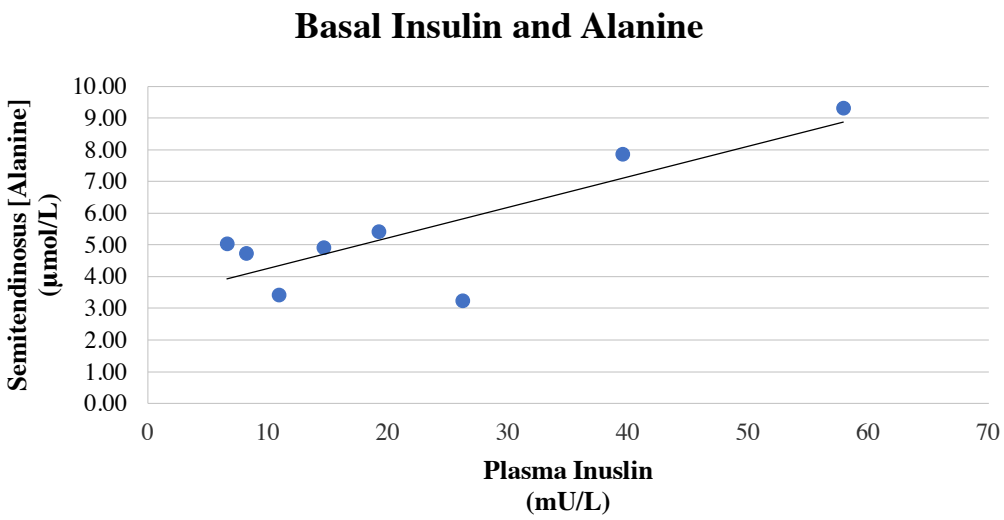
Methionine	Normal	69.9	112.0	109.9	82.2	79.9	11.7	0.99
	IR	69.0	110.0	106.6	77.0	73.3		
Ornithine	Normal	40.5	67.5	70.4	55.7	56.3	11.3	0.65
	IR	51.2	83.5	90.3	65.2	57.2		
Phenylalanine	Normal	91.5	135.8	116.4	89.2	94.0	9.8	0.90
	IR	93.4	134.2	122.8	95.8	87.3		
Proline	Normal	118.3	285.3	265.5	198.7	188.5	29.4	0.83
	IR	136.7	296.3	255.3	180.8	170.7		
Serine	Normal	270	432.7	428.5	336.8	373.0	58.5	0.66
	IR	395.3	584.6	572.7	448.8	431.2		
Taurine	Normal	71.9	96.5	90.0	74.3	81.1	13.0	0.37
	IR	58.6	91.5	92.5	66.6	53.7		
Threonine	Normal	104.4	181.4	181.3	130.4	134.5	24.2	0.78
	IR	116.2	192.0	200.6	138.2	115.5		
Tryptophan	Normal	17.1	29.2	24.8	20.5	17.6	5.0	0.65
	IR	15.8	24.5	31.4	17.5	15.1		
Tyrosine	Normal	153.4	210.7	191.4	154.5	161.1	23.7	0.92
	IR	159.2	215.0	212.9	163.7	161.8		
Valine	Normal	241.6	378.7	344.2	258.3	274.2	44.3	0.89
	IR	284.7	414.0	410.8	307.6	286.0		

Semitendinosus Muscle Free Amino Acids

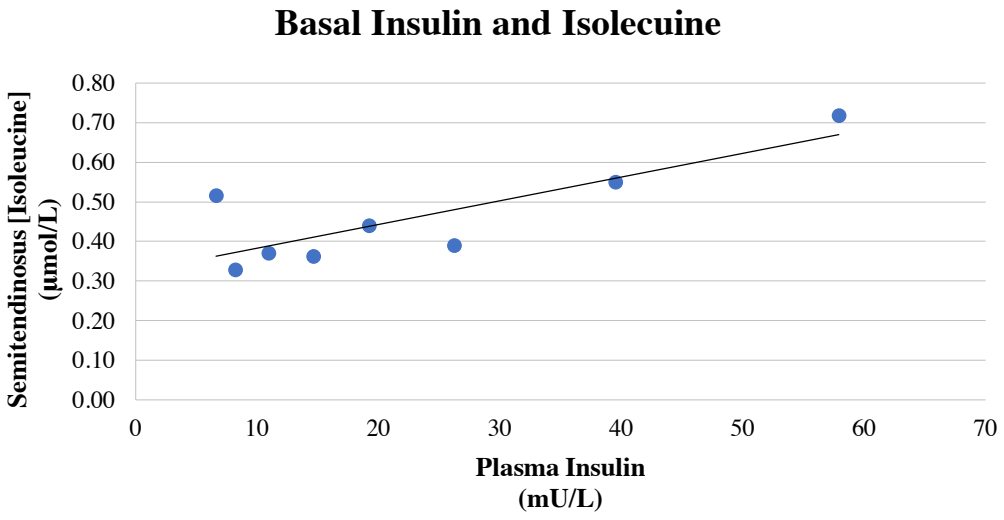
There were no differences in semitendinosus muscle free amino acids between Normal and IR horses ($p > 0.17$; Table 5.4). Strong, positive correlations were found between basal insulin concentrations and semitendinosus amino acids: alanine, leucine, isoleucine, phenylalanine, and serine (Figure 5.3 A, B, C, D, & E). Pearson's correlations between basal insulin concentrations and semitendinosus muscle free amino acids included Alanine ($R = 0.82, p = 0.013$), Isoleucine ($R = 0.82, p = 0.013$), Leucine ($R = 0.78, p = 0.027$), Serine ($R = 0.77, p = 0.025$) and Phenylalanine ($R = 0.71, p = 0.051$).

Table 5.4 Semitendinosus muscle free amino acids for Normal and IR horses

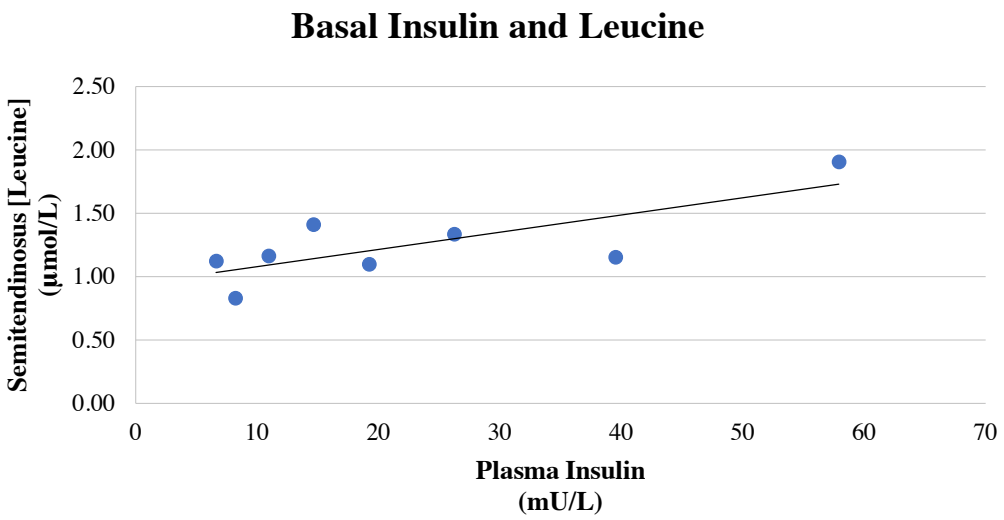
Amino Acid	Normal (nmol/g)	IR (nmol/g)	SE	*p*-value
Alanine	4.53	6.46	0.74	0.21
Arginine	0.9	0.87	0.06	0.85
Asparagine	0.4	0.45	0.03	0.45
Aspartate	0.68	0.57	0.07	0.68
Citrulline	0.91	0.92	0.07	0.93
Glutamine	8.67	5.92	0.71	0.43
Glutamate	5.69	5.24	0.71	0.77
Glycine	9.34	5.78	0.68	0.22
Histidine	0.5	0.54	1.28	0.81
Isoleucine	0.4	0.52	0.05	0.17
Leucine	1.13	1.37	0.04	0.31
Lysine	0.91	0.78	0.12	0.57
Methionine	3.07	2.16	0.06	0.21
Ornithine	0.2	0.21	0.35	0.83
Phenylalanine	0.55	0.63	0.02	0.57
Proline	1.37	1.05	0.06	0.22
Serine	1.64	1.97	0.08	0.37
Taurine	11.83	8.09	0.16	0.52
Threonine	0.83	0.83	1.64	0.99
Tryptophan	0.19	0.22	0.08	0.41
Tyrosine	0.97	1.06	0.01	0.56
Valine	0.62	0.82	0.07	0.36


Basal Insulin and Alanine
Semitendinosus [Alanine] (µmol/L)
10.00
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
10
20
30
40
50
60
70
Plasma Inuslin (mU/L)

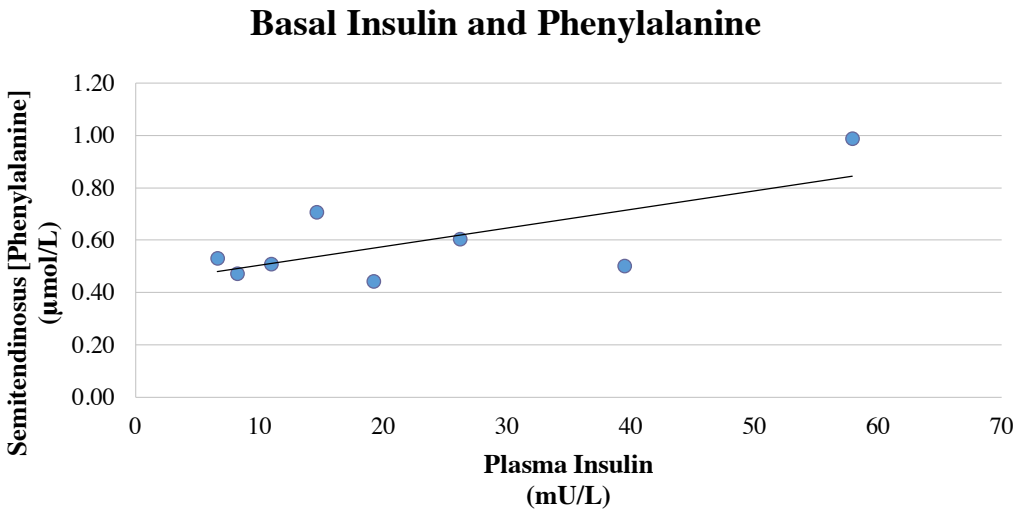
A


Basal Insulin and Isolecuine
Semitendinosus [Isoleucine] (µmol/L)
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0
10
20
30
40
50
60
70
Plasma Insulin (mU/L)

B

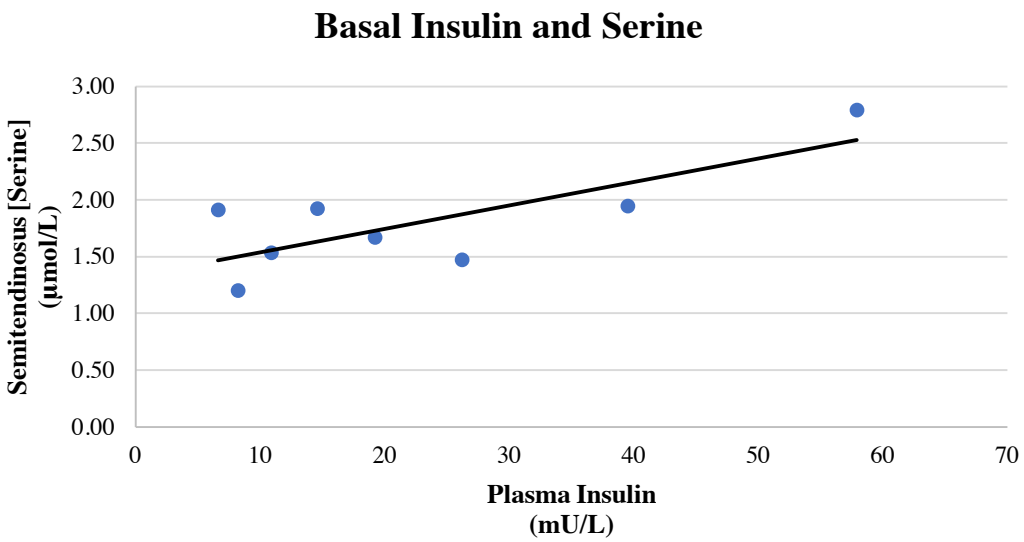


C



D

$R = 0.71, p = 0.051$

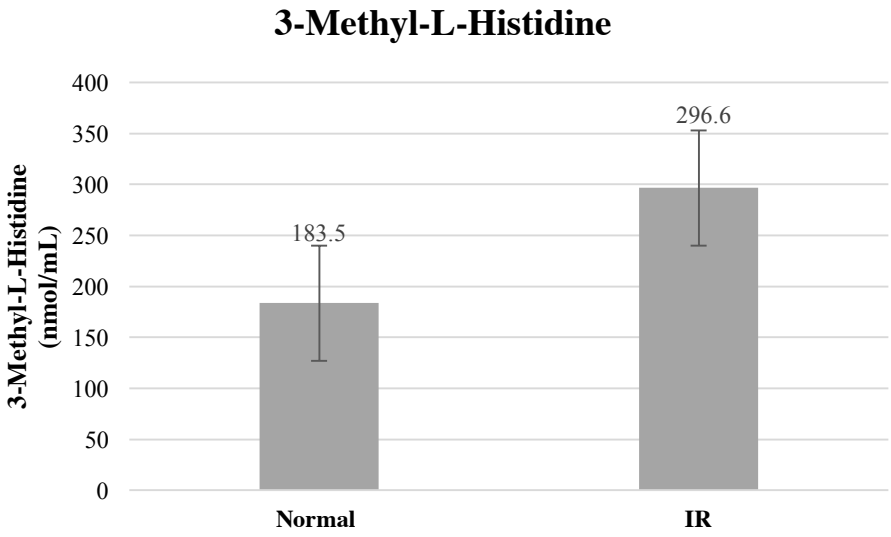


E

Figure 5.3 Pearson's correlations between basal insulin and semitendinosus AA.
(A) Basal insulin and alanine concentrations ($R = 0.82, p = 0.013$).
(B) Basal insulin and isoleucine concentrations ($R = 0.82, p = 0.013$).
(C) Basal insulin and leucine concentrations ($R = 0.78, p = 0.027$).
(D) Basal insulin and phenylalanine concentrations ($R = 0.71, p = 0.051$).
(E) Basal insulin and serine concentrations ($R = 0.77, p = 0.025$).

Urinary 3MH and Creatinine

Concentrations of 3MH were greater in IR horses compared to Normal horses ($p = 0.006$; Figure 5.4A). Concentrations of creatinine did not differ between IR and normal horses (Figure 5.4B). In addition, the ratio of 3MH: creatinine was not different ($p = 0.073$) in IR (0.96 ± 0.12) compared to Normal (0.64 ± 0.08) horses.



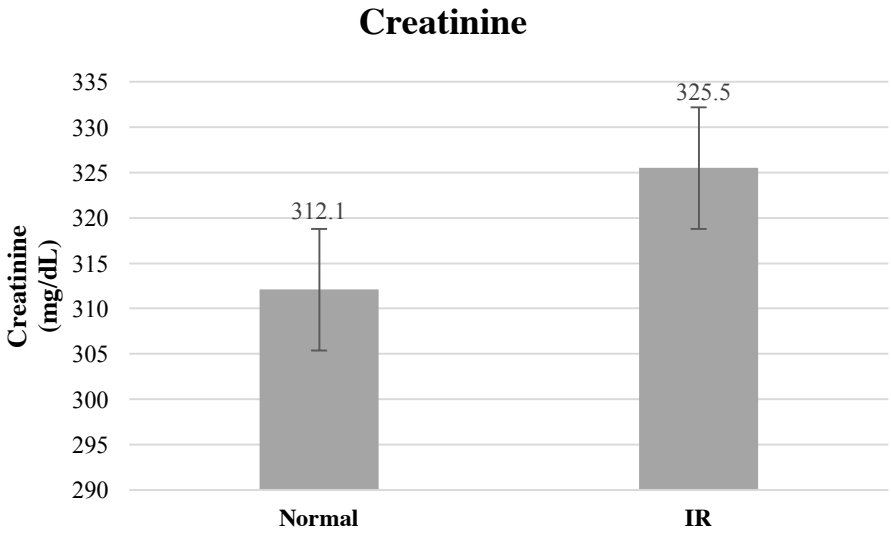


Figure 5.4 Urinary 3MH and creatinine concentrations for Normal and IR horses.
(A) Urinary 3MH ($p = 0.006$).
(B) Urinary Creatinine ($p = 0.88$).

Post-Prandial Insulin and Glucose

There were no difference between Normal and IR horses for post-prandial plasma glucose concentrations during the collection period ($p = 0.13$; Figure 5.5). Post-prandial insulin was higher in IR compared to Normal horses ($p = 0.046$; Figure 5.6). AUC for glucose and insulin concentrations during the post-prandial period were not different between Normal and IR horses (Table 5.5).

Table 5.5 AUC for glucose and insulin during the post-prandial period

	Normal	IR	*p*-value
Glucose, $mg/dL^{-1}*min^{-1}$	3367 ± 1357	9052 ± 4162	0.24
Insulin, $mU/L^{-1}*min^{-1}$	9471 ± 1213	25250 ± 13215	0.17

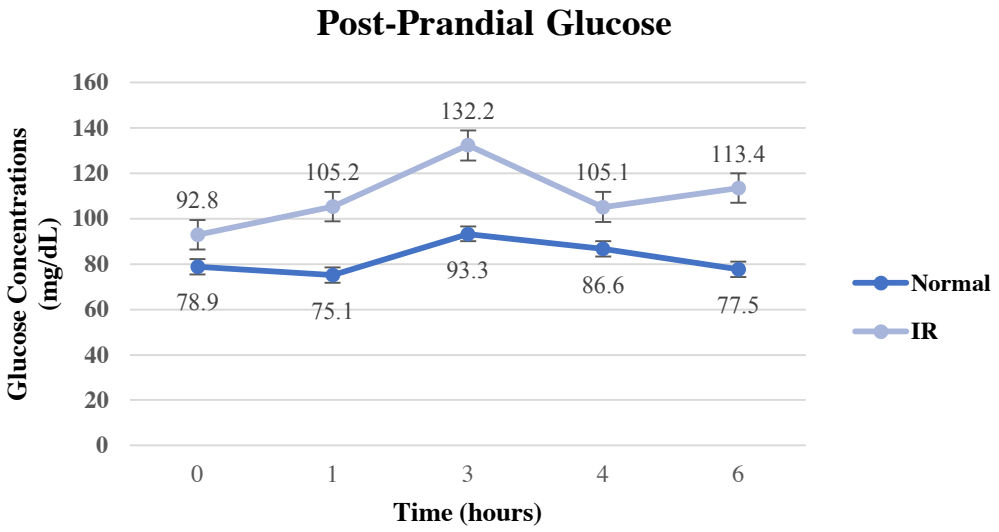


Figure 5.5 Post-prandial glucose means during sampling period for Normal and IR horses. ($p = 0.13$)

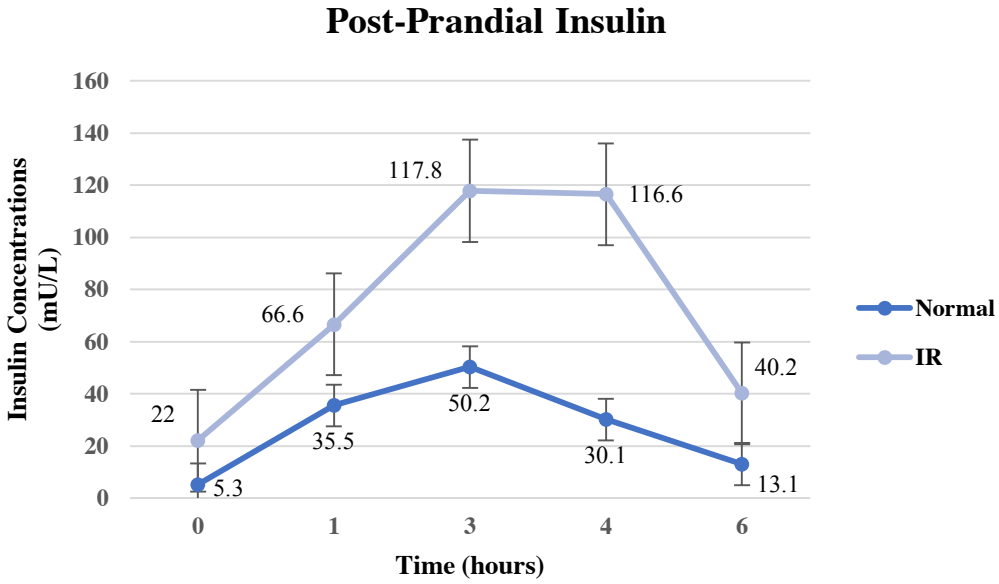


Figure 5.6 Post-prandial insulin means during sampling period of Normal and IR horses. ($p = 0.046$)

CHAPTER 6. DISCUSSION

The results of this study suggest that there were limited effects of insulin resistance on plasma and semitendinosus free amino acid status, 3-methyl histidine and creatine. This discussion will address these findings systematically, beginning with the horses themselves, followed by the variables measured.

Horse Demographics – Diet, Age, and BCS

The dietary treatment in this study was designed for horses to consume the same amount of nutrients, especially protein. Naturally, accommodations were made for body size. Horses were not exercised on the current study; therefore, exercise was not a factor to consider when formulating rations. Horses had no issues consuming the diet offered during both the dietary adaption phase or the collection period.

It has been found that mature horses have reduced insulin sensitivity compared to younger horses (Frank, 2009; Wagner and Urschel, 2012). Insulin resistance, which normally is a precursor to EMS is seen in middle-aged horses (Frank, 2009). In the current study, IR (17 $\pm$ 1 yrs) and Normal (15 $\pm$ 4 yrs) did not differ in age ($p = 0.30$). Therefore, age differences between IR and Normal horses were not a contributing factor to the results seen in the present study.

Obesity has been associated with reduced insulin sensitivity (Hoffman et al., 2003b). Vick et al. (2007) witnessed that as insulin sensitivity decreased, % Fat and BCS increased, which agrees with Hoffman et al. (2003b). Occasionally, IR can be seen in horses that are not obese (Frank, 2009). In this study, Normal (655.3 $\pm$ 68 kg) and IR (575 $\pm$ 79 kg) horses did not differ in mean body weight ($p = 0.17$). There was a

difference in BCS between Normal (4.75 ± 0.5) and IR (6.5 ± 0.6) horses ($p = 0.004$). This is to be expected with horses that have IR and agrees with Vick et al. (2007). Obesity is a known symptom of IR and can potentially influence the severity of IR. While measurements to quantify obesity's effects on IR horses were not performed in this study, the higher BCS in the IR horses supports the designation of IR for these subjects.

Confirmation of Normal and IR Groups

There are several methods for diagnosing IR in horses. Methodology for diagnosing IR horses in the current study were used due to practicality and validation in other studies (Frank 2009; Schuver et al., 2014). Other, more complex procedures control for physiological responses and allow for a more accurate quantification of IR; however, basal glucose and insulin concentrations and OST data confirmed the presence of two groups, Normal and IR horses, in the current study. The AAEP defines IR in horses having fasting insulin concentrations > 30 mU/L, while horses having fasting insulin concentrations of < 20 mU/L are said to have normal circulating insulin concentrations (Frank, 2013). In this study, IR horses (42.2 ± 15.9 mU/L) had higher plasma insulin concentrations that were above AAEP guidelines, as well as the Normal horses (10.1 ± 3.5 mU/L). Frank et al. (2006) reported similar resting plasma insulin concentrations in non-obese, non-IR horses (9.1 mU/L) compared to obese and IR horses (50.5 mU/L).

IR horses had a greater plasma insulin concentration ($p = 0.020$) and a trend for glucose ($p = 0.055$) compared to normal horses during the OST. The post-prandial measures also showed higher plasma insulin concentrations in IR horses compared to Normal horses ($p = 0.046$). AUC data for both the OST and post-prandial period revealed no difference in both insulin and glucose concentrations between the Normal and IR

horses. The raw data, however, shows that the IR horses had higher plasma insulin concentrations in both the OST and post-prandial period, which validates the use of those horses as the IR group. McAuley et al. (2001) diagnosed IR in humans with the combination of simple screening tests. Several determinants were used to evaluate what screening measurements would be most useful to determine IR. McAuley et al. (2001) found that fasting insulin and triglycerides were the best screening tests to determine IR in the general population. Therefore, the use of two simple screening tests proved to separate two different groups of horses to evaluate insulin resistance's effects on concentrations of plasma and muscle amino acids.

Plasma and Muscle Free AA, 3MH, and Creatinine

Both plasma and muscle free AA were not different between groups in the current study. This contradicts the findings of Urschel et al. (2014) who found that plasma amino acids decreased after 120 minutes of insulin infusion. A decrease in plasma AA is observed when AA are extracted from the plasma pool and subsequently utilized for protein and muscle protein synthesis. This was validated by Urschel et al. (2014) who found increases in phosphorylation of Akt/PKB, 4EBP1, and S6. Plasma insulin concentrations for the four infusion rates used by Urschel et al. (2014), were 12, 83, 327, and 1001 mU/L. In the current study, post-prandial insulin concentrations for the IR group peaked at 117 mU/L. Therefore, Urschel et al. (2014) had much higher plasma insulin concentrations than the current study and could explain why there were no differences in plasma and muscle free AA between Normal and IR horses. In addition, Urschel used an HEC procedure in healthy horses in order to mimic insulin resistance. Granted, the HEC accurately stimulates the secretion of insulin, it still serves as a

synthetic stimulator. In the current study, IR horses were used and simply fed grain and forage meals to stimulate insulin release. This natural stimulation would theortically capture the alterations in insulin's actions on protein metabolism. Urschel et al. (2014) concluded that insulin stimulates whole-body protein synthesis by activating mTORC1. Markers for protein synthesis were not measured in the current study. Therefore, the author cannot speculate that protein synthesis increased or decreased; however, no changes in plasma and muscle free AA between groups indicate that the AA pool was supplying the adequate amount of AA needed to maintain muscle mass.

Leucine flux is a procedure to quantify protein degradation. During a HEC, leucine flux was measured to determine if insulin's normal action of inhibiting protein degradation was altered (Halvastsiotis et al., 2002). Diabetics had higher acute insulin concentrations compared to non-diabetic patients; however, there AA concentrations were similar (Halvastsiotis et al., 2002). Therefore, increased, short-term insulin concentrations could be an explanation for the lack of a difference in plasma and muscle free AA between Normal and IR horses in the current study.

IR is associated with EMS (Frank, 2009). No difference between Normal and IR horses, in regards to free AA in plasma and muscle, could be contributed to horses not being at the status of EMS. However, this also contradicts findings in human studies that indicate AA to be indicators of IR. Felig et al. (1969) found increases in valine, leucine, isoleucine, tyrosine, phenylalanine in obese subjects that were matched by age and sex with controls. Increased AA correlated with increased in serum insulin (Felig et al., 1969). A more recent study, conducted by Wang et al. (2011) followed 2,422 patients over the course of 12 years. Out of the original patient pool, 201 patients developed

diabetes. Various metabolites were measured throughout the course of the 12 years, including AA. Five AA (isoleucine, leucine, valine, phenylalanine, and tyrosine) were associated with developing diabetes (Wang et al., 2011). Homeostatic model assessment (HOMA) quantifys insulin resistance, as well as assessing beta cell function by using equations dervied from data collected from phsyiological studies. HOMA-IR was associated with BCAA, aromatic AA, gluconeogenesis intermediates, and ketone bodies (Wurtz et al., 2012).

Correlations between insulin and BCAA, one of the aromatic AA, alanine, and serine were found in the present study. BCAA activate mTOR and its downstream signaling proteins in muscle, liver, and adipose tissue. Aromatic AA are metabolized to catecholamines, which can alter liver function producing hyperinsulinemia. In the liver during gluconeogenesis, alanine can be metabolized to pyruvate to maintain glucose homeostasis (Bi & Henry, 2017). The correlation between serine and plasma insulin concentrations; however, is not physiologically explainable at this time. These correlations were found in all horses in the present study; therefore, cannot be assumed as metabolites for predicting IR. It is more likely that these correlations between AA and plasma insulin relate to normal protein metabolism and its association with insulin.

Another plausible explanation for similar concentrations of plasma and muscle free AA found in Normal and IR horses is the utilization of plasma fatty acids. The cause of IR in the horses on this study is unknown. Therefore, IR could have resulted from age, environmental factors, or obesity. Although IR can be independent of obesity, fat within skeletal muscle can resultin IR as well. Increased plasma fatty acids or a reduction in β-oxidation results in increased concentrations of fatty acyl-CoAs, diacylglycerols (DAG),

and serine/threonine kinases in skeletal muscle. DAG is an activator for protein kinase C (PKC) isoforms. Activated PKC and serine/threonine kinases phosphorylate IRS1 and inhibit insulin-induced PI3K activity (Morino, Peterson, Shulman, 2006). Therefore, increased adiposity and fatty acids within the diet could have been the cause of IR in the horses studied; however, measures of plasma fatty acids were not measured in this study. Thus, this explanation is theoretical at best.

Creatinine is a marker for muscle mass (Finco, 1997). Creatinine concentrations were not different between Normal and IR horses, indicating that skeletal muscle mass was not different between horses. Graham-Thiers and Kronfeld (2005) found greater creatinine concentrations in AA supplemented groups, suggesting that horses increased muscle mass. Findings from Graham-Thiers and Kronfeld (2005) were examined in closer detail by Latham (2016), who found that AA availability may not have been limiting protein synthesis. This overall finding was concluded from no difference between controls and AA supplemented in regards to creatinine output, presence of mTOR proteins, nitrogen retention, or phenylalanine kinetics (Latham, 2016). In the current study, horses were being stalled during the trial period and were not exercised. Thus, muscle protein synthesis and degradation would be balanced in these horses, which was shown by a lack of a difference in creatinine concentrations between the Normal and IR horses.

3MH is marker for catabolism of skeletal muscle (Gallagher et al., 1999). 3MH was higher in the IR group, in the present study, suggesting skeletal muscle catabolism in IR horses. IR results in decreased stimulation of protein synthesis pathways and increased protein degradation, which is seen as muscle loss in patients suffering from Type 2

Diabetes (Kaylyani, Corriere, & Ferrucci, 2014). Insulin normally functions to activate mTOR signaling pathways and inhibit autophagy. These normal effects of insulin are altered in insulin resistant states and have potential to contribute to loss of muscle mass in diabetic patients (Kaushik & Cuervo, 2010). Graham-Thiers and Kronfeld (2005) found lower 3MH concentrations in the AA supplemented group, suggesting decreased muscle catabolism. These results indicated that the AA pool in the supplemented group provided the essential AA needed for maintenance of skeletal muscle (Graham-Thiers & Kronfeld, 2005). Horses in the current study were not being fed supplemental AA; however, their diet was balanced and met or exceeded their requirements based off the NRC. In comparison to Graham-Thiers and Kronfeld (2005), findings of the current study suggest that even with adequate supply of dietary protein, IR horses were still catabolizing skeletal muscle. One plausible explanation is that IR horses were using dietary AA for sources of energy, thus depleting their plasma pools. However, this was not corroborated by a loss of muscle mass when creatinine was measured or a decrease in plasma AA in IR horses. More precise measurements of protein synthesis and degradation would need to be used in order to determine IR's effect on protein and muscle protein synthesis and degradation.

Conclusion

The current study suggests insulin resistance does not affect the incorporation of amino acids into the plasma pool or muscle. While the cause of insulin resistance was not determined in the current study, insulin resistant status was confirmed with basal insulin concentrations, oral sugar test, and post-prandial insulin concentrations. Normal and Insulin-Resistant horses were fed a meal to stimulate the natural release of insulin,

theoretically capturing the physiological alterations in insulin's secretion and actions on protein metabolism. Correlations between basal insulin and semitendinosus amino acids were found in both groups suggesting that branched chain and aromatic amino acids may not be effective predictors of insulin resistance in horses due to finding the correlation in both Normal and Insulin-Resistant horses. Future research should use more robust measurements to precisely determine if protein synthesis and degradation are being altered by insulin resistance, and explore horses with more severe cases of insulin resistance, such as those seen in Equine Metabolic Syndrome or horses with pituitary dysfunction.

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APPENDICES

APPENDIX A

CURRICULUM VITAE

Experience

Graduate Student (M.S.) – Middle Tennessee State University August 2015-Present

Master of Science in Horse Science – Major advisor: Dr. Rhonda Hoffman

- Thesis project investigating plasma and muscle amino acid concentrations in insulin resistant compared to normal horses in the fed and fasted state.
  - Experience includes: venipuncture blood sampling, body condition scoring, urine collection, muscle biopsy collection, oral glucose tolerance test, and currently learning a variety of assays.
  - Location: Research performed in Bristol, VA in cooperation with Dr. Patricia Graham-Thiers at Emory and Henry College.

Graduate teaching assistant for:

- ABAS4090 – Equine Reproduction, taught by Dr. John Haffner
- ABAS3430 – Horse Production, taught by Dr. Rhonda Hoffman
- ABAS3040 – Stable Management, taught by Dr. Holly Spooner
- ABAS2400 – Fundamentals of Horsemanship, taught by Anne Brzezicki & Andrea Rego

MTSU barn experience:

- Daily activities: cleaning 60-stall horse barn, feeding, watering, cleaning, horse turnout, and overall stable management on a university level.

Other:

- Middle Tennessee State University's Journal: *Scientia et Humanitas,* Position: Peer Reviewer
- Coach of Middle Tennessee State University's Undergraduate Academic Quadrathlon Team

Membership:

- Phi Kappa Phi Honor Society
- Equine Science Society
- American Society of Animal Science

Undergraduate Research & Teaching Assistant December 2014-May 2015

Research

- Undergraduate research assistant with an academic research project: Modeling ammonia emissions from horses fed different concentrations of dietary crude protein. PI: Dr. Carissa Wickens, PhD student: Jessie Weir. December 2014-May 2015.
  - Experience includes: precise measurements of hay and grain for trial diets, collection of urine and feces, measuring representative samples, and drying and grinding of samples.

Teaching

- Undergraduate teaching assistant for ANS3006C: Introduction to Animal Science, taught by Dr. Jason Scheffler – assisted with laboratory activities, creation of exam material, and grading.

Membership

- Collegiate Leadership and Achievements Society

Education

Middle Tennessee State University August 2015-Present

Master of Science in Horse Science - Equine Physiology emphasis

Major Advisor: Dr. Rhonda Hoffman

Expected Graduation Date: May 2017

Current cumulative GPA: **3.89**

University of Florida Graduated May 2015

Bachelor of Science in Animal Sciences – Animal Biology emphasis

Graduation Date: May 2015

Cumulative GPA: **3.13**

Indian River State College Graduated December 2012

Associates of Arts

Graduation Date: December 2012

Cumulative GPA: **3.21**

Publications & Abstracts

Macon, E. L., R. Hoffman, and P. Graham-Thiers. 2017. Plasma and muscle amino acid concentrations in insulin resistant compared to normal horses in the fed and fasted state. Middle Tennessee State University Scholar's Week – University Wide Poster Session, Murfreesboro, TN, USA, March 31st, 2017. (abstract accepted)

Macon, E. L., R. Hoffman, and P. Graham-Thiers. 2017. Plasma and muscle amino acid concentrations in insulin resistant compared to normal horses in the fed and fasted state. Middle Tennessee State University Scholar's Week – College of Basic and Applied Science Poster Session, Murfreesboro, TN, USA, March 29th, 2017. (abstract accepted)

Weir, J.M., H. Li, L.K. Warren, **E. Macon**, and C. Wickens. 2017. Manure nitrogen characteristics from horses fed warm season grass hays. 25th Equine Science Society Symposium, Minneapolis, MN, May 30 – June 2, 2017. (abstract accepted)

Weir J., H. Li, L.K. Warren, **E. Macon**, and C. Wickens. 2017. Evaluating the impact of ammonia emissions from equine operations on the environment. Waste to Worth. (abstract accepted)

Macon, E.L., P.M. Graham-Thiers, K.L. Bowen. 2017. Plasma and muscle amino acid concentrations in insulin resistant compared to normal horses in the fed and fasted state. 25th Equine Science Society Symposium, Minneapolis, MN, May 30 – June 2, 2017. (abstract accepted)

Macon, E.L. 2017. Review of equine glycogen storage diseases. Scientia et Humanitas. Vol 7. (Manuscript accepted).

Weir, J., H. Lee, L.K. Warren, **E. Macon**, and C. Wickens. 2016. Modeling ammonia emission rate from horses fed different concentrations of dietary crude protein. Joint Annual Meeting, Salt Lake City, UT, July 19-23, 2016. J. Anim. Sci. 94 (E-Suppl. 5): 381.