

INVESTIGATION OF CITRULLINE-MALATE SUPPLEMENTATION ON PLASMA
AMINO ACIDS AND GLYCEMIC AND INSULINEMIC RESPONSES IN HORSES

By

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LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
AEC	Aminoethyl Cysteine Hydrochloride
AGC1	Aspartate-Glutamate Carrier 1
AHC	American Horse Council
apoB	Apolipoprotein B
ASS	Argininosuccinate Synthase
ASL	Argininosuccinate Lyase
ATP	Adenosine Triphosphate
BCS	Body Condition Score
BMI	Body Mass Index
BW	Body weight
CALS	College of Agriculture and Life Sciences
cGMP	Cyclic Guanosine Monophosphate
CIT	Citrulline-Malate – treatment group
CON	Control - Urea
CSP I	Carbamoyl-Phosphate Synthase I
DM	Dry Matter
EDRF	Endothelium-Derived Relaxing Factor
EDTA	Ethylenediaminetetraacetic acid, anticoagulant
EMS	Equine Metabolic Syndrome
ESC	Equine Sciences Center
FFA	Free Fatty Acid
FFAs	Free Fatty Acids
FSIGT	Frequently Sampled Intravenous Glucose Tolerance Test

GLUT	Glucose Transporter
GSIS	Glucose Stimulated Insulin Secretion
HCl	Hydrochloride
HOMA	Homeostasis Model Assessment
IFAS	Institute of Food and Agricultural Sciences
IRS-1	Insulin Receptor Substrate-1
ITT	Insulin Tolerance Test
IVGTT	Intravenous Glucose Tolerance Test
MIRG	Modified Insulin Response to Glucose
mRNA	Messenger Ribonucleic Acid
NAGS	N-Acetylglutamate Synthase
NAHMS	National Animal Health Monitoring System
NH ₄ ⁺	Ammonium
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NSC	Non-Structural Carbohydrate
OAT	Ornithine Aminotransferase
OCT	Ornithine Carbamoyltransferase
OGTT	Oral Glucose Tolerance Test
P5CS	L-Δ ¹ -Pyrroline-5 Carboxylate Synthase
PPID	Pituitary Pars Intermedia Dysfunction
QH	Quarter Horse
RBC	Red blood cell
RISQI	Reciprocal of the Insulin Square Root Index
scFOS	Short-Chain Fructo-Oligosaccharides

SSA	Sulfosalicylic acid
STZ	Streptozotocin
TAG	Triacylglycerol
TB	Thoroughbred
TNF	Tumor Necrosis Factor
VLDL	Very Low Density Lipoprotein
VMRCM	Virginia-Maryland Regional College of Veterinary Medicine
WSC	Water Soluble Carbohydrate

Abstract of Thesis Presented to the Graduate School
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Supplementation with arginine has been shown to improve insulin sensitivity in several species. Because it escapes splanchnic extraction, citrulline has been shown to be more effective at increasing arginine availability than direct supplementation with arginine. Therefore, citrulline could provide a novel dietary intervention for the management of insulin resistance horses. The objectives of this study were to: 1) investigate citrulline-malate (CIT) supplementation and its effects on availability of other amino acids; 2) assess whether oral CIT will act as a dietary precursor for arginine; and 3) determine if oral CIT will alter glycemic and insulinemic responses to a starch-rich meal in healthy horses. We hypothesized that supplementation of CIT would increase arginine availability and consequently alter insulin response to a meal. Twelve clinically normal horses (mean \pm SE, age 10.8 ± 2.5 y; BW 552.0 ± 31.2 kg; body condition score 5 to 6 on scale of 1 to 9) were randomly assigned to urea (isonitrogenous control; 25 mg/kg BW) or citrulline-malate (CIT; 86 mg/kg BW) supplementation for 14 d. Insulinemic and glycemic responses to a meal (0.25% BW) of grain mix concentrate and the daily allotment of urea or CIT supplement were evaluated on d 14 following an

overnight fast. Venous blood samples were taken 30 min and immediately before the meal, and then every 30 min after the meal for 5 h. Plasma was assayed for glucose, insulin and amino acids. Statistical analysis was a mixed model with repeated measures with time, treatment, and time by treatment as fixed effects. Plasma citrulline, arginine, ornithine and glutamate increased in response to a meal and were higher in horses supplemented with CIT compared to urea. Plasma urea, lysine, methionine and threonine were unaffected by CIT consumption. Glycemic response to a grain meal was similar between treatments; however, serum insulin was lower when horses consumed a meal containing CIT versus urea. Insulin sensitivity (estimated by the reciprocal of the insulin square root index and homeostasis model assessment) and pancreatic β -cell responsiveness (estimated by modified glucose to insulin ratio and homeostasis model assessment) were not affected by CIT supplementation. Results demonstrate that CIT can be used to increase whole-body arginine supply without negatively affecting amino acids that may be limiting in equine diets. In addition, supplementation with CIT may be useful for maintaining glycemic control while reducing hyperinsulinemia in insulin resistant horses, but deserves further study.

CHAPTER 1 INTRODUCTION

Veterinary researchers in Virginia reported that 51% of horses evaluated on farms were overweight and 19% were obese (Thatcher et al., 2007). Furthermore, 18% of the overweight horses and 32% of the obese horses were hyperinsulinemic. Insulin resistance has been implicated in several equine diseases as a pathogenesis, including equine metabolic syndrome (EMS), pituitary pars intermedia dysfunction (PPID; Cushing's disease), and laminitis (Kronfeld et al., 2005a, 2006; Treiber et al., 2006a; Pratt et al., 2009; Valberg and Fishman, 2009; Zimmel and McFarlane, 2009). Thus, there is great interest in finding ways to control insulin resistance in the horse.

Arginine is a potent secretagogue of the endocrine system, including insulin secretion by the pancreas (Sener et al., 1989). Arginine supplementation has been reported to increase insulin sensitivity in healthy and diabetic subjects (Jobgen et al., 2009; Clemmensen et al., 2011; Monti et al., 2012). Improvement in insulin sensitivity appears to result from improved glucose tolerance with arginine supplemented subjects usually having lower glucose concentrations. The effects on circulating insulin concentrations have been shown to vary with arginine supplementation. Although the exact mechanisms are unknown, arginine's effects on insulin dynamics and glycemic control have been attributed to enhanced production of nitric oxide (Calver et al., 1992) and accumulation of polyamines in pancreatic islet cells (Sener et al., 1989).

Dietary arginine is rapidly absorbed by the intestine and degraded by arginase, contributing little to plasma arginine concentrations (Böger and Bode-Böger, 2001). Furthermore, oral supplementation of citrulline has been shown to be more efficient at increasing whole body arginine supply (Schwedhelm et al., 2008). Therefore, citrulline

supplementation may be more effective than arginine for improving insulin responsiveness.

The objectives of this study were to: 1) investigate oral citrulline-malate supplementation and its effects on the availability of other amino acids; 2) assess whether citrulline-malate will act as a dietary precursor for arginine; and 3) determine if citrulline-malate will alter glycemic and insulinemic response to a starch-rich meal in healthy horses. We hypothesized that supplementation of citrulline-malate would increase arginine availability and consequently improve insulin response to a meal.

CHAPTER 2 LITERATURE REVIEW

Insulin Resistance

Insulin is a natural hormone secreted from the β -cells of the pancreas. Insulin acts as a major regulatory hormone in glucose and fat metabolism, vascular function, inflammation, and tissue remodeling (Treiber et al., 2006b). In a normal horse, insulin will stimulate the uptake of glucose by skeletal muscle, adipose tissue and liver. Insulin resistance is simply the failure of these tissues to respond appropriately to insulin. The pancreas will continue to secrete insulin to compensate for a decrease in tissue or cell effectiveness. Therefore, the resting serum insulin levels will be high in horses with moderate to severe insulin resistance. In humans, insulin resistance typically develops with obesity and can eventually result in the onset of type 2 (non-insulin dependent) diabetes (Valberg and Fishman, 2009). Few horses are thought to be truly diabetic, but they can be insulin resistant. Insulin resistance has been implicated in several equine diseases as a pathogenesis, including equine metabolic syndrome (EMS), pituitary pars intermedia dysfunction (PPID; Cushing's disease), hyperinsulinemia, pituitary adenoma, and forms of laminitis (Kronfeld et al., 2005a, 2006; Treiber et al., 2006a; Pratt et al., 2009; Valberg and Fishman, 2009; Zimmel and McFarlane, 2009). Insulin resistance is also thought to play a role in diseases such as hyperlipemia (Forhead, 1994), endotoxemia (Tóth et al., 2008), and osteochondritis dissecans in horses (Valberg and Fishman, 2009).

Insulin resistance happens over time as the horse loses its sensitivity to insulin and is unable to utilize glucose from the diet as effectively. Insulin resistance has been observed in fasting, obese, and inactive horses (Treiber et al., 2005a). Moreover, insulin

resistance also influences reproductive efficiency and probably exercise tolerance (Kronfeld et al., 2005a). Insulin resistance, especially when associated with PPID, can contribute to muscle loss, abnormal fat accumulation, laminitis, excessive water consumption, frequent urination and increased susceptibility to disease (Zimmel and McFarlane, 2009).

Insulin Sensitivity versus Insulin Effectiveness

Clarification of a few terms regarding insulin and its functions may be beneficial. Insulin's ability to facilitate insulin-mediated glucose transport into the cell of target tissues, such as adipose tissue and skeletal muscle, is referred to as insulin sensitivity (Treiber et al., 2006c; Pratt et al., 2009). Irrespective of the cause, insulin insensitivity is pertaining to reduced cell surface activity and glucose supply, whereas insulin ineffectiveness pertains to reduced intracellular glucose utilization (Kronfeld et al., 2005a). Insulin resistance however, involves a decreased response to circulating insulin by insulin-sensitive cells, which are primarily in skeletal muscle and adipose tissue, but also in liver (Treiber et al., 2005a). Insulin resistance alludes to insensitivity at the cell surface. The term resistance can refer to inefficient insulin signaling at the cell surface, which is low insulin sensitivity, or in the case of disruption of insulin signaling pathways within the insulin sensitive cells, such as insulin ineffectiveness (Kronfeld et al., 2005a; Treiber et al., 2005a).

Prevalence of Insulin Resistance in the current horse population

Currently, there is limited information on the prevalence of insulin resistance in horses. The Virginia-Maryland Regional College of Veterinary Medicine (VMRCM) along with the College of Agriculture and Life Sciences at Virginia Tech, conducted a perspective research study on the prevalence of obese and insulin resistant horses

(Thatcher et al., 2007). The study included 300 horses (4 to 20 years of age) from 114 farms chosen randomly from 1,000 horses examined by the VMRCM field service. Horses were evaluated prior to any grain or concentrate consumption to reduce the chance of glucose and insulin levels being altered. Horses were evaluated based on two independent body condition scores (BCS) to assess the amount of fat cover, and morphometric measurements were taken to calculate body weight and body mass index (BMI). The study examined each horse for signs of laminitis, as well as measured blood glucose, insulin and other hormones, cytokines and oxidative biomarkers. Researchers found that 51% of the horses in the study were overweight and 19% were found to be obese. Furthermore, 18% of the overweight horses and 32% of the obese horses were hyperinsulinemic (Thatcher et al., 2007). Interestingly, the researchers also suggests that equine obesity may result from natural grazing behavior in addition to the overuse of grains and other feed supplements. If these data are extrapolated to the 9.2 million horses reported to be in the United States (AHC, 2012), an estimated 560,000 to 846,000 horses (6 to 9% of the total population) could be affected by insulin resistance.

Insulin-mediated Glucose Transport

The two primary variables involved in glucose homeostasis are: 1) the insulin secretory response of β -cells within the islets of Langerhans of the pancreas to increases in blood glucose; and 2) the sensitivity of skeletal muscle and adipose tissues to serum insulin concentrations (Firshman and Valberg, 2007). Consumption of a meal containing starch and/or sugar will result in the absorption of glucose and an increase in circulating blood glucose. Elevated blood glucose subsequently triggers the secretion of insulin from the β -cells within the pancreas. Insulin mediates the transport and metabolism of glucose, as well as its storage, first in skeletal muscle then in adipose

tissue (Gould and Holman, 1993). Insulin also both inhibits glucagon secretion and lowers serum free fatty acid concentrations, consequently decreasing liver glucose production (Shepherd and Kahn, 1999). Toxic effects on cells begin to result from prolonged elevation of blood glucose concentrations (Yki-Jarvinen, 1992). In addition, low blood glucose concentrations can result in seizures (Valberg and Fishman, 2009). Therefore, maintaining blood glucose concentrations through hormonal regulation is extremely important.

Glucose transport, the initial step in glucose utilization, is considered rate determining in glucose utilization in muscle (Kahn, 1992). However, there evidence from clinical studies (Yki-Järvinen et al., 1987; Kelley et al., 1996) and studies from cultured human muscle cells (Jacobs et al., 1990) that glucose phosphorylation by hexokinase also affects the rate of glucose utilization, especially under high glucose concentrations or stimulation by insulin (Perriott et al., 2001). Because vertebrate muscle has little glucose-6-phosphatase activity (Surholt and Newsholme, 1981), the phosphorylation of glucose is irreversible in this tissue. Irreversibility is a feature of rate-determining steps in metabolism and supports the notation that phosphorylation aids in determining the overall rate of glucose utilization (Perriott et al., 2001). The lipid bilayers in cell membranes are naturally impermeable to glucose; thus, glucose needs a special transport system to enter the cell. Facilitated diffusion down a glucose concentration gradient is mediated by transmembrane proteins known as glucose transporters (GLUT). Several GLUT isoforms, distinguished by their tissue distribution have been identified in most species and include GLUT-1, GLUT-2, GLUT-3, and GLUT-4 (Gowrishankar et al., 2011). Not all GLUT are influenced by insulin. Skeletal muscle,

myocardium, and adipose tissue express the GLUT-4 and GLUT-1 isoforms. In these tissues, GLUT-1 is largely responsible for basal glucose transport, because a significant amount of this transporter isoform is present at the cell surface in the absence of insulin (Giorgino et al., 2000). In contrast, the majority of GLUT-4 localizes to intracellular tubulovesicular structures clustered in the cytoplasm, often close to the plasma membrane (Giorgino et al., 2000). In adipocytes, GLUT-4 is slowly exocytosed and rapidly endocytosed, and a very small percent of the total GLUT-4 is in the plasma membrane (Blot and McGraw, 2008). The GLUT-4 transporter is sequestered intracellularly and only translocates to the cell membrane under the influence of either insulin or exercise (Holman and Kasuga, 1997). Insulin increases the number of GLUT-4 transporters in the plasma membrane, resulting in substantial increases in both cell surface GLUT-4 levels and glucose transport rates (Giorgino et al., 2000). Translocation of GLUT-4 to the cell membrane occurs via a complex process initiated as insulin binds to the GLUT-4 receptor on the cell's plasma membrane (Valberg and Fishman, 2009). Insulin binding causes phosphorylation of the receptor and insulin receptor substrate proteins (Valberg and Fishman, 2009). The substrates produced form complexes with docking proteins in order to activate phosphoinositide-3 kinase, a major pathway for the mediation of insulin-stimulated glucose transport and metabolism (Shepherd et al., 1998). The functionally important targets further downstream in the phosphoinositide-3 kinase signaling cascade are still being studied and identified, but are thought to include proteins that help regulate the docking of GLUT-4 containing vesicles at the plasma membrane and their fusion to the membrane (Rea and James, 1997).

Insulin signaling involves numerous substances and affects the transport and utilization of glucose, particularly in correlation to hexokinase, glycogen synthase, and other key enzymes in glucose and lipid metabolism (Kronfeld et al., 2005a). Tyrosine kinase is involved in the main insulin receptor on the cell membrane (Kronfeld et al., 2005a). The activation of tyrosine kinase leads to translocation of glucose transporter, mainly GLUT-4 in muscle, from the interior to the cell surface (Kronfeld et al., 2005a). Two main chains of proteins link the tyrosine kinase receptor to the GLUT-4 transporters and therefore is critical to the biologic response of insulin (Kronfeld et al., 2005a). Other insulin receptor signals affect enzymes involved in glucose and lipid metabolism inside the cell in order for proper function (Kronfeld et al., 2005a). Therefore, the insulin receptor itself is then capable of integrating insulin sensitivity and effectiveness such as glucose transport into the cell and its subsequent intracellular utilization (Kronfeld et al., 2005a). Although insulin is the foremost stimulus for glucose uptake into cells via GLUT-4, other stimuli, such as thyroid hormone and leptin, can also activate the translocation of GLUT-4 into muscle and fat cell membranes (Valberg and Fishman, 2009).

Non-Insulin Mediated Glucose Uptake

The GLUT-1 glucose transporter is constantly present in the plasma membranes of cardiac and skeletal muscle and adipocytes and provides basal, non-insulin dependent uptake of glucose (Gaster et al., 2001). The GLUT-3 transporter has a similar function to GLUT-1, but is expressed in neural tissue (Taha et al., 1995). In contrast to GLUT-4 and GLUT-1, the GLUT-2 facilitative glucose transporter is found primarily in liver and pancreatic β -cells (Giorgino et al., 2000). Also in contrast to GLUT-4 and GLUT-1, GLUT-2 in the liver has been shown both *in vivo* and *in vitro* to be

upregulated by glucose and downregulated by insulin (Giorgino et al., 2000). Further, GLUT-2 has been suggested to serve as a glucose sensor in the liver portal vein, indicating its essential nature for the liver's rapid response to glucose (Giorgino et al., 2000). Regulation of GLUT-2 is at the transcriptional level and yet it permits a rapid, dose-dependent response to glucose by the liver (Giorgino et al., 2000).

Exercise stimulates glucose transport through the use of pathways that are independent of phosphoinositide-3 kinase and may involve 5'-AMP-activated kinase (Shepherd and Kahn, 1999). Basal and insulin-stimulated glucose uptake into muscle cells and adipocytes are increased by thyroid hormone, partially as a result of an increase in GLUT-4 expression (Kahn, 1992; Abel et al., 2004). In horses, levothyroxine is known to improve insulin sensitivity and decrease blood lipid concentrations (Frank et al., 2005). Adipocytes secrete leptin and in the brain it signals a response to change energy stores (Berti et al., 1997). Leptin improves insulin response in skeletal muscle and insulin-stimulated glucose disposal, which is largely attributed to an increase in fatty acid oxidation and a decrease in intramuscular triacylglycerol (Stefanyk et al., 2011).

Insulin Resistance: Obesity and Fatty Acids

Insulin secreted after a meal acts as an anabolic hormone, promoting fuel storage and favoring weight gain (Pittas and Roberts, 2006). From an evolutionary standpoint, insulin hypersecretion in response to a meal may have conferred a survival advantage in increasing the efficiency of energy storage in adipose tissue when it was in abundance, which is also known as the thrifty gene hypothesis (Southam et al., 2009). Few studies have examined the prevalence of obesity in horse and pony populations. The 1998 National Animal Health Monitoring System study estimated that 4.5% of the horse population in the United States was overweight or obese (Geor, 2008). Thatcher

and coworkers (2007) more recently predicted that the prevalence of overweight (51%) and obese (19%) horses supersedes that of the National Animal Health Monitoring System in 1998, indicating that obesity in the equine industry is greatly increasing. Unlike their ancestors, today's horses are often presented with an abundance of readily available food and are kept in relative confinement. Some horse owners also lack understanding of the proper dietary management of horses and overfeed them. Collectively, this has led to excessive weight gain and fat accumulation.

Some studies in humans have found an association between insulin secretion and weight gain (Hodge et al., 1996; Odeleye et al., 1997; Sigal et al., 1997), whereas others have not found the same association (Weyer et al., 2000; Mayer-Davis et al., 2003; Silver et al., 2006). In horses, diet-induced weight gain occurred concurrently with decreased insulin sensitivity that was effectively compensated for by an increase in insulin secretory response (Carter et al., 2009). The study fed 200% of the horse's digestible energy requirements for maintenance for 16 wk to induce weight gain, which resulted in hyperinsulinemia and hyperleptinemia. Thus, prevention of obesity is a potential strategy to help avoid insulin resistance, hyperinsulinemia, and hyperleptinemia in the horses. Another study used obese geldings (BCS 7 to 8) to estimate glucose effectiveness and insulin sensitivity and found that these geldings were insulin resistant and seemed to rely primarily on glucose effectiveness for glucose disposal (Hoffman et al., 2003), indicating they had reduced insulin sensitivity due to high serum insulin when compared to other non-obese horses. One mechanism that may explain the association between insulin hypersecretion and future weight gain is the development of hypoglycemia in the post-absorptive period, which has been shown

to result in a pattern of increased hunger, frequent snacking, and increased energy intake in humans (Pittas and Roberts, 2006). This is thought to be the primary cause of weight gain in medical or iatrogenic conditions associated with hyperinsulinemia (Sinha et al., 1996; Dizon et al., 1999). There is also evidence to suggest that relative hypoglycemia contributes to increased intake in healthy, non-obese people (Pittas et al., 2005; Pittas and Roberts, 2006). In healthy subjects, short-term hypoglycemia increases morning food intake (Schmid et al., 2008). Similar effects of hypoglycemia on increased voluntary intake are likely present in horses, but this has not been thoroughly investigated.

It is well established that adipose tissue is not only involved in energy storage, but functions as an endocrine organ that secretes various bioactive substances such as adipokines (Ouchi et al., 2003; Berg and Scherer, 2005). Adipose tissues in obese individuals and in animal models of obesity are infiltrated by a large number of macrophages, whose recruitment is linked to systemic inflammation and insulin resistance (Weisberg et al., 2003; Xu et al., 2003). Functionally, M2 macrophages are associated with repair of injured tissues and resolution of inflammation (Gordon, 2003). It has been suggested that M1 macrophages promote insulin resistance and M2 macrophages protect against obesity-induced insulin resistance (Odegaard and Chawla, 2011). Insulin resistance can be driven by a chronic, low-grade inflammatory state characterized by elevated serum levels of proinflammatory cytokines [e.g. interleukin (IL)-1, IL-6, IL-8, IL-12, and tumor necrosis factor α (TNF α)], chemokines (e.g. monocyte chemoattractant protein 1 and macrophage inflammatory protein 1), insulin resistance associated adipokines (e.g. retinol binding protein 4 and resistin), and insulin

sensitivity-associated adipokines (e.g. adiponectin, visfatin, omentin, and vaspin) (Odegaard and Chawla, 2011).

Free fatty acids (FFA) are important to the pancreatic β -cell for its normal function, its capacity to compensate for insulin resistance, and its failure in type 2 diabetes (Nolan et al., 2006). Depriving pancreatic islets of fatty acids in the pancreas causes loss of glucose-stimulated insulin secretion, a process that can be reversed by supplying exogenous FFA (Nolan et al., 2006). In contrast, high FFA supply augments glucose-stimulated insulin secretion. However, chronic excess of saturated fatty acids, especially coupled with elevated glucose, reduces insulin biosynthesis (Poitout et al., 2006) and secretion (Prentki et al., 2002) and induces β -cell apoptosis (Lee et al., 1994; Prentki et al., 2002; El-Assaad et al., 2003). Saturated and certain monounsaturated fats have been implicated as factors causing insulin resistance, whereas polyunsaturated fatty acids (PUFAs) and, particularly omega-3 fatty acids, appear to have no adverse effects or even fairly positive effects on the action of insulin (Manco et al., 2004). Polyunsaturated fatty acids regulate fuel partitioning within the cells by inducing their own oxidation through the reduction of lipogenic gene expression and the enhancement of the expression of those genes controlling lipid oxidation and thermogenesis (Manco et al., 2004). Moreover, PUFAs prevent insulin resistance by increasing membrane fluidity and GLUT-4 transport (Manco et al., 2004). In contrast, saturated fatty acids are stored in non-adipocyte cells as triacylglyceride leading to cellular damage as a sequence of their lipotoxicity.

Although it is not entirely clear how obesity produces insulin resistance or type 2 diabetes, elevated plasma FFA are thought to play a major role (Boden, 2003). FFA

directly stimulate insulin secretion and decrease metabolic clearance of insulin (Boden, 2005). In healthy, young women, an increase in plasma FFA from approximately 0.5 to 1.1 mmol/L raised insulin secretion by 17% under euglycemic conditions without a change in insulin clearance (Hennes et al., 1997). However, when blood glucose concentration was raised to approximately 7 mmol/L and plasma FFA to approximately 1.1 mmol/L, the subsequent rise in serum insulin was attributed to both increased insulin secretion and decreased metabolic clearance of insulin (Hennes et al., 1997). When blood glucose was further increased to approximately 11 mmol/L, the rise in serum insulin was almost entirely due to a decrease in insulin clearance. Currently, data suggests that when healthy individuals have elevated levels of FFA, it stimulates long term insulin secretion precisely to the degree needed to compensate for the FFA-induced insulin resistance (Boden, 2005). In contrast, in individuals genetically predisposed to type 2 diabetes, FFA stimulation of insulin secretion is not sufficient to compensate for the FFA-induced insulin resistance, which may lead to diabetes (Boden, 2005).

Mechanisms of Insulin Resistance

A cell's resistance to the stimulatory effect of insulin on glucose utilization is considered an important pathogenic feature of obesity, metabolic syndrome, and most human forms of type 2 diabetes (Shepherd and Kahn, 1999). The precise mechanisms that cause insulin resistance are not known in either humans or horses. Several mechanisms may be involved in the gradual process of becoming insulin resistant. Four possible mechanisms are discussed below.

The first mechanism that might be involved in insulin resistance involves aberrations in GLUT-4 trafficking. The GLUT-4 transporter is responsible for an insulin-

mediated increase in glucose uptake by skeletal and cardiac muscle and adipocytes. Uptake of glucose by the cell is the rate-limiting step for glucose metabolism in these cell types, and therefore the appropriate regulation of GLUT-4 is critical to maintenance of normal whole-body glucose homeostasis. Insulin stimulates the recruitment of GLUT-4 from intracellular compartments to the plasma membrane; thus if GLUT-4 translocation is hindered, it may contribute to insulin resistance.

Much effort has been expended on identifying the amino acid motifs in GLUT-4 that are responsible for its insulin-regulated subcellular trafficking. A decrease in insulin-stimulated glucose uptake in obese humans and obese mice was associated with the impairment of GLUT-4 translocation in both skeletal muscle and adipocytes (Zierath et al., 1998; Miura et al., 2001). However, not all studies have found the insulin-stimulated translocation mechanism to be impaired; therefore, the role of GLUT-4 trafficking in the pathogenesis of type 2 diabetes is not absolute (Gaster et al., 2001).

Under normal conditions, a greater amount of GLUT-4 present at the cell surface appears to largely account for the concomitant increase in glucose transport activity (Song et al., 2008); thus, an adequate presence of GLUT-4 within the membrane is important for the correct biological response to insulin. However, GLUT-4 trafficking in the fasting and insulin-stimulated states is still the subject of controversy.

Possible mutations in GLUT-4 could also be a mechanism of insulin resistance. Mutations of glutamic acids of the acidic cluster TELEY motif has been shown to affect GLUT-4 movement from the endosomal recycling pathway to a subcompartment of the trans-Golgi network in both basal and insulin-stimulated adipocytes (Blot and McGraw, 2008). There are several motifs that regulate different steps of GLUT-4 traffic (Blot and

McGraw, 2008), and if mutated or hindered it would affect insulin and whole body glucose homeostasis.

The second mechanism that may be involved in insulin resistance is the expression of GLUT-4. Reduced GLUT-4 expression on the cell surface limits the activation of phosphoinositide-3 kinase (Zierath et al., 1998), which is the major pathway for the mediation of insulin-stimulated glucose transport and metabolism (Shepherd et al., 1998). Muscle GLUT-4 overexpression in transgenic animals ameliorates insulin resistance associated with obesity or diabetes as it increases glucose uptake without high concentrations of insulin (Zorzano et al., 2005).

The third mechanism that may be involved in insulin resistance is the impairment of insulin-stimulated glucose transport by circulating or paracrine factors (Valberg and Fishman, 2009). Tumor necrosis factor (TNF)- α is a major pro-inflammatory cytokine which has been implicated in metabolic disorders, such as obesity and insulin resistance (Qin et al., 2008). Apolipoprotein B (apoB100) is a major protein component of plasma lipoproteins and is required for the synthesis and secretion of triacylglycerol-rich circulating lipoproteins such as very low density lipoproteins (VLDL) (Qin et al., 2008). Qin and coworkers (2008) provided evidence that TNF- α induces whole-body insulin resistance and impairs hepatic insulin signaling, which is accompanied by the overproduction of apoB100-containing VLDL particles, an effect likely mediated via TNF receptor 2. The chronic elevation of serum FFA concentrations, such as those that occur in obese or diabetic humans or horses with equine metabolic syndrome, may also contribute to the decreased uptake of glucose into peripheral tissues (Valberg and Fishman, 2009). Elevated FFA concentrations are linked with the onset of peripheral

and hepatic insulin resistance as the high FFA and intracellular lipid appear to inhibit insulin signaling, leading to a reduction in insulin-stimulated glucose transport, decreased muscle glycogen synthesis and glycolysis and liver hyperglycemia (Boden and Shulman, 2002).

Lastly, the fourth mechanism that is a highly probable cause of insulin resistance is chronic hyperglycemia. Glucose in excess causes toxic effects on the structure and function of organs, including the pancreatic islets where insulin is secreted from (Yki-Jarvinen, 1992). A potential mechanism for glucose toxicity is the formation of excess reactive oxygen species, which cause oxidative stress over time, defective insulin gene expression, and insulin secretion impairment (Robertson, 2004).

Other possible mechanisms of insulin resistance could pertain to the breakdown of insulin signaling receptors (such as tyrosine kinase), which could reduce circulating insulin or downregulate insulin downstream (Treiber et al., 2006c), affecting downstream kinases such as phosphatidylinositol-3 kinase or serine/threonine kinase. Tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) by the insulin receptor, promotes association of the docking protein with effector proteins, such as phosphatidylinositol-3 kinase (Ozes et al., 2001). The phosphatidylinositol-3 kinase and its downstream target, Akt, promote insulin-induced movement of GLUT-4 to the cell membrane, glucose uptake, glycolysis, glycogen synthesis, and protein synthesis (Ozes et al., 2001). The most common causes of insulin resistance are thought to be alterations in signal transduction associated with decreased insulin receptor-autophosphorylation, decreased IRS-1 phosphorylation and reduced activation of phosphatidylinositol-3 kinase (Treiber et al., 2006c). Subsequently, the disruption of

intracellular glucose metabolism regulated by enzymes such as hexokinase and glycogen synthase could reduce insulin-mediated glucose uptake and storage (Treiber et al., 2006c).

Another possible mechanism could be the decrease in insulin response due to rapid insulin degradation or possibly to the neutralization by antibodies (Kronfeld et al., 2005a). Interference at the insulin receptor on the cell surface, such as a glycoprotein associated with receptor tyrosine kinases could be a cause of insulin resistance (Kronfeld et al., 2005a), as discussed above.

Ultimately, a diminution in the intracellular capacity to utilize glucose in the muscle, liver, or adipose tissue, subsequently leads to insulin resistance, regardless of glucose transport and availability, where insulin can become partially or completely ineffective. Compensations for insulin insensitivity may be achieved by either an increase in the release and secretion of insulin from pancreatic β -cells or increasing concentration of plasma and extracellular glucose by an increase in glucose-mediated glucose uptake (Kronfeld et al., 2005a).

Assessment of Insulin Resistance

The amount of insulin that the pancreas secretes in response to glucose and the sensitivity of skeletal muscle and adipose tissue to insulin, affects whole body insulin response. Therefore, both need to be assessed to provide an accurate measure of insulin resistance (Firshman and Valberg, 2007). There are currently 7 different methods to measure insulin resistance in horses, which are described below.

Oral glucose tolerance test

The oral glucose tolerance test (OGTT) is used to assess small intestinal absorption of glucose, hepatic glucose uptake and, to a lesser extent, the endocrine

function of the pancreas and peripheral insulin resistance (Fishman and Valberg, 2007; Valberg and Fishman, 2009). The test requires the horse to fast overnight and then 1 g/kg body weight (BW) of glucose is administered orally via a nasogastric tube (Valberg and Fishman, 2009). Blood glucose is measured before glucose administration and every 30 to 60 minutes thereafter (Valberg and Fishman, 2009). Blood glucose levels generally peak 90 to 120 minutes after glucose administration then return to baseline around 240 to 360 minutes after glucose administration. A sustained high level of blood glucose in response to an OGTT might suggest reduced pancreatic function or possible insulin resistance (Ralston, 2002). However, the test can be affected by the stress of intubation. Further, the test may be affected by variable rates of glucose administration, gastric emptying, and intestinal absorption (Valberg and Fishman, 2009), and is therefore somewhat limited for evaluating insulin resistance. It also does not account for endogenous insulin secretion and therefore is limited in subjects with altered pancreatic function (Kim, 2008)

Intravenous glucose tolerance test

An intravenous glucose tolerance test (IVGTT) is somewhat similar to an OGTT with the exception that the glucose is administered intravenously. This test is used to avoid the variable absorption of glucose by the intestinal tract as can occur with an OGTT (Kronfeld et al., 2005b). Horses are fasted for 12 to 24 hours and then administered 0.5 g glucose per kg/BW intravenously over a 10 minute time period (Valberg and Fishman, 2009). Blood glucose and insulin concentrations are determined at 0, 2, 15, 30, 60, and 90 minutes and then hourly for 5 to 6 hours after injection (Valberg and Fishman, 2009). Half-life of glucose disposal and the fractional turnover rate, which is a measure of glucose utilization and the peripheral insulin resistance can

then be calculated (Valberg and Fishman, 2009). A normal horse will show an immediate increase in blood glucose concentration and will return to normal levels within one hour (Valberg and Fishman, 2009). The insulin response curve should parallel the glucose response curve with a peak in insulin at approximately 30 minutes post glucose injection (Ralston, 2002; Valberg and Fishman, 2009). Horses with insulin resistance would potentially produce a higher peak in blood glucose and a consistent delay before returning to a baseline after 2 hours post injection (Valberg and Fishman, 2009). Insulin concentrations must also be measured to determine if the pancreas secretion of insulin is impaired or if insulin-stimulated glucose disposal is impaired (Valberg and Fishman, 2009). If the pancreatic β -cell is impaired there could be a delay in both glucose clearance from circulation and the appearance of insulin (Valberg and Fishman, 2009). Although the IVGTT overcomes some of the limitations encountered with an OGTT, the IVGTT is still not a sensitive means to measure diminished pancreatic response (Fishman and Valberg, 2007).

Insulin tolerance test

An insulin tolerance test (ITT) measures blood glucose response to an intravenous injection of insulin. The amount of insulin administered to horses has been highly variable, ranging from 0.2 IU/kg to 0.6 IU/kg (Valberg and Fishman, 2009). An ITT test is also used to assess the response of the horse to an insulin-induced hypoglycemia (Valberg and Fishman, 2009). Depending on the dose of insulin used for injection, blood glucose levels will drop to 50% of the original value within 20 to 30 minutes of injection, then return to fasting level within 1.5 to 2 hours (Valberg and Fishman, 2009). An ITT test is usually used for an animal that is known to be insulin insensitive; thus, it has limited use in the screening for insulin resistance. In an insulin resistant horse, blood

glucose levels will not fall as dramatically as a healthy horse and will return to basal levels more quickly compared to a healthy horse (Valberg and Fishman, 2009).

Frequently sampled intravenous glucose tolerance test

The frequent sampling intravenous glucose tolerance (FSIGT) test uses the combination of intravenous injections of glucose and insulin to test both pancreatic insulin response to elevated blood glucose and peripheral insulin resistance (Pratt et al., 2005; Treiber et al., 2005a). With the FSIGT test, horses are not fasted. Blood is drawn prior to administration of glucose and then horses are rapidly administered 300 mg/kg of glucose solution intravenously (Valberg and Fishman, 2009). Blood is then taken via a catheter at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 minutes afterward (Valberg and Fishman, 2009). Twenty minutes after glucose has been administered, a small intravenous dose of insulin (20 mIU/kg) is given and blood is then taken at 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180 minutes (Valberg and Fishman, 2009). Glucose and insulin dynamics are evaluated via minimal model analysis described by the following equations: $G'(t) = -G(t) \times [S_g + X(t)] + S_g \times G_b$, where $G'(t)$ is the net rate of change in plasma glucose ($\text{mg} \cdot \text{dL}^{-1} \cdot \text{min}^{-1}$) (Treiber et al., 2005a). Glucose effectiveness (S_g) describes one component of the plasma disposal rate (min^{-1}), which is the capacity of the cells to take up glucose without insulin mediation. The plasma glucose concentration (mg/dL) at time = t is $G(t)$; G_b is the basal glucose concentration (mg/dL), maintained primarily by hepatic production. Insulin action ($X(t)$) represents the insulin mediated component of plasma glucose disposal rate via the acceleration of glucose uptake in response to an increment change in the insulin concentration. This component is further described by $X'(t) = p_3 \times [I(t) - I_b] - p_2 \times X(t)$, where $X'(t)$ is the rate of change of the insulin action (min^{-1}), p_3 describes the delivery of

insulin to the interstitium, and p_2 describes the disposal of insulin from the interstitial fluid, possibly reflecting hepatic extraction of plasma insulin. Insulin sensitivity (SI) is the ratio of the parameters: $SI = p_3/p_2$ ($L \cdot \text{min}^{-1} \cdot \text{mIU}^{-1}$) and represents the efficiency of insulin to accelerate glucose uptake by the cells. Insulin sensitivity (SI) is estimated from the reciprocal of basal insulin concentration: $SI = (7.93(1/(\sqrt{[\text{insulin}]}) - 1.03$ (Treiber et al., 2005b; Valberg and Fishman, 2009).

Responsiveness of β -cells to the glucose load is described by the acute response of insulin to glucose (AIR_g ; $\text{mIU}/[\text{L} \cdot \text{min}]$), which is the increase in plasma insulin above basal concentration integrated from 0 to 10 minutes after the glucose dose (Treiber et al., 2005a). The product of ARI_g ($\text{AIR}_g = (70.1 [\text{MIRG}]) - 13.8$) uses the modified insulin response to glucose (MIRG ; calculated as $800 - 0.30[\text{insulin} - 50]^2 / (\text{glucose} - 30)$) and SI to determine the disposition index (DI) or the appropriateness of the β -cell response relative to the degree of insulin resistance in the tissue ($\text{DI} = \text{AIR}_g \times \text{SI}$).

Unlike the glucose clamp, which is discussed below and depends on steady-state conditions, the minimal model approach uses dynamic data. Valid minimal model analysis of the FSIGT requires several assumptions (Muniyappa et al., 2008).

Muniyappa and coworkers (2008) describe several assumptions to be considered:

- First assumption: instantaneous distribution of the glucose bolus in a monocompartmental space is assumed to occur.
- Second assumption: glucose disappearance in response to glucose or insulin is assumed to occur at a monoexponential rate.
- Third assumption: glucose concentration at the end of the FSIGT is assumed to be identical to the beginning concentration.
- Fourth assumption: insulin is assumed to act through extravascular (interstitial or extracellular space where insulin directly exerts metabolic actions) activity to promote glucose disappearance.
- Fifth assumption: the minimal model lumps together effects of insulin to promote glucose disposal in skeletal muscle and suppressed hepatic glucose production.

- Sixth assumption: to obtain a valid estimate of S_1 the minimal model assumes that total insulin secretion (endogenous plus exogenous) during the FSIGT is above a certain threshold

The FSIGT and the minimal model analysis is generally easier to perform than the glucose clamp method because though both are labor intensive, the FSIGT it is slightly less labor intensive, steady-state conditions are not required, and there are no intravenous infusions that require constant adjustment (Muniyappa et al., 2008). In addition, information on insulin sensitivity, glucose effectiveness, and β -cell function can be derived from a single dynamic test. Moreover, the minimal model generates excellent predictions of glucose disappearance during the FSIGT. A major limitation to the FSIGT/minimal model approach is that it involves intensive blood sampling over a 3-h period, making it labor intensive, when compared to other model approaches.. Further, the minimal model oversimplifies the physiology of actual glucose homeostasis and it lumps together the effects of insulin to promote peripheral glucose utilization and suppress hepatic glucose production.

Hyperglycemic and hyperinsulinemic clamps

The glucose clamp technique is a method for quantifying insulin secretion and resistance, which was developed in 1979 (DeFronzo et al., 1979). The hyperinsulinemic-euglycemic clamp involves intravenous infusion of insulin at a constant rate to raise and maintain systemic insulin levels causing a hyperinsulinemic state (Kim, 2008). The hyperglycemic clamp is used to fix plasma glucose at an acutely elevated level for two hours to suppress endogenous hepatic glucose production (DeFronzo et al., 1979). Glucose intravenously infused at variable rates is used to maintain euglycemia. The rate of glucose infusion directly correlates with insulin sensitivity. This is accomplished by an intravenous glucose infusion consisting of two

phases: 1) a 15 min priming dose needed to raise the glucose level in plasma and extravascular compartments to the desired plateau and 2) a maintenance dose that is computed at 5 min intervals throughout the study (DeFronzo et al., 1979). Periodic adjustments in the glucose infusion rate made every 5 min are based on the negative feedback principle: if the actual glucose concentration is higher than the goal, the infusion is decreased and vice versa (DeFronzo et al., 1979). In this manner, the glucose infusion rate becomes a measure of pancreatic insulin secretion, allowing quantification of the pancreatic β -cell's sensitivity to glucose (Valberg and Fishman, 2009). This procedure is followed by the hyperinsulinemic- clamp , which provides a supramaximal but steady state level of insulin (Valberg and Fishman, 2009). Insulin is infused to raise the plasma insulin concentration acutely to a new plateau and maintain it at that level (DeFronzo et al., 1979). The rate of glucose infusion required to maintain euglycemia during the hyperinsulinemic clamp acts as a measure of insulin sensitivity for skeletal muscle and adipose tissues (Valberg and Fishman, 2009).

Incorporation of radioactive-labeled glucose during a euglycemic clamp can further be used to measure glucose metabolism by individual organs. One advantage of this test is that it is a more sensitive measure of insulin action and can be used to assess insulin sensitivity in individual organs. Another advantage of this test is that endogenous insulin secretion can be controlled and any potential fluctuations during the test that might alter glucose homeostasis are minimized (Valberg and Fishman, 2009).

A disadvantage of clamps is that they are ill-suited for large studies because of the extensive requirements for cost, labor, and technical expertise (Lee et al., 2011). Operator-induced variability and site-specific differences in clamp methods can pose a

problem as well. This test tends to require more technical expertise due to the constant infusion and mitigation of hormone levels at specific times and doses in order to maintain goal plasma concentrations. Nonetheless, the clamping technique remains one of the most accurate means to measure sensitivity of tissues to hyperglycemia and hyperinsulinemia (Firshman and Valberg, 2007).

Basal glucose and insulin measurement

Single fasting glucose and insulin measurements have been commonly used by veterinarians to identify horses that are suspected to be insulin resistant (Kronfeld et al., 2005a; Treiber et al., 2006c). However, the accuracy of a single fasting sample has been questioned due to the wide variation in both glucose and insulin concentrations noted within an individual horse over a short time period (Treiber et al., 2005a; Treiber et al., 2006c).

Proxies and reference quintiles for basal glucose and insulin

Proxies for insulin sensitivity and pancreatic β -cell responsiveness that are derived from basal glucose and insulin concentrations are commonly used to screen potential diabetic patients. They minimize sampling and stress to the animal and require less labor and materials, yet are thought to be an improvement above fasting glucose and insulin concentrations for assessing insulin resistance.

Treiber et al. (2005a) evaluated insulin sensitivity and responsiveness in a small number of normal and insulin resistant-suspect horses and ponies via a FSGIT and compared the outcome to several proxies based on basal values of insulin and glucose that are routinely used in public health. The authors found that the proxies that best represented insulin sensitivity and insulin responsiveness according to the minimal model were the reciprocal of the insulin square root index using basal insulin

concentrations of mIU/L (RISQI; calculated as $1/\sqrt{\text{insulin}}$), and the modified insulin response to glucose using basal glucose concentrations of mg/dL and basal insulin concentrations of mIU/L (MIRG; calculated as $800 - 0.30[\text{insulin} - 50]^2 / (\text{glucose} - 30)$), respectively. The RISQI estimates the amount of insulin compensation required to chronically maintain glucose homeostasis, whereas MIRG estimates the capacity of the pancreatic β -cells to increase insulin secretion and compensate for exogenous glucose. The MIRG capacity is limited by chronic basal insulin secretion, while chronic decompensation is indicated by increasing basal glucose levels. The combined use of RISQI and MIRG have enabled assessment of compensatory insulin secretion in apparently healthy horses and insulin signaling failure in hyperglycemic horses (Treiber et al., 2005b). In addition, these proxies have been used to document insulin resistance in ponies and predict the likelihood of developing laminitis (Treiber et al., 2006c). It should be noted that proxies are less accurate than the specific quantitative parameters they predict and can have resting blood sample variability as described by Pratt and coworkers (2009). However, they are superior to nonspecific indicators, such as basal hyperinsulinemia, glucose intolerance, or analogies to diseases in other species (Treiber et al., 2005b).

The homeostasis model assessment (HOMA) is a test used to determine the degree to which insulin resistance and deficient β -cell function can be assessed from a subject's fasting plasma insulin and glucose concentrations (Matthews et al., 1985). The calculation for HOMA is: $(\text{basal glucose (mg/dL)} - \text{insulin (mU/L)}) / 22.5$ (Treiber et al., 2005b). The HOMA is used to take advantage of the reciprocal relationship between both basal insulin and basal glucose concentrations and to avoid ambiguity of low

insulin concentrations that could indicate high insulin sensitivity or insulin secretion failure (Matthews et al., 1985). High HOMA scores denote low insulin sensitivity or insulin resistance (Bonora et al., 2000). In humans, the HOMA index shows a correlation to the euglycemic-hyperinsulinemic clamp technique and, to a lesser extent, the hyperglycemic clamp technique (Treiber et al., 2005b). However, the estimation of the set of fasting insulin values by HOMA are unlikely to be precise, in part because the range over which insulin is measured is small and results depend on precision of the insulin radioimmunoassay (Matthews et al., 1985). Equine pancreatic β -cells appear particularly resistant and in insulin resistant horses a high basal insulin concentration is usually seen (Treiber et al., 2005b). Therefore, the usefulness of HOMA in identifying insulin resistant horses may not be any more accurate than other proxies, such as MIRG and RISQI.

Equine Metabolic Syndrome

The term equine metabolic syndrome (EMS) is a label for horses whose physical examination and laboratory testing suggest a heightened risk for developing laminitis as a result of underlying insulin resistance (Johnson et al., 2010). Equine metabolic syndrome is not considered a disease, but rather a collection of clinical abnormalities that are combined to identify a patient with the likelihood of developing laminitis as opposed to individuals lacking EMS characteristics. An examination that shows positive characteristics of EMS is characterized by obesity, regional adiposity, insulin resistance, hypertriglyceridemia, and hyperleptinemia (Frank, 2009). The term “equine metabolic syndrome” was first introduced into veterinary medicine in 2002 when it was proposed that obesity, insulin resistance, and laminitis were all a component of a clinical syndrome recognizable in both horses and ponies (Johnson, 2002). Usage of the term

EMS was adopted based on its similarities to the metabolic syndrome in humans, which is a collection of risk factors used to predict type 2 diabetes mellitus and the incidence of coronary artery disease (Frank et al., 2010). Equine metabolic syndrome usually develops in horses less than 15 years of age, which differs from PPID that affects horses who are over 15 years of age (McFarlane, 2011). However, both EMS and PPID share the commonality of underlying insulin resistance.

Equine Metabolic Syndrome and Obesity

Most horses diagnosed with EMS are obese with enlarged adipose tissue within the nuchal ligament of the neck, commonly referred to as a cresty neck (Frank et al., 2010; Tadros and Frank, 2011). Fat pads will also develop around the tailhead and within the prepuce or mammary gland area in horses considered to have EMS (Frank et al., 2010; Tadros and Frank, 2011). Occasionally, horses that are affected with EMS will also have subcutaneous adipose tissue deposits randomly distributed on their body (Tadros and Frank, 2011). It should be noted that some obese and overweight horses and ponies do have normal insulin sensitivity. Not all EMS-affected horses are obese and not all obese horses develop insulin resistance (Johnson et al., 2010). Whether obesity induces insulin resistance or the insulin resistant horse is predisposed to obesity has not been determined.

In humans, the development of type 2 diabetes mellitus is thought to have a higher correlation with mesenteric and omental adipose tissues when compared to fat accumulation elsewhere (Frank et al., 2010). The fatty acids and adipokines released into portal circulation from these visceral sites have been shown to cause a more profound effect on hepatic metabolism and insulin clearance. Whether a similar

difference exists between adipose tissue from the neck crest or abdomen and fat tissues collected from other locations in the horse is currently under investigation.

The two primary theories linking insulin resistance and obesity are: 1) the down regulation of insulin signaling pathways induced by adipokines and cytokines produced in adipose tissue; and 2) the accumulation of intracellular lipids in insulin-sensitive tissues such as skeletal muscle, which is also known as lipotoxicity (Frank et al., 2010). A typical equine diet is low in fat; however, excess dietary carbohydrate can be converted to fat via *de novo* lipogenesis (Frank et al., 2010). Fats are stored in adipose tissue cells as triacylglycerol and are used for energy. Once the storage of the cells have reached capacity, fats are repartitioned towards nonadipose tissues such as skeletal muscle, liver, and pancreas. These tissues will attempt to utilize the fats by increasing β -oxidation. Consequently, if too much lipid accumulates in these tissues it alters normal cellular functions, including insulin signaling.

Equine Metabolic Syndrome and Laminitis

With EMS usually comes the onset of some form of laminitis. Laminitis literally means the inflammation of the laminae in the hoof, the interdigitating plates that lock the hoof wall to the third phalangeal bone (Kronfeld et al., 2006). The lamellar structure confers weight carrying strength through a combination of rigidity and resilience. In horses with EMS, laminitis appears to occur spontaneously without a history of a bacterial disease, retained placenta, or grain overload (Tadros and Frank, 2011).

Insulin resistance represents a risk factor for laminitis; consequently the development of laminitis is used to support the diagnosis of EMS in horses (Johnson et al., 2010). Although the physical or radiographic appearance of the hoof indicates laminitis in an EMS horse, the owner may report that lameness or pain has not been

evident. Therefore, structural changes in the hoof-lamellar interface may occur in the absence of laminitic pain in EMS horses.

The insulin resistance component of EMS most likely predisposes a horse to laminitis, because insulin possesses vasoregulatory properties (Frank, 2009). Activation of the insulin receptor stimulates two different signaling pathways within the vascular endothelial cell. One of the pathways is mediated by phosphatidylinositol-3 kinase (PI3K), which promotes vasodilation, whereas the second pathway is the mitogen-activated protein kinase (MAPK) pathway, which promotes vasoconstriction (Frank, 2009). These two pathways are critical in the regulation of blood flow to the hoof, especially the sensitive laminae.

The vasodilatory effects of insulin and insulin-dependent stimulation of glucose uptake are both mediated by the PI3K pathway, which becomes disrupted when insulin resistance develops (Tadros and Frank, 2011). Consequently, vasoconstriction is then promoted in an insulin resistant animal because the MAPK pathway still remains fully functional. PI3K is one of the most important regulatory proteins involved in different signaling pathways and controlling key functions of the cell (Krasilnikov, 2000). The PI3K pathway leads to the phosphorylation and activation of phosphoinositide dependent kinase-1 (PDK-1), which in turn phosphorylates and activates Akt (also known as protein kinase B) (Kim et al., 2006). The Akt will directly phosphorylate endothelial nitric oxide (NO) synthase (eNOS), which results in increased eNOS activity and NO production for vasodilation that regulates vascular tone (Iwakiri et al., 2002; Kim et al., 2006). Although insulin is a key regulator to induce the PI3K pathway, various cytokines and growth factors have also been shown to induce the PI3K and subsequent

phosphorylation of eNOS (Iwakiri et al., 2002). Because the PI3K pathway is the one that stimulates glucose uptake, this pathway is likely to be compromised in an animal that is insulin resistant (Frank, 2009).

The MAPK pathway is the second pathway of insulin signaling that generally regulates growth and mitogenesis and controls the secretion of endothelin-1 (ET-1) in the vascular endothelium which leads to vasoconstriction (Kim et al., 2006). Insulin resistance is associated with hyperinsulinemia that has been linked to the activation of the endothelin system (Yang and Li, 2008). Insulin-induced elevation of ET-1 contributes to abnormal cell growth, atherosclerosis and hypertension in insulin resistant humans. In 2007 an increase in plasma ET-1 concentration was detected in blood collected from the digital veins 12 hours after carbohydrate was administered to induce laminitis in healthy horses (Eades et al., 2007). These findings suggest that digital vessels undergo vasoconstriction as a result of carbohydrate overload in horses, which may contribute to the development of laminitis. Vasoconstriction is already promoted in horses with chronic insulin resistance and may be more likely to develop laminitis (Tadros and Frank, 2011).

Hyperinsulinemia and Vascular Dynamics

Laminitis can be induced by prolonged hyperinsulinemia in healthy young, lean ponies, independent of changes in blood glucose concentration or insulin sensitivity (Asplin et al., 2007; Nourian et al., 2009). This evidence suggests that the laminae of the hooves have a sensitivity to the effects of elevated glucose or insulin concentrations. In horses, hyperinsulinemia is defined by a serum/plasma insulin concentration of greater than 20 $\mu\text{L/mL}$ ($\mu\text{U/mL}$) (Frank et al., 2010; Tadros and Frank, 2011). Experimentally-induced laminitis has been performed in healthy ponies and

Standardbred horses by inducing hyperinsulinemia (Asplin et al., 2007; De Laat et al., 2010). In both studies, glucose and insulin were infused intravenously according to the euglycemic-hyperinsulinemic clamp procedure, with mean serum insulin concentrations exceeding 1000 $\mu\text{U}/\text{mL}$. Mean time to onset of Obel grade 2 laminitis was 46 h in horses and 55 h in ponies (Asplin et al., 2007; De Laat et al., 2010). An increase in the temperature of the hoof surface in response to the insulin infusion was noted, suggesting that hyperinsulinemia actually induces laminitis through a mechanism involving vasodilation. Hyperinsulinemia-induced vasodilation would then overcome the MAPK-driven vasoconstriction, which is promoted by insulin resistance (Tadros and Frank, 2011). Also proposed is that the hyperinsulinemia-induced vasodilation causes an increase in glucose delivery to the hoof tissues, consequently causing local glucotoxicity (De Laat et al., 2010).

Management of Equine Metabolic Syndrome and Insulin Resistance

Key strategies for managing insulin resistance and EMS may be differ based on the presence or absence of laminitis. However, whether laminitis has been noticed or not, promoting weight loss in obese or overweight horses and improving insulin sensitivity through dietary management and exercise should be taken seriously (Tadros and Frank, 2011). Horses and ponies that are affected with EMS seem to be particularly sensitive to ingested non-structural carbohydrates (NSC) (Johnson et al., 2010).

Diet

Diets that are high in NSC such as a high grain diet or *ad libitum* access to very lush pasture tend to exacerbate insulin resistance because the diet further stimulates the production of insulin (Frank, 2008). In fact, the term pasture-associated laminitis is becoming more common and is given to horses that develop laminitis whilst grazing

pasture without any other known concurrent disease or problem to have caused the onset of laminitis. Grazing lush pasture is the most common cause for laminitis recognized by practicing veterinarians (Johnson et al., 2010). Exposure to pasture forage high in NSC (15 to 20% DM) exacerbated hyperinsulinemia in insulin-resistant ponies and this diet-associated alteration in insulin dynamics may contribute to increased risk of laminitis (Treiber et al., 2005b; Bailey et al., 2008).

The total carbohydrate content of pasture grass is characterized by the carbohydrates that constitute the cell wall structure of plant cells (structural carbohydrates or fiber, such as cellulose and hemicellulose), which are indigestible by mammalian enzymes (Johnson et al., 2010). The NSC (starches, soluble sugars, and fructans) in pasture grass can be high, and when ingested can cause both glycemic and insulinemic spiking, which seems to be associated with aggravated laminitis. It is also possible that certain types of fructans undigested by the small intestine may cause perturbations in hindgut microflora leading to colonic acidulation, increased epithelial permeability, and the absorption of other laminitis triggers such as endotoxins and vasoactive amines. The horse with EMS is similar to a person with diabetes, so excessive sugar should be avoided as it may exacerbate insulin resistance. Due to the quantity consumed, pasture grass is usually one of the largest sources of sugar in the diet of a horse (Frank, 2008). The carbohydrate content of a pasture varies between regions and depends on soil type, climate, hours of sunlight, and grass species (Frank, 2008). Therefore, horses with EMS and/or insulin resistance should be restricted from pasture, depending on the severity of their condition.

A forage based diet, with hay that has a NSC content of less than 10% (on a dry matter (DM) basis) has been recommended for an insulin resistant horse (Tadros and Frank, 2011). Pratt and coworkers (2006) found that physical conditioning lessened the impact of a high NSC diet on insulin sensitivity. In addition to NSC, intake of water soluble carbohydrates (WSC), which comprise the simple sugars (glucose, fructose and sucrose) and more complex oligo- or polymeric fructans need to be restricted (Longland et al., 2011). It is believed that WSC constituents promote the development of laminitis through potentiation rapid fermentation of the fructan fraction. It has been suggested that diets for horses prone to insulin resistance or laminitis should contain diets low in WSC with approximately 100 g WSC/kg DM as a maximum for such animals. Longland and coworkers (2011) demonstrated the variability of different hays when soaked for 0, 20, and 40 min and 3 and 16 h in water. Results from this study concluded that soaking of hay is not a reliable way to lower the water soluble carbohydrate (WSC) content as previously thought. Overweight and obese horses should be placed on a weight management diet composed of hay at 1.5% BW (e.g., 7.5 kg for a 500-kg horse) and a protein/vitamin/mineral supplement (Tadros and Frank, 2011). Body condition score (BCS) should be assessed and recorded. If the horse does not begin to lose weight after one month on this diet, the amount of hay fed should be lowered to 1% of the horse's ideal BW (e.g., 5 kg for a 500-kg horse). A reduced meal should be fed until an ideal BCS of 5 or 6 is reached. Any type of grain should be eliminated from the diet and access to pasture should be eliminated or strictly limited, or horses may be equipped with a grazing muzzle while the horse is trying to lose weight (Tadros and Frank, 2011).

There has been very limited exploration of the effects of insulin secretion on weight loss response to hypocaloric diets in humans (Pittas and Roberts, 2006) or horses. Weight loss is historically difficult to achieve and maintain in humans and can also be difficult in horses. There is animal and human evidence that suggests that the dynamics of insulin sensitivity and secretion play a role in body weight regulation (Pittas and Roberts, 2006). Therefore, the parameters may affect individual responses to hypocaloric or low calorie diets. Moreover, specific dietary factors that influence these parameters may interact with subject-specific characteristics of insulin dynamics to influence the effect of the hypocaloric diet with varied macronutrient composition on weight loss and maintenance.

Initial evidence for an important role of insulin secretion in energy balance and weight loss comes from pharmacologic studies in which insulin secretion was suppressed with pharmacologic agents and the patient's weight loss response assessed (Pittas and Roberts, 2006). In one study obese participants received a hypocaloric diet and then randomized to either placebo or diazoxide, a K^+ (ATP) channel agonist that decreased the secretion of insulin and is used in the medical management of insulinomas (Alemzadeh et al., 1998). Compared with the placebo group, the diazoxide group lost more weight (4.6 vs. 9.5 kg, respectively) while on the hypocaloric diet. This evidence suggests an important role for insulin secretion in modifying weight loss in response to caloric restriction. In a more recent pharmacologic study insulin secretion was suppressed in obese individuals by octreotide-LAR, a somatostatin analog used in various endocrine hypersecretory conditions (Velasquez-Mieyer et al., 2003). For the entire cohort, significant insulin suppression was achieved with

accompanied weight loss and decreased self-reported carbohydrate craving, without a concomitant lifestyle intervention. In a post hoc analysis, participants who lost the most weight exhibited the highest suppression of pancreatic β -cell activity and the highest reduction in carbohydrate cravings and intake.

In summary, the contribution of insulin secretion to weight and response to hypocaloric diets, supplements, including those that vary in macronutrient composition, is still controversial. As an example, a current study examined insulin resistance in unrelated ponies where the variation between their insulin sensitivity was reduced by feeding a diet moderately high in NSC diet with increased amounts of free glucose for 32 days (Tinworth et al., 2011). These ponies remained clinically healthy with no significant changes in body weight and laminitic effects were not found, suggesting possible adaptation or different tolerance thresholds to high NSC diets may vary between horses. The effects are also difficult to isolate from insulin resistance.

Exercise

Weight management alone may not be effective enough to improve insulin resistance. Weight management combined with exercise is a healthier and more effective option to reduce weight on a horse.

Long-term exercise programs have been shown to improve insulin sensitivity and reduce fasting and glucose-stimulated plasma insulin levels in obese humans (Brown et al., 1997). However, intensive training is not required to improve insulin sensitivity. Studies have shown that low-intensity exercise can improve insulin sensitivity and increase glucose uptake into the muscle in obese horses, even in the absence of weight loss (Powell et al., 2002). In one study, 31 stabled horses underwent three different exercise regimes: turnout, light exercise, and moderate exercise, while being fed a diet

containing 60% concentrate (Turner et al., 2011). Blood was sampled monthly and analyzed for insulin. Insulin sensitivity was assessed and compared across months; the study concluding that insulin was higher during periods of moderate and light physical activity when compared with turnout. Moderate exercise intensity performed 5 days a week seemed to benefit insulin sensitivity, even in insulin sensitive horses in this study. Results indicate that turnout alone may not be adequate to improve insulin sensitivity in horses fed high amounts of concentrates.

A program to increase physical activity should be implemented to promote weight loss, which has been shown to be effective therapeutic intervention to increase insulin sensitivity in obese insulin resistant people (Frank et al., 2010). It has been shown that exercise increases glucose uptake from the plasma with and without insulin-mediation (Treiber et al., 2006a). In addition, exercise decreased insulin secretion, which could promote mobilization and utilization of fatty acids as an additional energy source in order to induce weight loss. Hyperinsulinemic obese ponies subject to training had improved insulin sensitivity after only 2 weeks (Ralston, 2002). Another study looked at age related training on insulin sensitivity as insulin sensitivity declines with age in horses (Malinowski et al., 2002) and found that exercise improves pancreatic β -cell function and insulin sensitivity in old and young horses (Liburt et al., 2011).

It has been suggested that an exercise regime should include at least 200 minutes of moderate intensity exercise per week (Frank et al., 2010). The exercise can start with 2 to 3 exercise sessions per week of either riding or longeing for at least 20 to 30 minutes per session with a gradual increase in intensity and duration building to 5 sessions per week. It is thought that exercise improves insulin sensitivity by 3

mechanisms: 1) reduced muscle and liver glycogen; 2) increased binding of insulin to insulin receptors; and 3) increased glucose uptake by muscle cell glucose transporters (Sternlicht et al., 1989; Powell et al., 2002).

Pharmacologic intervention

Medical management can also be used through pharmaceutical products. Weight loss can be induced and insulin sensitivity improved through the administration of levothyroxine sodium (Frank et al., 2010). Metformin is a drug used in human medicine to increase insulin sensitivity as it reduces glucose concentrations by improving hepatic and peripheral tissue insulin sensitivity without affecting insulin secretion (Dunn and Peters, 1995). Hyperinsulinemic horses have had positive responses to metformin (Frank et al., 2010).

Dietary Supplements

Dietary supplementation may improve insulin sensitivity instead of having to control the health problem with a drug; however, there is a lack of scientific proof establishing significance of supplementation improvement of EMS or insulin resistance. Common supplements containing leucine, cinnamon extract or fish oil are believed to improve insulin resistance and EMS. However, studies have shown that leucine does not affect plasma glucose or insulin (Etz et al., 2011), and neither cinnamon extract nor fish oil supplementation affected insulin sensitivity of mares of known reduced insulin sensitivity (Earl et al., 2011). If supplements are proven in humans or rats to work to improve insulin sensitivity, such as cinnamon extract (Anderson, 2008), does not mean that this crosses over and works in the horse as well.

A recent study found that supplementation with short-chain fructo-oligosaccharides had no effect on plasma glucose, but moderately improved insulin sensitivity in

overweight Arabian geldings (Respondek et al., 2010). Psyllium supplementation affected glycemic and insulinemic responses as it resulted in lower mean postprandial blood glucose and insulin concentrations (Moreaux et al., 2011). Psyllium fed daily for 60 d altered post-prandial glycemia and insulinemia in normal, nonobese, and unexercised horses. According to studies performed in humans, the mechanism of action of psyllium is related to its ability to increase the viscosity of digesta, which results in altered transit time and absorption of nutrients (Sierra et al., 2001, 2002). A study in endurance horses supplemented with psyllium concluded that the psyllium lowered pack cell volume and the authors hypothesized this was due to the availability of sequestered water in the gastrointestinal tract (Cinotti et al., 1997). The water-retaining capacity of feeds and potentially the rate of gastric emptying is related to the ratio of insoluble to soluble fiber entering the stomach of mammals (Bach Knudsen, 2001). The viscosity and passage rate of digesta through the small intestine of humans and swine is affected by soluble dietary fiber. It has been suggested that the viscosity and rate of digesta flow through the small intestine of the horse alter uptake of nutrients (Ellis and Hill, 2005). Small intestinal luminal pH also affects the active transport of nutrients across the intestinal mucosa (Hoffman, 2003; Ellis and Hill, 2005), which is suggested to contribute to altered transit time of psyllium.

Arginine

Arginine was first isolated in 1886, and in 1932 scientists learned that L-arginine was needed to create urea, a waste product produced when toxic ammonia is removed from the body (Mayo Clinic, 2010). Arginine is known by several names such as (S)-2-Amino-5-guanidinopentanoic acid or more commonly, L-arginine with a molecular formula of $C_6H_{14}N_4O_2$.

L-Arginine is a basic amino acid and one of the 20 most common, naturally occurring amino acids in nature. Arginine is also considered among the 10 essential amino acids for the horse and other mammals (NRC, 2007; Wu et al., 2009). Arginine is a component of dietary protein and body fluids (Wu et al., 2009). In animals and humans, the amino acid fulfills versatile physiological functions that are critical to normal regulatory properties.

The requirements of arginine in most mammalian species are frequently met through either dietary intake or endogenous synthesis (Deusdelia et al., 2002). In the species studied, arginine is considered “essential” for young and growing animals and is “conditionally essential” for the adult mammal. Based on nitrogen balance or growth and the functional needs beyond tissue protein synthesis, arginine is nutritionally essential for: 1) development of the mammalian conceptus; 2) neonates; 3) birds, cats, ferrets, and fish; and 4) adults under certain conditions, such as intestinal resection or dysfunction, burns, or renal dysfunction associated with NO deficiency (Wu et al., 2009). In comparison to other species, there has been no research on arginine requirements in nonruminant herbivores, such as the horse.

Dietary Arginine Supply

Several foods are naturally rich in arginine, including seafood, watermelon juice, nuts and seeds, algae, meats, rice protein concentrate, and soy protein (Wu et al., 2009). In feedstuffs provided to livestock and horses, those with a higher protein content will generally have a higher amount of arginine. For example, alfalfa has a higher percentage of arginine (0.77% of DM) compared to a warm-season grass such as bermudagrass (0.04%) (NRC, 2007). Grains such as oats and corn have approximately

0.85% and 0.17% arginine, respectively. Within common oilseed meals, cottonseed meal has by far the most arginine (5.01%), whereas canola meal has the least (2.42%).

Arginine tends to be low in the milk of most mammals including cows, humans, and pigs (Wu et al., 2009). However, stage of lactation may influence milk arginine content. In a comparative study, arginine was 38% lower in milk than colostrum in humans, but 29% higher in milk than colostrum in the horse (Davis et al., 1994). Compared to milk from the cow and sheep (34 mg/L), llama (36 mg/L), pig (44 mg/L) or the elephant (48 mg/L), arginine seems to be the highest in milk from the horse (60 mg/L) and the cat (64 mg/L). This suggests that neonates of some species may have a higher requirement for arginine than others, perhaps due to reduced or delayed ability to synthesize arginine endogenously.

Role of Arginine in the Body

Arginine is one of the most versatile amino acids as it serves as a precursor for the synthesis of protein, NO, urea, polyamines, proline, glutamate, creatine, and agmatine (Guoyao and Morris, 1998). Furthermore, arginine has been shown to stimulate the secretion of different hormones, such as insulin, growth hormone, glucagon and prolactin. Thus, arginine availability might affect the homeostasis of different biochemical pathways in the horse and other mammals due to its important role in the body.

Arginine has several metabolic functions including involvement in the transport, processing and excretion of nitrogen, and urea synthesis in the liver (McConell, 2007). Arginine is critical to the detoxification of ammonia, which is highly toxic to the central nervous system (Wu et al., 2009). In addition, experimental and clinical trials have produced evidence that arginine regulates interorgan metabolism of energy substrates

and the function of multiple organs, and is nutritionally essential for spermatogenesis, embryonic survival, fetal and neonatal growth, as well as for the maintenance of vascular tone and hemodynamics. More recent studies provide evidence that dietary supplementation or intravenous administration of arginine improves reproductive, cardiovascular, pulmonary, renal, gastrointestinal, liver, and immune functions, as well as facilitates wound healing, enhances insulin sensitivity, and maintains tissue integrity.

Arginine Metabolism

A prominent feature of arginine metabolism is the complex differential expression of relevant enzymes within the major organs (Wiesinger, 2001). In essence, there is not a single organ or cell type that expresses all the enzymes involved in arginine metabolism; thus, many of the various aspects of arginine synthesis and degradation occur in site-specific tissues. Subcellular compartmentalization and expression of various isoenzymes further complicates arginine metabolism.

Specific aspects of arginine metabolism are discussed in the following sections. It is worth noting that most studies on arginine metabolism have been conducted in humans, pigs, sheep, and rodents. While arginine metabolism is presumably similar in horses, further research is needed in this species.

Endogenous Synthesis of Arginine

In mature animals, the synthesis of endogenous arginine involves the intestinal-renal axis (Wu and Morris, 1998) (Figure 2-1A). The process begins in the small intestine, with the conversion of glutamine, glutamate and proline into citrulline in the mitochondria of enterocytes (Wu et al., 2009). Citrulline is then released from the small intestine into circulation and taken up primarily by the kidneys. Within the kidneys,

citrulline is converted into arginine and the arginine is released back into circulation (Guoyao and Morris, 1998; Sidney, 2004).

Biosynthesis of arginine differs between mature animals and the neonate (Figure 2-1B). Glutamine serves as the main precursor for citrulline formation in the mitochondria of enterocytes in adults. Adults release citrulline into the circulation so it can be taken up by the kidney to be converted to arginine, which is different from the neonate where citrulline is converted into arginine locally in the cytosol of the enterocytes (Wu et al., 2007b). Studies in neonatal rats have shown that the enzymes responsible for metabolizing citrulline to arginine are located only in the upper part of the small intestinal villi, whereas the enzymes involved in the synthesis of citrulline from glutamine are concentrated within the crypts of the intestine (Marini et al., 2010a). The absence of arginase (an arginine-degrading enzyme) in neonatal enterocytes allows maximal output of arginine from the small intestine into the portal circulation, as was demonstrated in pre-weaning pigs (Wu et al., 2007b). These metabolic strategies are thought to help maximize the supply of arginine from the mother to fetus and from maternal milk.

Independent of age, the small intestine is essential for arginine uptake and/or biosynthesis. Citrulline derived from glutamine, glutamate and proline in the gut is equally effective as dietary arginine as a source of arginine for the body (Wu et al., 2007b). Glutamine and proline metabolism are interconnected via glutamate and L- Δ^1 -pyrroline-5 carboxylate synthase (P5CS), which is a nexus between the tricarboxylic acid and urea cycles (Bertolo and Burrin., 2008). Because of these pathways, both amino acids serve as dietary precursors for arginine and urea synthesis. The metabolic

pathway for arginine synthesis in mammals is via P5CS and proline oxidase (Wu and Morris, 1998). Some of the enzymes in this pathway are present in a variety of cell types, while others are highly restricted. Phosphate-dependent glutaminase ornithine aminotransferase (OAT), argininosuccinate synthase (ASS), argininosuccinate lyase (ASL) and aspartate aminotransferase are widely distributed in animal tissues. Whereas, carbamoyl-phosphate synthase 1 (CPS 1), ornithine carbamoyltransferase (OCT), and *N*-acetylglutamate synthase (NAGS) are restricted to the liver and intestinal mucosa. Proline oxidase is mainly in the small intestine, liver, and kidneys, but PCS is located almost exclusively in the intestinal mucosa, with only trace amounts in other tissues. Enteric synthesis of citrulline from the amino acid precursors is catalyzed by P5CS and NAGS in the small intestine (Wu et al., 2009). Proline oxidase and OAT allow proline to serve as a precursor for citrulline synthesis in the intestine (Bertolo and Burrin., 2008). As a result of citrulline synthesis in the intestine, citrulline has been used as a biomarker of intestinal failure in infants and adults (Rhoads et al., 2005; Crenn et al., 2008). Interestingly, chickens, cats, and ferrets cannot produce citrulline from glutamine and glutamate due to a lack of P5C synthase in enterocytes (Wu and Morris, 1998). The enzyme NAGS is important in regulating citrulline production in enterocytes as it is an allosteric activator of both P5CS and CSP 1 (Wu et al., 2004). Thus, when dietary levels of arginine are high, intestinal synthesis of citrulline from glutamine and glutamate may be inhibited, thereby sparing these amino acids for use in other metabolic pathways (Wu et al., 2009).

Synthesis of arginine in the liver depends on the presence of OCT, which together with CPS 1 is located in the mitochondrial matrix of the liver (Wiesinger, 2001; Wraith,

2001). However, expression of both OCT and CPS 1 enzymes are restricted to the periportal hepatocytes in liver, the epithelial cells in the mucosa of the small intestine and to a smaller extent, the colonocytes of the large intestine (Wiesinger, 2001).

Citrulline released from the small intestine has not been shown to be extracted by the liver to any great extent, and is instead utilized for arginine synthesis by extrahepatic tissues (Wu et al., 2007b). Curis and coworkers (2005) explain that the main reason for citrulline metabolism split between the intestine and kidney is related to the efficacy of the capture of arginine by the liver. Without metabolic adaptation, all of the arginine from dietary supply would be withdrawn from the portal blood by the liver, leaving very low amounts of available arginine for other organs. Curis and coworkers (2005) further explain that as arginine is a positive regulator of ureagenesis, other amino acids could be inappropriately degraded. Citrulline is considered nature's solution to this problem as it acts as a masked form of arginine that essentially bypasses the liver. Citrulline is then converted to arginine by the kidney and released into the blood to make it available for the whole body.

It was found that nearly 100% of the citrulline and 90% of the arginine derived from the gut, bypass the liver in pigs (Wu et al., 2007c). Citrulline is readily converted into arginine in nearly all cell types, including adipocytes, endothelial cells, enterocytes, macrophages, neurons, and myocytes (Wu and Morris, 1998). The kidney has perhaps the highest rate of arginine synthesis from endogenous and exogenous citrulline compared to other tissues. Studies with macrophages and endothelial cells demonstrated that citrulline is transported into the cells by the N system, which is selective for amino acids with a side-chain amide group (e.g., glutamine and

asparagine) (Wu et al., 2009). Once inside the cell, the only pathway for citrulline utilization is through the conversion of citrulline to arginine via ASS and ASL.

Arginine and the Urea Cycle

The urea cycle is the first biochemical cycle to be described for the body's route of disposal of surplus nitrogen, thus providing a means of detoxification of ammonia (Wiesinger, 2001) (Figure 2-2). The liver is the main organ that has a full complement of enzymes necessary to convert ammonia and aspartate to urea (Walker, 2009); however, there is evidence for a low level of urea cycle activity in enterocytes of post-weaning pigs (Wu, 1995). The substrates in the urea cycle are ammonia, bicarbonate and aspartate (Walker, 2009). With each turn of the cycle, two atoms of nitrogen (N) are converted to urea.

N-acetylglutamate is synthesized from glutamate and acetyl Co-enzyme A by the hepatic mitochondrial enzyme NAGS (Mew and Caldovic, 2011). Carbamyl phosphate from NH_4^+ , ATP, and bicarbonate help to form CPS 1 in the mitochondrial matrix (Jackson et al., 1986). The initial step in the urea cycle is localized in the mitochondria where CPS 1 catalyzes a reaction with NAGS as an allosteric activator (Morris, 2002). The next step in the mitochondrial matrix is when OCT catalyzes the synthesis of citrulline from ornithine and carbamoyl phosphate, and then citrulline is shuttled into the cytosol (Jackson et al., 1986). The mitochondrial aspartate-glutamate carrier (AGC1; citrin) is an important isoform that catalyzes an exchange between intra-mitochondrial aspartate and cytosolic glutamate (Napolioni et al., 2011). The AGC1 is exclusively mitochondrial (Ramos et al., 2004), and is a component of the malate-aspartate shuttle transferring glutamate and aspartate between the cytosol and mitochondrial matrix in order to proceed with the urea cycle (Napolioni et al., 2011). Next ASS catalyzes the

condensation of aspartate and citrulline in the cytoplasm to form argininosuccinate with the concomitant hydrolysis of ATP to AMP and PP_i (Jackson et al., 1986). This step in the cytoplasm is followed by argininosuccinate being converted to arginine and fumaric acid by the enzyme ASL. Also in the cytoplasm, the next step is when arginase catalyzes the cleavage of arginine to ornithine and urea. Ornithine is then transferred back into the mitochondria via the mitochondrial ornithine transporter, which has specificity of only the L-forms of ornithine (Monné et al., 2012). Ornithine is then used again to condense with OCT to form citrulline.

There is a common misconception that arginine is formed in the liver and released into circulation. Although arginine can be synthesized in the liver, there is no net synthesis of arginine via the hepatic urea cycle (Wu and Morris, 1998). This is because the liver contains high levels of the enzyme arginase, which hydrolyzes arginine into urea and ornithine. It is the input of ornithine, the ability to convert ornithine into citrulline, and the catabolism of arginine (mainly by arginase and NOS) that determine the role of an organ or cell as an arginine producer or consumer (Wiesinger, 2001).

Arginine Degradation

Arginine uptake by cells involves the system y⁺ (a high-affinity, Na⁺-independent transporter) and Na-dependent transporters in a cell-specific manner (Wu et al., 2009). Once arginine is inside the cell, there are multiple pathways for arginine degradation to produce NO, ornithine, urea, polyamines, glutamine, creatine, and/or agmatine (Wu and Morris, 1998). Many of these byproducts have biological importance. Arginine degradation is initiated by either arginase I or II, the three isoforms of NOS, arginine:glycine amidinotransferase, and arginine decarboxylase (Wu et al., 2009). There are two distinct isoforms of mammalian arginase (arginase I and II), which are

encoded by different genes and differ in molecular and immunological properties, tissue distribution, subcellular location, and regulation of expression (Wu and Morris, 1998). Arginase I (a cytosolic enzyme) is highly expressed in the liver and to a much lesser extent in a few other cell types, whereas expression of arginase II (a mitochondrial enzyme) is widespread (Wu et al., 1997; Morris, 2002). Arginine turns over rapidly in mammals, with a half-life in circulation of 1.06, 0.75, and 0.65 h for adult, pregnant, and neonatal pigs, respectively (Wu et al., 2007a). In short, arginine degradation in mammals involves multiple organs and complex compartmentalization at cellular and systemic levels (Wu and Morris, 1998). The understanding of arginine catabolism is still limited due to its complexity.

Regulation of Arginine Metabolism

Arginine metabolism is regulated by multiple factors that include nutrients such as lysine, manganese, and omega-3 fatty acids; hormones such as glucocorticoids, growth hormone, leptin; cytokines; endotoxins; and endogenously generated substances such as creatine, lactate ornithine, P5CS, and methylarginines (Wu et al., 2009).

Lysine competes with arginine for entry into cells and also inhibits arginase activity (Wu and Morris, 1998). Lysine was also shown to reduce insulin-stimulated NO production by inhibiting arginine transport (Kohlhaas et al., 2011). Therefore, the dietary arginine:lysine ratio is a critical factor to consider when supplementing with arginine (Wu et al., 2009). Based on data in pigs, the total amount of arginine in the diet should not exceed 150% than that of lysine (Wu et al., 2009).

Glucocorticoids play a major role in upregulating arginine degradation via the arginase pathway in many cell types, particularly hepatocytes and enterocytes (Wu et al., 2009). These hormones also inhibit NO generation by suppressing NOS expression.

Cytokines such as interleukin 4 and interferon- γ , other inflammatory stimuli such as lipopolysaccharides, and cyclic adenosine monophosphate (cAMP) can greatly stimulate the expression of arginase I, arginase II, and ornithine decarboxylase in many cell types. As a result, concentrations of arginine in plasma are reduced markedly in response to infection or inflammation.

Castillo and colleagues (1993) studied the kinetics of arginine metabolism in healthy adult young men and established that arginine homeostasis is achieved by coupling the net rate of arginine degradation to the intake of arginine. Deusdelia and colleagues (2002) reported that whole body arginine catabolism is decreased in arginine-deprived adult mice. Inadequate intake of dietary arginine has been associated with several health issues, such as impaired spermatogenesis, alterations in the urea cycle causing orotic aciduria and transitory hyperammonemia, and reductions in tissue and circulating levels of arginine and ornithine (Castillo et al., 1993; Deusdelia et al., 2002)

Citrulline

Citrulline is a nonprotein amino acid, and therefore is not commonly found in food (Curtis et al., 2007). As a result, there is little to no citrulline present in the diet of most mammals, with the exception of watermelon. In fact, citrulline received its name from the Latin word for watermelon, *Citrullus vulgaris*, which contains large amounts of this amino acid (Curis et al., 2005). Interestingly, red flesh watermelons have slightly less citrulline than yellow or orange flesh watermelons (7.4, 28.5, and 14.2 mg/g DM, respectively) (Rimando and Perkins-Veazie, 2005). In addition, the rind contained more citrulline than flesh of the watermelon (24.6 and 16.7 mg/g DM, respectively) (Rimando and Perkins-Veazie, 2005). It has been proven that plasma concentration of arginine can be increased through intake of citrulline from watermelon (780 g watermelon juice

per d for 3 wk) (Collins et al., 2007). Remarkably, citrulline is one of the most potent scavengers of hydroxyl radical, and the watermelon accumulates citrulline simply as the plant has no other way to allow the specific decomposition of the free radical (Moinard and Cynober, 2007). Therefore, unless watermelon is a part of the diet, citrulline found in the body is almost entirely of endogenous origin (Marini et al., 2010a).

Role of Citrulline in the Body

Citrulline's primary role is as precursor for endogenous arginine synthesis in the small intestine, particularly as part of the urea cycle (Curis et al., 2005). Pioneering studies have shown that citrulline is the end product of intestinal glutamine metabolism, and accounts for 27.6% of the metabolized glutamine nitrogen (Windmueller and Spaeth, 1981). Citrulline used in a very important reaction with aspartate as nucleophilic compound; the reaction produces argininosuccinate and constitutes a step in the urea cycle (Curis et al., 2005). Citrulline can form peptide bonds; hence it can therefore be present in proteins. However, there is no known codon in the genetic table for this amino acid and thus must result from post-transcriptional modification of the protein. Furthermore, the supplementation of citrulline can lead to a dramatic improvement of nitrogen balance and protein status (Moinard and Cynober, 2007).

Despite citrulline's fairly limited role in the body, it has been studied for its potential health benefits. Citrulline at 3 g/kg d has been shown to accelerate the clearance of ammonium and lactate from plasma, possibly contributing to the improvement of muscle function during exercise (Giannesini et al., 2011). Additionally, citrulline supplementation (6 g/d) in young, normotensive men resulted in reduced brachial and aortic blood pressure (Figuroa et al., 2010). Citrulline was also fed to malnourished rats (5 g/kg d) as a means to increase whole body nitrogen status after dietary restriction, and was

reported to increase protein synthesis and protein content in skeletal muscle (Osowska et al., 2006).

Citrulline Biosynthesis

Citrulline was originally thought to be mainly produced from glutamine in the small intestine. More recent research in mice and other species has indicated that arginine and ornithine likely serve as the main precursors for citrulline synthesis in the small intestine (Marini et al., 2010b; Marini, 2012). Marini and coworkers (2010a) concluded that dietary glutamine is a poor carbon skeleton precursor for the synthesis of citrulline; however, this is still under investigation. Additional sources of endogenous citrulline include turnover of citrullinated proteins and the action of NOS on arginine and dimethylarginine dimethylaminohydrolase on dimethylarginine.

The metabolism of free citrulline can be classified into three pathways (Curis et al., 2005). The first metabolic pathway is arginine biosynthesis, which involves citrulline exchange at the whole body level. The second pathway is the NO cycle, which can involve local recycling of citrulline. The third pathway is the complete urea cycle, taking place in the liver. The use of citrulline for arginine biosynthesis was discussed previously in the section on arginine metabolism. The other metabolic fates of citrulline are discussed in more detail below.

Citrulline is produced when arginine is acted upon by any of the NOS enzymes, a reaction that occurs in all tissues. Within the NOS family, there are three different enzymes that differ in their level of expression within different tissues (Curis et al., 2005). The naming of the NOS enzymes is based on where these enzymes are most prevalent; where nNOS is mainly present in neural cells, iNOS in macrophages and eNOS in endothelial cells (Curis et al., 2005). All these enzymes share a common

outcome, whereby citrulline is generated from arginine with the release of NO. The reaction also requires nicotinamide adenine dinucleotide phosphate (NADP⁺), flavin mono-nucleotide and biopterin as cofactors (Meulemans, 2000).

There is currently no known citrulline-specific transporter for cellular uptake of citrulline (Curis et al., 2005). However, various types of cells appear able to take up or release citrulline, with several studies demonstrating that citrulline can be transported by common, generic amino acid transporters. The nervous system may employ a citrulline-specific transport, but the mechanism of citrulline extraction by neural cells has not yet been fully elucidated. The uptake of citrulline by endothelial aortic cells has been suggested to have a different transport system than the γ^+ system used for arginine, since this system does not carry citrulline. In rat aortic smooth muscle cells, citrulline transport appears to be partially Na⁺-dependent and pH-insensitive. In macrophages, there are two transport systems; one is a saturable system for neutral amino acid transport, while the other presents a competitive inhibition of arginine transport by citrulline. The uptake of citrulline by enterocytes appears to require sodium. A citrulline carrier in the kidney, where a large proportion of citrulline is converted to arginine, has yet to be identified.

Citrulline degradation is catalyzed via the urea cycle and within the small intestine by ASS and ASL in a reversible ATP-dependent condensation of citrulline with aspartate to form argininosuccinate (Husson et al., 2003). However, ASS and ASL are in low concentrations within the intestine (Osowska et al., 2004) Argininosuccinate is the immediate precursor of arginine leading to the production of urea in the liver and that of NO in many other cells (Husson et al., 2003). The ASS enzyme is almost ubiquitous to

all cells, with high levels found in the liver and kidney and the lowest levels found in the intestine in adults (Curis et al., 2005). The expression of ASS can be limited to certain cell subpopulations or certain regions (Husson et al., 2003).

The effects of citrulline loading on anabolic hormones were determined in an interesting study, which found that renal arginine synthesis become saturated with high dosages of citrulline. Eight fasting males underwent four separate oral loading tests (2, 5, 10, or 15 g CIT) in random order (Moinard et al., 2008). Blood was drawn 10 times over an 8 h period. Plasma insulin in this study was not affected. By comparison, citrulline seemed to be cleared extremely rapidly from plasma. The pharmacokinetic parameters suggest that saturation of CIT begins to occur at a load of 15 g, which was confirmed by the increase in urinary arginine excretion and the decrease in both citrulline retention percentage and fractional reabsorption rate.

Citrulline-Malate

Malate is an intermediate of the tricarboxylic acid cycle (TCA) and its supplementation has been shown to enhance energy production (Giannesini et al., 2009). Recently, the combination of citrulline and malate (i.e., citrulline-malate) has been promoted as a performance-enhancing supplement. Results from a limited number of studies in humans and rats have indicated that citrulline-malate supplementation improved muscle performance by reducing muscle fatigability and weakness (Giannesini et al., 2009, 2011). It has also been shown that oxidative stress was reversed and the antioxidative defense system strengthened by dietary supplementation with L-malate (Wu et al., 2008).

Arginine and Citrulline Supplementation and Insulin Resistance

Arginine and Metabolic Disease

Obesity in humans and animals occurs because of a chronic imbalance between energy intake and expenditure (Wu et al., 2009). Not only is obesity a crisis worldwide in humans, but also becoming more prevalent in the equine industry. Growing evidence in humans indicates that arginine supplementation may be a novel therapy for obesity and metabolic syndrome. Dietary supplementation of arginine decreased plasma levels of glucose, homocysteine, fatty acids, and triglycerides, and improved insulin sensitivity in chemically-induced diabetic rats (Kohli et al., 2004). Kohli and coworkers (2004) found that daily oral administration of L-arginine-hydrochloride (HCl) (1.51 %) or alanine (isonitrogenous control, 2.55%) added to drinking water for nondiabetic rats and arginine (0.43%) and alanine (0.73%) in the drinking water for diabetic rats (which consumed more water) for 14 d reduced plasma glucose concentration and resulted in the loss of BW in streptozotocin (STZ)-induced diabetic rats, regardless of food intake. Oral administration of L-arginine-HCl (0.62 g/kg BW) to diabetic hamsters (Popov et al., 2002) or intraperitoneal administration of arginine (10 μ mol) to rats with alloxan-induced diabetes (Mendez and Balderas, 2001), also reduced plasma glucose levels by 65%. Kohli and coworkers (2004) also found that arginine supplemented to nondiabetic and diabetic rats increased plasma concentrations of insulin in both groups compared to isonitrogenous supplementation with alanine. The underlying mechanisms for the decrease in plasma glucose and BW in diabetic rats are unknown, but may involve an increase in insulin release. Arginine has been shown to stimulate the secretion of insulin by pancreatic β -cells (Guoyao and Morris, 1998; Adeghate et al., 2001; Flynn et al., 2002; Kohlhaas et al., 2011).

Interestingly, both diabetic rats (Bronsnan et al., 1983; Pieper and Dondlinger, 1997) and humans (Grill et al., 1992) have markedly decreased concentrations of plasma arginine. One of the hallmarks of diabetes mellitus is endothelial dysfunction, which may result from a deficiency of NO (Kohli et al., 2004). Kohli and coworkers (2004) found that the intracellular concentration of arginine (0.48 mmol/L) in diabetic rats was substantially lower than in nondiabetic rats. This finding suggests that endothelial NO synthase was saturated with intracellular arginine in both diabetic and nondiabetic rats and that arginine was not a limiting substrate for endothelial NO synthesis. Kohli and coworkers (2004) did find that dietary arginine supplementation markedly increased *in vitro* NO production in the coronary endothelial cells of both diabetic and nondiabetic rats. Further findings were that arginine treatment normalized endothelial NO synthesis in STZ-diabetic rats to the values of nondiabetic rats. Therefore, these results support that intracellular or extracellular arginine concentrations are indeed critical for endothelial NO production (Wu and Meininger, 2002)

Endothelial cells synthesize endothelium-derived relaxing factor (EDRF) from L-arginine (Pollock et al., 1991). The pharmacological and biochemical properties of EDRF are mimicked by NO or NO-containing compounds, which activate soluble guanylyl cyclase to increase the second messenger of NO, cyclic guanosine monophosphate (cGMP). In turn, cGMP causes relaxation of vascular smooth muscle. Both clinical and experimental studies have shown beneficial effects of arginine administration improving vascular function in diabetic subjects. Intravenous arginine, the precursor of NO, was measured in 10 male type 1 diabetic patients and 10 nondiabetic patients and was found to increase cGMP and citrulline and decreased blood pressure

in both (Smulders et al., 1994). A more recent crossover clinical trial on 6 patients with type 2 diabetes mellitus and mild hypertension were given 3 g of arginine per hour, for 10 hours on either d 2 or d 3 of the three-day trial (Huynh and Tayek, 2002). Results from this study showed an increase in plasma citrulline, which may reflect an increase in the conversion of arginine into NO and citrulline. Systolic blood pressure was reduced from 135 ± 7 to 123 ± 8 mmHg. These data suggest that oral arginine may increase endothelial NOS to increase vascular NO to reduce blood pressure. Serum insulin, mean blood glucose, cortisol, epinephrine, norepinephrine, glucagon, growth hormone (GH), or insulin-like growth factor 1 (IGF-1) concentrations were not affected by arginine supplementation in this study.

The supplementation of citrulline or arginine has shown positive treatment effects in overweight subjects. The supplementation of arginine (2.0% in drinking water) retarded the progression of atherosclerosis induced by a high fat diet in obese rabbits and improved NO-dependent vasodilator function (Böger et al., 1997). In rabbits fed a high-cholesterol diet, endothelium-dependent vasorelaxation in isolated thoracic aorta and blood flow of the ear artery *in vivo* were impaired (Hayashi et al., 2005). Rabbits administered arginine or citrulline, alone or in combination with antioxidants, improved endothelium-dependent vasorelaxation and blood flow, with the most notable increase in the ingestion of arginine, citrulline and antioxidants.

Supplementing either 0% or 1.0% arginine into conventional diets for growing-finishing pigs for 46 days reduced body fat accretion, enhanced muscle gain, and improved the metabolic profile (He et al., 2009). He and coworkers (2009) also found concentrations of low density lipoprotein (LDL), VLDL, and urea were lower in the

arginine supplemented pigs and concentrations of lipid signaling molecules were reduced. These findings suggest that dietary arginine supplementation alters catabolism of fat and amino acids in the whole body. Another study in pigs found similar results with the same level (1.0% of DM) of arginine supplementation (Tan et al., 2009) were arginine was shown to beneficially promote muscle gain and reduce body fat accretion in growing-finishing pigs. These findings suggest arginine may be a potential therapy to treat obesity.

A distinct advantage of arginine or citrulline over drugs is that dietary arginine supplementation reduces adiposity, while improving insulin sensitivity. Fu and coworkers (2005) found that arginine-HCl (1.51%) increased serum concentrations of arginine and NO were in arginine supplemented rats compared to control rats. Body weight was 6, 10, and 16% lower at wk 4, 7, and 10 in arginine supplemented rats in comparison to control rats. Arginine reduced abdominal and epididymal adipose tissues (45 and 25%, respectively) when compared to control rats. Arginine treatment enhanced NO production (71 to 85%), lipolysis (22 to 24%), and oxidation of glucose (34 to 36%). However, arginine did not increase serum levels of insulin or growth hormone in the ZDF-rats, which is in contrast to results observed for STZ-induced diabetic rats (Kohli et al., 2004). These results indicate that the response of ZDF rats to dietary manipulation likely depends on target tissues of individual nutrients. The supplementation of watermelon pomace juice was fed as it is high in arginine and citrulline, to ameliorate the metabolic syndrome in ZDF rats (Wu et al., 2007c). In this study, the drinking water contained 0% or 0.24% arginine-HCl, 63% watermelon pomace juice, 0.01% lycopene, or 0.05% citrus pectin for 4 weeks. The diets with arginine or watermelon pomace juice

increased serum concentrations of arginine, reduced fat accretion, lowered serum concentrations of glucose, free fatty acids, and enhanced vascular relaxation. However, similar to Fu and coworkers (2005), serum concentrations of insulin and growth hormone did not differ between the groups of rats in this study (Wu et al., 2007c). Jobgen and coworkers (2009) tested the effectiveness of arginine in diet-induced obesity. In this study 4 wk old male Sprague-Dawley rats were fed a high fat (40% energy) or a low-fat (10% energy) diet for 15 wk resulting in an 18% higher BW gain and 74% greater weight of major white fat pads (retroperitoneal, epididymal, subcutaneous, and mesenteric adipose tissue). At 19 wk of age rats in each dietary group were supplemented for 12 wk with 1.51% arginine-HCl or 2.55% alanine (isonitrogenous control) in drinking water. Despite similar energy intake, weights of white fat pads increased by 98% in control rats over a 12 wk period, but only 35% in arginine supplemented rats. Arginine reduced relative weights of white fat pads by 30% and enhanced those of soleus muscle by 13%, extensor digitorum longus muscle by 11%, and brown fat by 34% compared with control rats. Arginine seems to regulate the repartitioning of dietary energy to favor muscle over fat gain in the body. Also in agreement with Fu et al. (2005) and Wu et al. (2007c), Jobgen and coworkers (2009) found no difference in the serum concentrations of insulin or growth hormone between arginine supplemented rats and controls, but did observe lower serum glucose in response to arginine supplementation. The possible underlying mechanisms for the effect of arginine may involve multiple NO-dependent pathways that favor whole-body oxidation of fatty acids and glucose (Wu et al., 2009).

Interestingly, there are contradicting effects of whether arginine is actually the cause of increased insulin secretion. Studies suggest that arginine increased serum insulin concentrations (Guoyao and Morris, 1998; Adeghate et al., 2001; Flynn et al., 2002; Kohlhaas et al., 2011), and other studies have found no increase in serum insulin concentrations with arginine supplementation (Huynh and Tayek, 2002; Fu et al., 2005; Wu et al., 2007c; Jobgen et al., 2009).

The studies discussed suggest that arginine or citrulline supplementation may provide novel and effective target therapy for obesity, diabetes, and metabolic syndrome. The beneficial effect of arginine in treating many developmental and health problems is unique among amino acids. Arginine has been previously reported and thought to increase insulin secretion (Guoyao and Morris, 1998), possibly through the increase of NO, as NO is known to stimulate the release of anabolic hormones such as insulin (Jobgen et al., 2006).

Arginine Mechanisms that Improve Insulin Sensitivity

Arginine has been shown to increase insulin secretion from pancreatic β -cells and improve insulin sensitivity in tissues via enhanced production of NO (Calver et al., 1992). In another study, 10 normal subjects underwent euglycemic-hyperinsulinemic clamp procedures after administration of arginine (3 g consumed three times/d) for 1 month, which was considered the lowest dosage possible in order to create endothelial effects without changing insulin secretion (Piatti et al., 2001). This study found an increased NO availability induced by arginine, which resulted from an increase in cGMP. However, even if arginine treatment normalized NO activity, it was not able to completely overcome the defect of insulin sensitivity in type 2 diabetic patients. This suggests that insulin resistance is multifactorial. The mechanisms of the insulinotropic

action of arginine and of the glucose/arginine interaction are largely unknown (Pueyo et al., 1994). It has been suggested that the accumulation of arginine, a positively charged molecule, inside β -cells leads to depolarization of the plasma membrane and eventually to insulin secretion as purely biophysical effect (Blachier et al., 1989). Arginine has been shown to depolarize the plasma membrane in a way which is potentiated by glucose (Hermans et al., 1987), and to stimulate Ca^{2+} influx through voltage-sensitive Ca^{2+} channels (Hermans et al., 1987; Smith et al., 1997; Weinhaus et al., 1997). Other data have questioned membrane depolarization as the sole mechanism in arginine stimulation of insulin secretion from pancreatic β -cells showing in rat islets that insulin secretion persists by depolarized high K^+ (Blachier et al., 1989), suggesting that arginine in addition to stimulation of Ca^{2+} influx may stimulate insulin secretion by other mechanisms (Blachier et al., 1989; Sener et al., 1990).

Kohli and coworkers (2004) suggested they increased insulin sensitivity with supplementation of arginine through increased NO production since they found increased plasma concentrations of insulin in rats fed arginine. A possible mechanism for arginine's effect on insulin sensitivity is through selective overexpression of GLUT-4 in adipose tissue, which enhances glucose uptake by adipocytes, thereby improving whole-body insulin sensitivity (Shepherd et al., 1993). Another possible mechanism for arginine to increase insulin sensitivity is a study that found that in the presence of arginine ($0.52 \text{ mg/kg}^{-1} \text{ min}^{-1}$), steady state plasma glucose was lowered significantly (Wascher et al., 1997). However, in this same study arginine did not elicit an increase in blood flow in response to insulin, suggesting that arginine does not stimulate intact vascular effects of insulin.

Advantage of Citrulline over Arginine Supplementation

Citrulline supplementation may be a better vehicle for increasing arginine delivery, as substantial amounts of orally administered arginine never enter the systemic circulation in adult humans, pigs and rats (Wu et al., 2009). Over 40% of dietary arginine is degraded by the small intestine in first pass metabolism (Wu et al., 2009). Studies have shown that oral supplementation of citrulline is more efficient than giving arginine orally (Curtis et al., 2007). Oral L- citrulline supplementation has proven to raise plasma L-arginine concentration and augment NO-dependent signaling in a dose-dependent manner in a cross-over study with 20 healthy volunteers (Schwedhelm et al., 2008). This randomized, double-blind, placebo-controlled cross-over study gave participants either 0.75 , 1.5 or 3 g of citrulline twice daily, 1.0 g of immediate-release arginine three times a day, or 1.6 g of sustained-release arginine twice daily and compared it to a placebo. The researchers found that citrulline given at 0.74 g increased plasma arginine to the same extent as 1.6 g of sustained-release arginine and 1.0 g immediate-release arginine. Both higher doses of citrulline elevated plasma arginine. This strongly suggests that oral citrulline is at least as efficient as arginine supplementation for increasing plasma arginine concentrations. Bendahan and coworkers (2002) and Sureda and coworkers (2009) found that 6 g/d of citrulline increased plasma arginine in men.

Supplementation of citrulline, rather than arginine may be a more efficient and safer way of increasing plasma and tissue levels of arginine in mature animals and such supplementation may be particularly appropriate in horses who are obese or have EMS. By comparison, arginine supplementation could cause a problem as arginine is taken up and metabolized by the liver to yield urea, which raises questions about the safety of

arginine supplementation (Curis et al., 2005). However, a study with artificially reared piglets fed arginine (0.2, or 0.4%) in milk replacer showed a decrease in plasma concentrations of ammonia (20 and 35%, respectively) and urea (19 and 33% respectively) (Kim et al., 2004). Unfortunately, eightfold the dose of arginine was fatal for a 21-month old girl, who died from cardiac arrest and myelinolysis (Garard and Luisiri, 1997). This may not be the same reaction in mature mammals. Arginine supplementation has also been associated with nausea and vomiting (Boyd and Olin, 1984) and abdominal cramping and bloating in humans (Kattwinkel et al., 1972). Arginine is liable to cause excessive urea production, since it acts as a catalyst for ureagenesis (Curis et al., 2005). This effect on urea production is not generally observed with citrulline supplementation. The effects of citrulline loading on anabolic hormones was determined in a study with eight fasting males who underwent 4 separate oral loading tests (2, 5, 10, or 15 g CIT) in random order (Moinard et al., 2008). Blood was drawn 10 times over an 8 h period. None of the volunteers suffered nausea or diarrhea or any other side effect with any dosage of citrulline.

Citrulline-Malate's Potential to Ameliorate Insulin Resistance

Although strong epidemiological data are not available, the prevalence of obesity in horse and pony populations seems to be on the rise. The “fat and happy” ideology of horse owners, where provision of food is equated with care and concern, regrettably is a very poor and unsafe choice for the horse. Further, improvements in plant breeding to support faster tissue gains in cattle have led to forages rich in NSC that are incompatible with the “thriftiness” of some equines. Ongoing research is investigating the link between obesity and the onset of glucose intolerance or insulin resistance in horses, similar to that observed with type 2 diabetes in humans. An insulin resistant

horse has fat deposits due to the body's inability to use glucose, and thus it inappropriately stores the extra glucose as fat. The high level of insulin secreted also suppresses fat metabolism and supports fat deposition (Frayn et al., 2004).

Arginine and/or citrulline may provide novel and effective therapies in the horse for health problems such as obesity, insulin resistance, and blood circulation issues. The effect of arginine and citrulline in treating different developmental and health related issues is unique among amino acids, which can offer great promise for the improved health and wellbeing of horses. Understanding the relationship between arginine and citrulline may lead to a more defined and scientifically based nutritional intervention for horses with EMS, insulin resistance, or laminitis issues. Most arginine research has been performed in mice, pigs and humans, with very little research being conducted in the horse. To the author's knowledge, citrulline supplementation has never been investigated in horses.

Citrulline-malate supplementation may be a useful means to manage horses with EMS or to mitigate insulin resistance and future bouts of laminitis. Therefore, citrulline-malate could be used as a potential therapeutic target to decrease the continuous secretion of insulin as it tries to compensate for a decrease in tissue cell effectiveness in those horses found to be insulin resistant and overweight. Simultaneously, citrulline-malate may induce a weight loss effect, since a lower level of insulin will not promote the same level of fat storage.

Although pharmacologic agents have been successfully used to suppress insulin secretion and elicit weight loss in overweight horses with EMS, there are usually side-effects associated with chronic drug administration. Arginine or citrulline

supplementation may offer a safer, less expensive alternative to drugs for suppressing insulin secretion and/or improving insulin sensitivity. Further, supplement-induced suppression of pancreatic β -cell activity in the horse may induce weight loss. However, the ability of arginine or citrulline to suppress insulin production in horses is unknown, and the approach must be cautious to avoid starving cells of glucose. It is possible that the careful regulation of insulin through the supplementation of citrulline will reduce the risk of horses becoming less sensitive to insulin due to the overproduction of insulin. Suppressing the pancreatic β -cell activity may not only induce weight loss but also keep the pancreatic β -cell cells from being exhausted.

The objectives of this study were to: 1) investigate citrulline-malate supplementation and its effects on the availability of other amino acids; 2) assess whether oral citrulline-malate will act as a dietary precursor for arginine; and 3) determine if oral citrulline-malate will alter glycemic and insulinemic responses to a starch-rich meal in healthy horses. We hypothesized that supplementation of citrulline-malate would increase arginine availability and consequently improve insulinemic response to a meal.

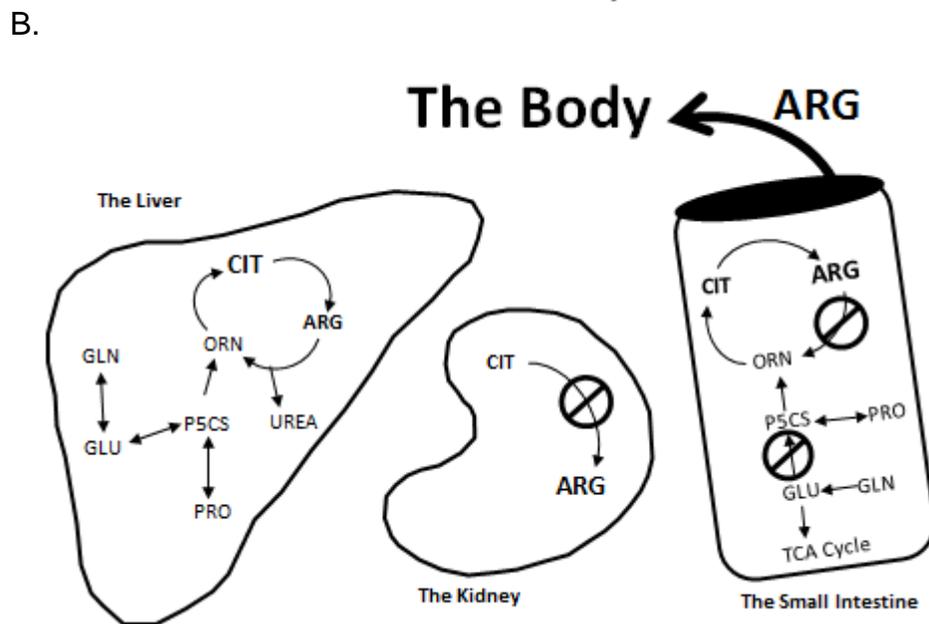
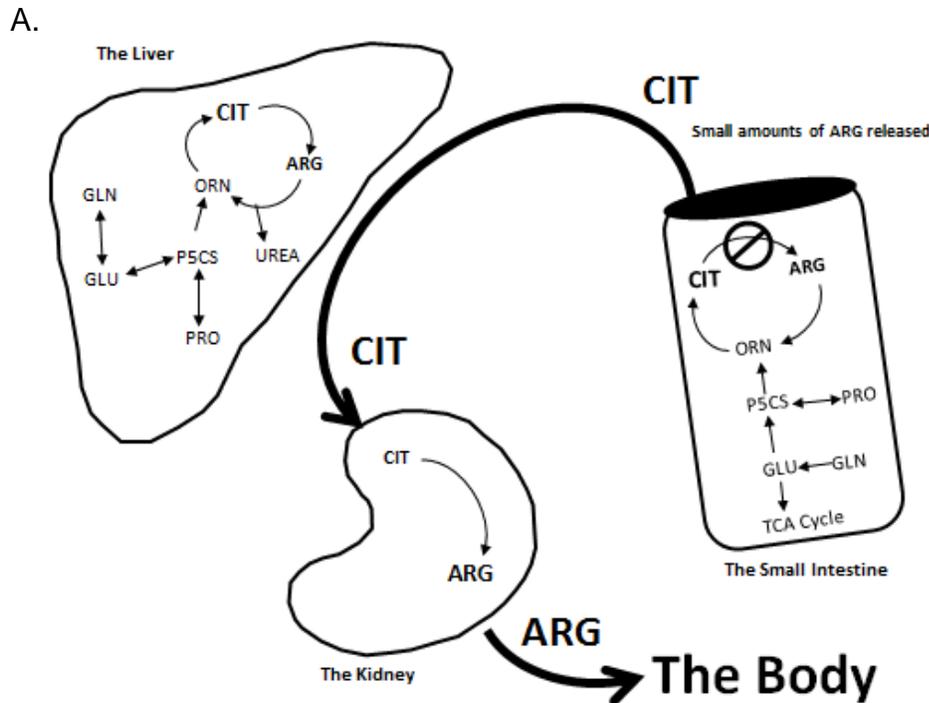


Figure 2-1. Arginine synthesis. A) In the adult citrulline is synthesized from glutamine (GLN), glutamate (GLU), proline (PRO) and arginine (ARG) in the small intestine and released to the portal vein where it bypasses the liver and is taken up by the kidney to be converted to arginine. B) In neonates arginine is synthesized predominantly from proline in the small intestine and released into the portal vein. The kidney of neonates is not capable of synthesizing arginine from citrulline (CIT) and thus relies on intestinal synthesis and supply. Other abbreviations: ornithine (ORN); pyrroline-5 carboxylate synthase (P5C); tricarboxylic acid cycle (TCA). (Adapted from Brosnan and Brosnan (2004) and Bertolo and Burrin (2008)).

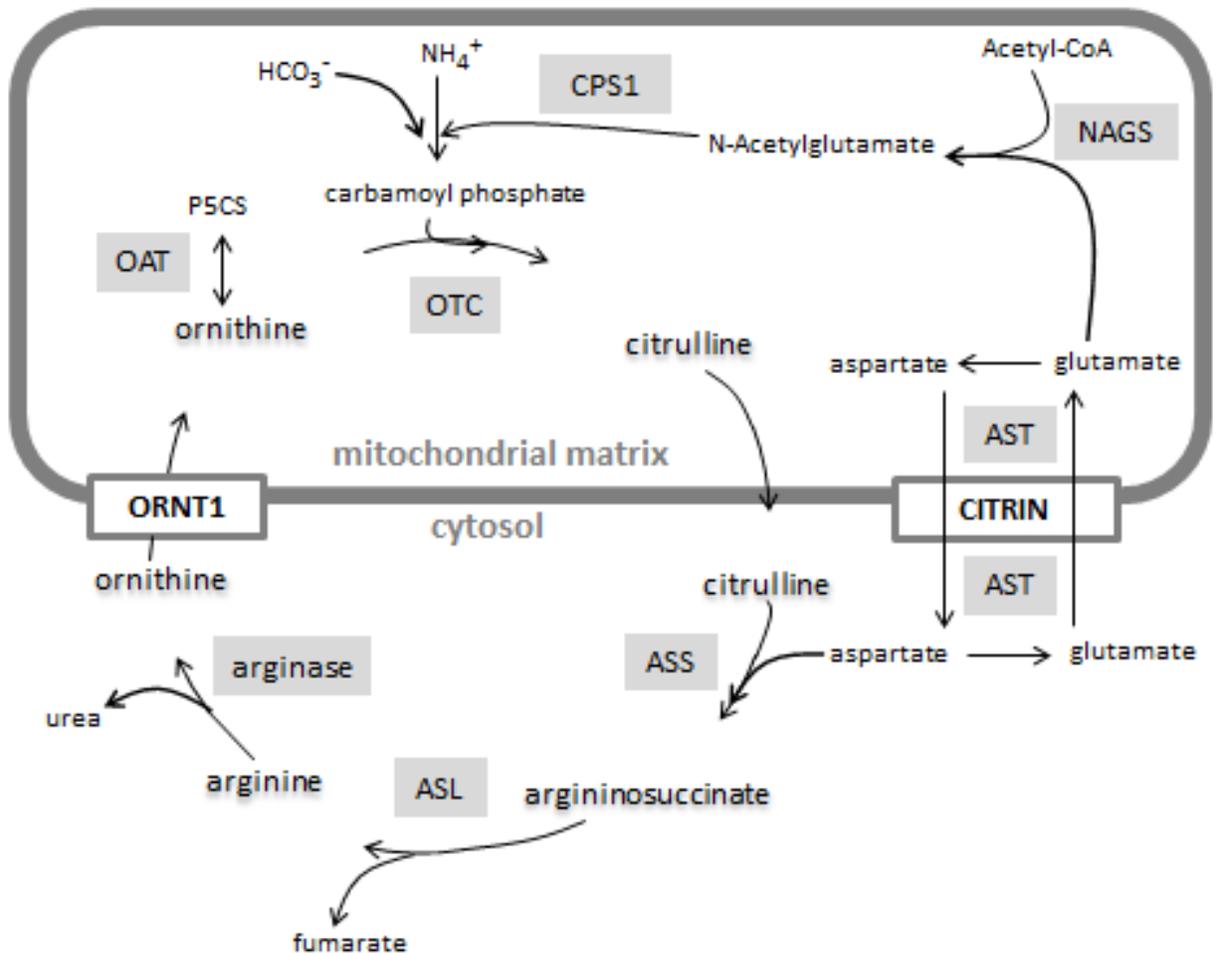


Figure 2-2. Metabolism of citrulline and arginine in the hepatic urea cycle and related pathways. Abbreviations: ASL, argininosuccinate lyase; ASS, argininosuccinate synthetase; CPS 1, carbamoylphosphate synthetase 1; OAT, ornithine δ -aminotransferase; ORNT1, the mitochondrial ornithine transporter (SLC25A15); OTC, ornithine transcarbamylase (also called ornithine carbamoyltransferase); P5C = Δ^1 -pyrroline 5-carboxylate synthase. (Adapted from Mandel et al. (2005) and Walker (2009)).

CHAPTER 3 MATERIALS AND METHODS

Horses

Twelve mature, non-gravid mares (6 Thoroughbreds and 6 Quarter Horses) with a mean \pm SE body weight (BW) of 552.0 ± 31.2 kg and age of 10.8 ± 2.5 y were utilized in this study. All horses had a BCS of 5 to 6 (Henneke et al., 1983). Horses were group-housed on a 4-ha pasture at the Institute of Food and Agricultural Sciences (IFAS) Equine Sciences Center (ESC) in Ocala, Florida. Pastures were equipped with outdoor, 3 m x 3 m pens that allowed individual feeding of concentrate and supplements once daily. Insulinemic response to a grain meal was performed with horses confined indoors in individual 3.7 m x 3.7 m stalls. All horses received routine healthcare, including vaccination, anthelmintic treatment and hoof care established in the standard operating procedures for the IFAS ESC. All animal protocols and procedures were reviewed and approved by the IFAS Animal Care and Use Committee at the University of Florida.

Dietary Treatments

After blocking for age and breed, horses were randomly assigned to one of two dietary treatments: urea supplementation (CON; n = 6) or citrulline-malate supplementation (CIT; n = 6). Citrulline-malate (99.1%; UniChem Enterprises, Inc., Neward, NJ) was supplemented at a rate of 86 mg/kg BW. The level of CIT supplementation was chosen based on studies in humans where 6 g CIT/d was shown to increase plasma arginine concentration (Bendahian et al., 2002; Sureda et al., 2009). Feed-grade urea was supplemented at a rate of 25 mg/kg BW and served as an isonitrogenous control. To facilitate ease of sample collection, horses were further divided into 3 groups of 4 horses, with 2 horses from each dietary treatment

represented in each group. Dietary treatments were initiated in one group of horses per day, over 3 consecutive days. As intended, this arrangement also staggered each data collection over a 3-d period. Horses received their respective dietary treatments for 14 days.

Horses were fed a basal diet consisting of *ad libitum* access to Coastal bermudagrass hay and a grain mix concentrate (Ocala Breeders' Feed and Supply, Ocala, FL) fed at 0.5% BW/d. The CIT and CON supplements were hand-mixed into the concentrate and fed individually once daily at 0730 h. Although horses were housed on pasture during the supplementation period, the study was conducted in February; thus, the majority of the pasture forage was dormant and intake was assumed to be minimal. Nutrient analysis of the hay and grain mix making up the basal ration is provided in Table 3-1.

Insulinemic Response to a Meal

Glycemic and insulinemic responses to a grain meal containing CIT or CON were evaluated on d 14 after an overnight fast. Horses were transferred from pasture housing to indoor, individual stalls on the afternoon of d 13 to facilitate fasting and sample collection. Body weights were obtained prior to the start of supplementation and on d 13 using a livestock scale accurate to ± 0.5 kg (MP800, Tru-test, Inc., Mineral Wells, TX). On d 14 at 0700 h, a 16 ga x 9 cm catheter (Abbocath-T, Hospira, Inc., Lake Forest, IL) was placed in the right jugular vein under local anesthesia using aseptic technique. At 0800 h, horses were fed 0.25% BW of the same grain mix concentrate used in their basal ration, along with the daily allotment of their respective dietary treatment. The time it took the horse to consume the meal was recorded, with the clock stopping when the entire meal was consumed. Blood samples were obtained 30 min (-0:30) and

immediately (0:00) before the meal was offered, and every 30 min for 5 h after the meal was consumed. Patency of catheters and extension tubing was maintained by flushing with heparinized (2,000 U/L) saline (0.9% NaCl) after each blood sample was collected. Blood was placed into tubes (Vacutainer, Becton Dickinson Co., Franklin Lakes, NJ) containing sodium heparin (for plasma amino acids), EDTA (for plasma glucose) or no anticoagulant (for serum insulin). Samples with anticoagulant were immediately placed on ice and were processed within 2 h of collection by centrifugation at 2000 g for 15 min at 4°C. Samples with no anticoagulant were kept at room temperature (approximately 20°C) for 5 h to permit clot formation prior to centrifugation. Serum and plasma were harvested and stored in polypropylene cryogenic vials in 1.0 mL aliquots at -80°C until analyses were performed.

Sample Analyses

Plasma Glucose

Plasma glucose concentration was determined using a commercially available kit (Cayman's Glucose Assay Kit, Cayman Chemical Company, Ann Arbor, MI). The kit determined glucose concentration based on an oxidase-peroxide reaction. Briefly, glucose was oxidized to δ -gluconolactone with flavin adenine dinucleotide (FAD)-dependent glucose oxidase. The reduced form of glucose oxidase, glucose oxidase-FADH₂, was converted back to glucose oxidase-FAD form by adding oxygen, yielding hydrogen peroxide. Finally, with horseradish peroxidase as a catalyst, hydrogen peroxide reacted with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminoantipyrine to generate a pink dye with an optimal absorption at 514 nm. Glucose concentration in plasma samples was determined colorimetrically using a BioTek PowerWave XS (BIO-TEK Instruments, Inc., Winooski, VT) plate reader at a wavelength

of 500 nm. Samples were analyzed in duplicate and compared to a set of glucose standards ranging from 0 to 250 mg/dL. Intra- and interassay coefficients of variation (CV) of pooled samples were 1.5 and 2.8%, respectively.

Glycemic response to a grain meal was evaluated as plasma glucose concentration over time, area under the curve (AUC), time to peak glucose, and peak glucose concentration.

Serum Insulin

Serum insulin was measured by radioimmunoassay using a commercially available kit (Coat-A-Count[®] Insulin, Siemens, Los Angeles, CA) previously validated for use in horses (McGowan et al., 2008). Each sample was analyzed in duplicate. Detection limits for the insulin assay was approximately 1.53 to 371 μ IU/L. Intra- and interassay CV of pooled samples were 1.6 and 11.4%, respectively.

Insulinemic response to a meal was evaluated as serum insulin concentration over time, AUC, time to peak insulin, and peak insulin concentration. In addition, proxies based on baseline (fasting) glucose and insulin concentrations were used for screening insulin sensitivity (SI) and pancreatic β -cell responsiveness. As described for horses by (Treiber et al., 2005b), the reciprocal of the insulin square root index (RISQI), calculated as $1/\sqrt{\text{insulin}}$ (with insulin as mU/L), was used to reflect the amount of insulin required to chronically maintain basal glucose homeostasis and served as a proxy for insulin sensitivity. The modified insulin response to glucose (MIRG), calculated as $(800 - 0.30[\text{insulin} - 50]^2) / \text{glucose} - 30$ (with insulin as mU/L and glucose as mg/dL), was used to estimate the capacity of pancreatic β -cells to increase insulin secretion and compensate for exogenous glucose (Treiber et al., 2005b). The homeostasis model assessment (HOMA) was used to derive an estimate of insulin sensitivity from the

mathematical modeling of fasting plasma glucose and insulin concentrations ($[\text{basal glucose (mg/dL)} - \text{insulin (mU/L)}] / 22.5$), where a higher HOMA indicates insulin resistance (Matthews et al., 1985). The glucose-to-insulin ratio was used to estimate the effect of insulin on glucose concentrations (or insulin sensitivity of peripheral tissues), as it is positively correlated with insulin sensitivity (Firshman and Valberg, 2007).

Plasma Amino Acids, Urea and Ammonia

Plasma citrulline and arginine in response to a grain meal with CIT or CON supplementation were of primary interest in this study, as well as amino acids that are linked with their metabolism, including glutamate, ornithine, and proline. To verify the effect of CIT or CON supplementation on amino acids most likely to be limiting in equine diets, plasma lysine, methionine, and threonine were also evaluated. Plasma urea and ammonia concentrations were simultaneously assessed with amino acid analysis.

Plasma samples were prepared for analysis by precipitating amino acids with 35% sulfosalicylic acid (SSA). Aminoethyl cysteine hydrochloride (AEC) was included in the SSA solution as an internal standard and used to calculate the efficiency of amino acid recovery. Samples were incubated at 4°C for 20 min followed by centrifugation at 11,000 g for 10 min at 4°C. Supernatant was harvested and filtered using a 0.22 µm syringe filter (Fisherbrand, MCE membrane, Fisher Scientific, Houston, TX), and then diluted with 0.02 N hydrochloric acid. Precipitates were then placed in 2-mL clear glass vials (Snap-It™, National Scientific, Rockwood, TN), capped and immediately analyzed.

Plasma amino acid concentrations were determined using classic ion-exchange separation followed by post-column derivatization with ninhydrin using an L-8900 Amino Acid Analyzer (Hitachi-High Technologies, Pleasanton, CA). The 60 mm X 4.6 mm (i.d.) chromatographic column consisted of polystyrene cross-linked by divinylbenzene, with

sulfone (SO_3^-) groups as active exchange sites and was equipped with a 40 mm X 4.6 mm (i.d.) guard column. Column temperature was 57°C and the reactor temperature was 135°C. While awaiting injection, sealed sample vials were maintained at 4 ± 2 °C in the autosampler rack. All buffers and the ninhydrin reagent were purchased from Hitachi High Technologies (Pleasanton, CA). Amino acid detection was by spectrophotometry at 570 and 440 nm with the ninhydrin reaction. Based on AEC concentrations, mean \pm SE recovery of amino acids was $111 \pm 3\%$.

Statistical Analyses

Differences in plasma glucose, amino acids, urea, and ammonia and serum insulin were analyzed using the PROC MIXED procedure in SAS with repeated measures (version 9.3, SAS Institute, Inc. Cary, NC). Dietary treatment, time, and time by treatment were included in the model as fixed effects and horse within treatment was included as a random variable. Based on Akaike's Information Criterion and Bayesian Information Criterion, heterogeneous autoregressive covariance structure was used for plasma glucose and serum insulin and the autoregressive covariance structure was used for all plasma amino acids. Type 3 fixed effects, least squared means, and differences of least squared means were used to determine statistical significance and the slice function of the LSMEANS statement was used as a means separation technique. Glucose and insulin data from one horse in the CON group was incomplete (missing at the 2.5 and 3 h time points) due to a labeling mistake during collection. Therefore, the Kenward-Rodger denominator degrees of freedom method (ddfm=kr) was used to evaluate the unbalanced data sets for unknown distributions (Kenward and Rodger, 1997; Gomez et al., 2005). Bartlett's test was performed with PROC GLM to determine heterogeneous variance in insulin concentrations and error variances were

greater for CON than CIT. Therefore, insulin concentrations were not normally distributed and were consequently normalized with logarithmic transformations in SAS ($\log[\text{INS}]$) prior to evaluation.

The trapezoidal rule was used to estimate the area under the plasma glucose and serum insulin level-time curve. The formula used for calculating AUC was $((m_2 + m_1) \cdot t_1) / 2 + ((m_3 + m_2) \cdot t_2) / 2 + ((m_4 + m_3) \cdot t_3) / 2 + ((m_5 + m_4) \cdot t_4) / 2 + ((m_6 + m_5) \cdot t_5) / 2 + ((m_7 + m_6) \cdot t_6) / 2 + ((m_8 + m_7) \cdot t_7) / 2 + ((m_9 + m_8) \cdot t_8) / 2 + ((m_{10} + m_9) \cdot t_9) / 2 + ((m_{11} + m_{10}) \cdot t_{10}) / 2 + ((m_{12} + m_{11}) \cdot t_{11}) / 2$, where m = repeated measures over time and t = time interval between measures (Pruessner et al., 2003). Differences in plasma glucose and serum insulin AUC were analyzed using the PROC GLM procedure in SAS. Treatment effects were evaluated among all time points collectively, as well as accumulated AUC for hours 1, 2, 3, and 4 after meal consumption.

Differences between treatments in peak glucose and insulin concentrations, time to peak glucose and insulin, RISQI, MIRG, HOMA, glucose-to-insulin ratio, meal consumption time, and BW were analyzed by ANOVA using the PROC MIXED function of SAS.

All data are expressed as least square means \pm SE. Differences were considered significant at $P \leq 0.05$ with trends for significance acknowledged at $P \leq 0.10$.

Table 3-1. Nutrient composition of feeds included in the basal ration

Nutrient ^{1,2}	Bermudagrass hay	Grain mix
DE, Mcal/kg	1.90	3.24
Crude protein, %	10.4	20.3
Crude fat, %	1.7	3.8
NDF, %	72.4	23.1
ADF, %	38.2	13.5
Starch, %	2.4	27.0
ESC, %	4.6	7.6
Ca, %	0.31	0.89
P, %	0.22	0.66
Cu, mg/kg	6	62
Zn, mg/kg	29	224
Arginine, %	0.40	1.22
Citrulline, %	0.00	0.00
Glutamic Acid, %	0.86	3.19
Lysine, %	0.40	1.03
Methionine, %	0.13	0.27
Ornithine, %	0.01	0.01
Proline, %	0.55	1.02
Threonine, %	0.35	0.69

¹ DE = digestible energy, NDF = neutral detergent fiber, ADF = acid detergent fiber, ESC = ethanol soluble carbohydrates.

² All values are presented on a 100% DM basis.

CHAPTER 4 RESULTS

All horses remained in good health throughout the observational period and willingly consumed both the urea and citrulline-malate supplements. Body weight of horses did not differ among treatments before supplementation began (546 ± 13 kg CON, 557 ± 13 kg CIT), nor after 14 d of treatment with CIT (570 ± 13 kg) or CON (563 ± 13 kg).

The amount of time it took the horses to consume the grain meal containing CIT (14.5 ± 2.7 min) or CON (15.3 ± 2.7 min) did not differ between treatments and there were no feed refusals in response to the meal. As expected, plasma glucose concentration was affected by sampling time ($P = 0.0001$) in response to the meal (Figure 4-1). Across treatments, plasma glucose increased within 30 min after the meal was consumed and returned to baseline values by 4.5 h. Plasma glucose was not affected by CIT supplementation ($P = 0.80$) or the time*treatment interaction ($P = 0.63$) (Figure 4-1). In addition, treatment had no effect on glucose AUC ($P = 0.43$) (Table 4-1). Although the time to peak glucose concentration following a grain meal was not affected by CIT supplementation ($P = 0.79$), there was a trend ($P = 0.09$) for peak glucose concentration to be higher in CON vs. CIT (Table 4-1).

Serum insulin, in response to a grain meal containing CON or CIT, also changed with respect to time ($P < 0.0001$) and mirrored the rise in plasma glucose (Figure 4-1). There was a trend for serum insulin to be affected by dietary treatment ($P = 0.06$), but not the time*treatment interaction ($P = 0.86$). Serum insulin increased above baseline ($P < 0.01$) within 30 min of meal cessation in CON and CIT and was lower ($P < 0.05$) in CIT at 2.5, 3, 3.5, 4 and 4.5 h postprandially compared to CON (Figure 4-1). Serum

insulin returned to baseline concentration at 4.5 h in CIT, but failed to return to baseline during the 5 h observation period in CON. As a result, insulin AUC was also lower ($P = 0.05$) in CIT than CON (Table 4-1). Although the time to peak insulin concentration was similar among treatments, there was a trend for peak insulin concentration to be higher ($P = 0.09$) in CON than CIT (Table 4-1). Proxy measurements for insulin sensitivity (RISQI, $P = 0.330$; HOMA, $P = 0.922$), pancreatic β -cell responsiveness (MIRG, $P = 0.345$), and the glucose-to-insulin ratio ($P = 0.384$) were not affected by 14 d of CIT supplementation (Table 4-1).

Plasma citrulline and arginine in response to a grain meal containing CON or CIT are presented in Figure 4-2. The concentration of citrulline in plasma was affected by time ($P < 0.0001$), treatment ($P < 0.0001$), and the time*treatment interaction ($P < 0.0001$). Plasma citrulline increased ($P < 0.0001$) in horses supplemented with CIT, with concentrations remaining higher in CIT than CON horses from 1 to 5 h after meal consumption ($P < 0.0001$). Additionally, plasma citrulline remained elevated above baseline in CIT at the end of the 5 h observation period ($P < 0.0001$). In contrast, plasma citrulline remained unchanged in response to the grain meal in CON horses. Plasma arginine was similarly affected by time ($P < 0.0001$), treatment ($P < 0.0001$), and the time*treatment interaction ($P = 0.0094$) (Figure 4-2). Plasma arginine concentration had increased ($P < 0.0001$) above baseline values 1 h postprandially in both treatments. It continued to increase in CIT horses at 2 h ($P = 0.0001$) and plateaued thereafter through 5 h post meal consumption. By comparison, plasma arginine gradually declined from 2 to 5 h in CON horses and was no different than baseline concentration 5 h after meal consumption ($P = 0.094$). As a result, plasma

arginine concentration was higher ($P < 0.0001$) in CIT horses at 2, 3, 4 and 5 h after meal cessation (Figure 4-2).

Changes in plasma ornithine, proline and glutamate in response to a grain meal containing CIT or CON are presented in Figure 4-3. Plasma ornithine concentration was affected by time ($P < 0.0001$) and treatment ($P = 0.0119$), but not the treatment*time interaction ($P = 0.0659$). Plasma ornithine was elevated above baseline within 1 h after meal cessation in CIT horses ($P = 0.0001$), but not until 2 h postprandially in CON horses ($P = 0.001$). In CON and CIT, plasma ornithine remained above ($P < 0.01$) baseline concentrations through the 5-h observation period. Plasma ornithine concentration was higher from 1 to 5 h after meal cessation when horses were supplemented with CIT compared to CON ($P < 0.0001$). Plasma proline concentration was affected by time ($P < 0.0001$), but not treatment ($P = 0.7686$) or treatment*time ($P = 0.1012$) (Figure 4-2). Across treatments, plasma proline increased gradually above baseline concentration through 3 h post meal consumption ($P < 0.0001$), and then sharply declined below baseline concentration at 4 h ($P < 0.0001$), where it remained through 5 h following the grain meal ($P < 0.0001$).

Plasma glutamate concentration was affected by time ($P = 0.0037$) and treatment ($P = 0.0290$), but not the treatment*time interaction ($P = 0.4991$). In CIT horses, plasma glutamate was elevated above baseline at 1 h ($P = 0.0188$) and 2 h ($P = 0.0134$) after meal cessation, but had returned to baseline concentration 3 h ($P = 0.1441$) after the meal had been consumed. By comparison, in CON horses plasma glutamate remained elevated above baseline concentrations from 1 to 4 h postprandially ($P < 0.01$). Plasma

glutamate concentration was greater at 1, 3, and 4 h after meal cessation in CON than CIT ($P < 0.05$).

Figure 4-4 shows the response of plasma lysine, methionine and threonine to a grain meal containing CIT or CON. Plasma lysine ($P < 0.0001$), methionine ($P < 0.0001$), and threonine ($P < 0.0001$) were affected by time. Plasma lysine was elevated above baseline concentration from 1 to 4 h following meal consumption ($P < 0.001$), but had returned to baseline by 4 h ($P = 0.2289$). Plasma methionine was elevated 1 h after meal consumption ($P < 0.0001$), but declined thereafter such that plasma concentrations were below baseline measurements at 4 and 5 h postprandially ($P < 0.0087$). Plasma threonine was elevated 1 h after meal consumption ($P < 0.0001$) and was no different than baseline concentrations by 5 h ($P = 0.7186$). Supplementation with CIT had no effect on plasma lysine ($P = 0.3607$), methionine ($P = 0.2855$), or threonine ($P = 0.2200$).

Plasma urea concentration in response to a grain meal containing CON or CIT was unaffected by time ($P = 0.1224$) or treatment ($P = 0.9175$) (Figure 4-5). Plasma NH_3 concentration was highly variable among horses, but was also unaffected by time ($P = 0.3348$) or treatment ($P = 0.8803$) (Figure 4-5).

Table 4-1. Measures of glucose and insulin response to a grain meal containing citrulline-malate (CIT) or an isonitrogenous amount of urea (CON).

Measurement ¹	Treatment		SEM	P-value
	CON	CIT		
Basal insulin, $\mu\text{IU/mL}$	5.27	4.08	0.91	0.38
Basal glucose, mmol/L	4.68	4.65	0.37	0.95
AUC				
Glucose, mmol/L	34.17	31.27	2.50	0.43
Insulin, $\mu\text{IU/mL}$	276.57	151.38	40.21	0.05
Peak glucose				
Concentration, mmol/L	9.64	8.43	0.45	0.09
Time to peak, h	2.1	2.1	0.17	0.79
Peak insulin				
Concentration, $\mu\text{IU/mL}$	95.37	55.28	15.24	0.09
Time to peak, h	2.7	2.5	0.32	0.58
RISQ, $\text{mIU/L}^{-0.5}$	0.44	0.57	0.09	0.33
MIRG, ($\text{mU}_{\text{insulin}}^2 / [10 \cdot \text{L} \cdot \text{mg}_{\text{glucose}}]$)	4.12	3.19	0.66	0.34
HOMA, ($\text{mg/dL}_{\text{glucose}} - \text{mU/L}_{\text{insulin}} / 22.5$)	3.57	3.53	0.30	0.92
Glucose:Insulin ($[\text{mg/dL}] / [\text{mU/L}]$)	18.48	28.17	7.47	0.38

¹ AUC = area under the curve; RISQ = reciprocal of the square-root of insulin index; MIRG = modified insulin response to glucose; HOMA = homeostasis model assessment.

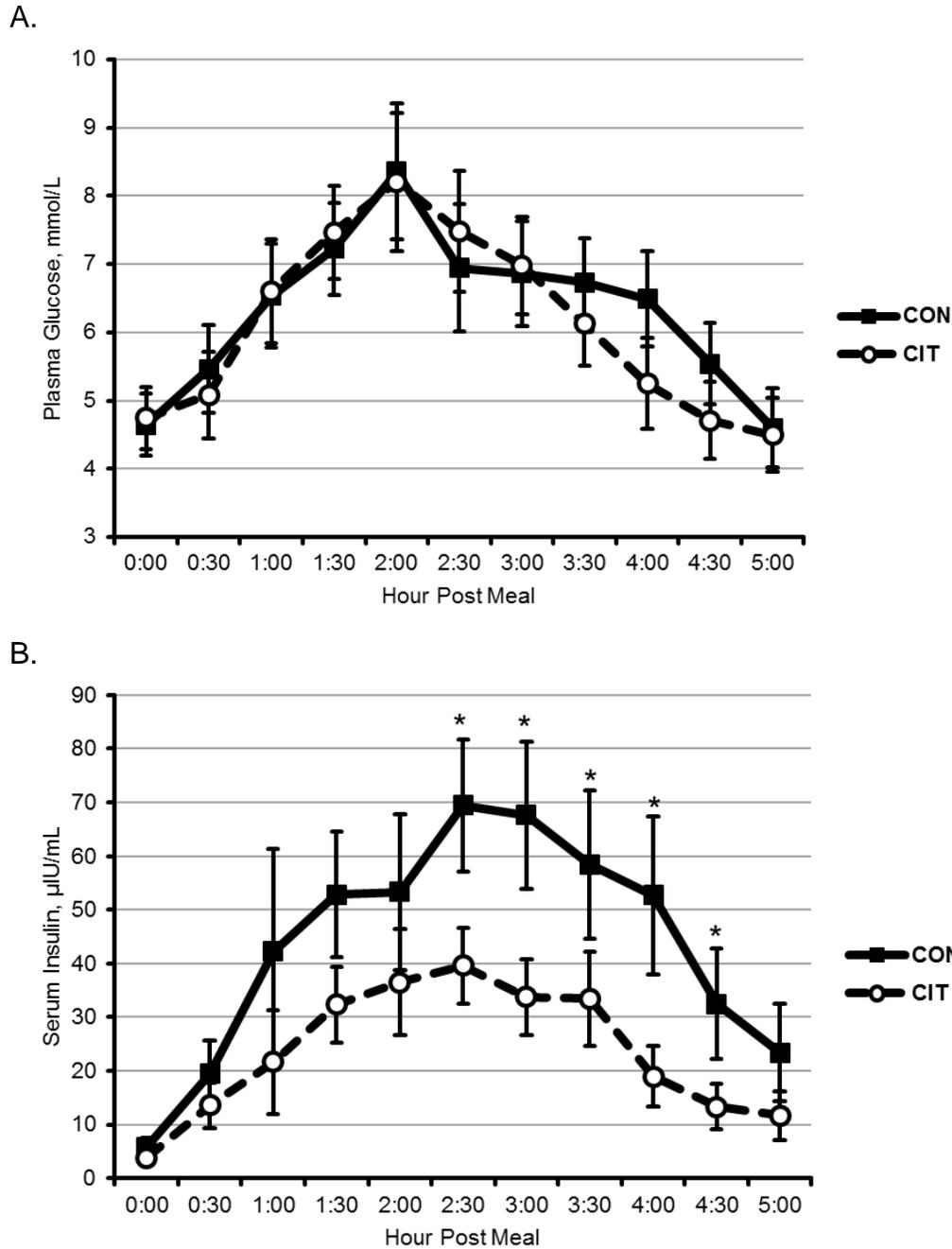
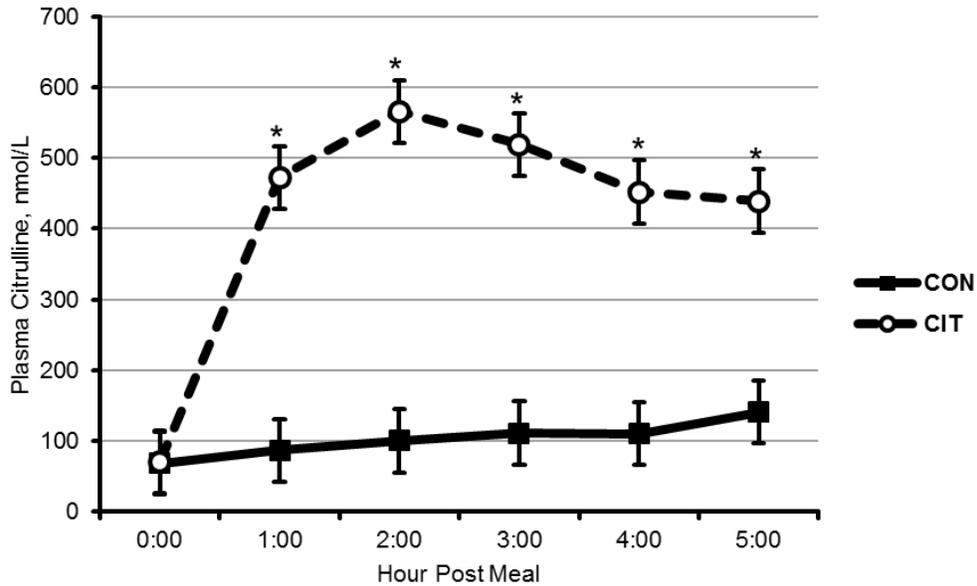


Figure 4-1. Plasma glucose and serum insulin responses before (0:00 h) and after horses consumed a grain meal containing citrulline-malate (CIT) or an isonitrogenous amount of urea (CON). A) Plasma glucose: overall effect of time ($P = 0.0001$), treatment ($P = 0.8036$), and time*treatment ($P = 0.6361$). B) Plasma insulin: overall effect of time ($P < 0.0001$), treatment ($P = 0.0635$), and time*treatment ($P = 0.8593$). An asterisk (*) indicates a difference between treatments at a specific time point ($P < 0.05$).

A.



B.

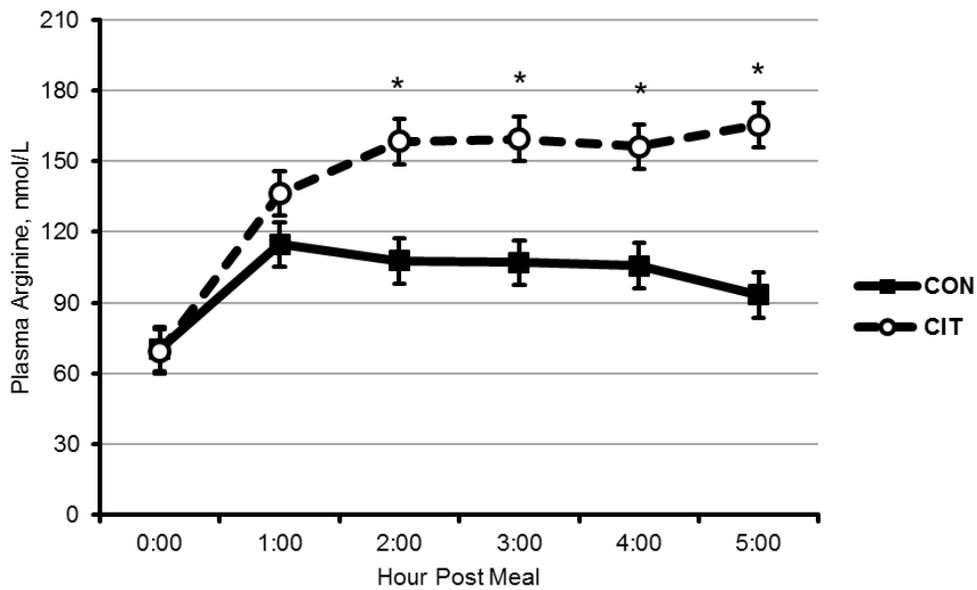


Figure 4-2. Plasma citrulline and arginine concentrations before (0:00 h) and after horses consumed a grain meal containing citrulline-malate (CIT) or an isonitrogenous amount of urea (CON). A) Plasma citrulline: overall effect of time ($P < 0.0001$), treatment ($P < 0.0001$), and time*treatment ($P < 0.0001$). B) Plasma arginine: overall effect of time ($P < 0.0001$), treatment ($P < 0.0001$), and time*treatment ($P = 0.0094$). An asterisk (*) indicates a difference between treatments at a specific time point ($P < 0.01$).

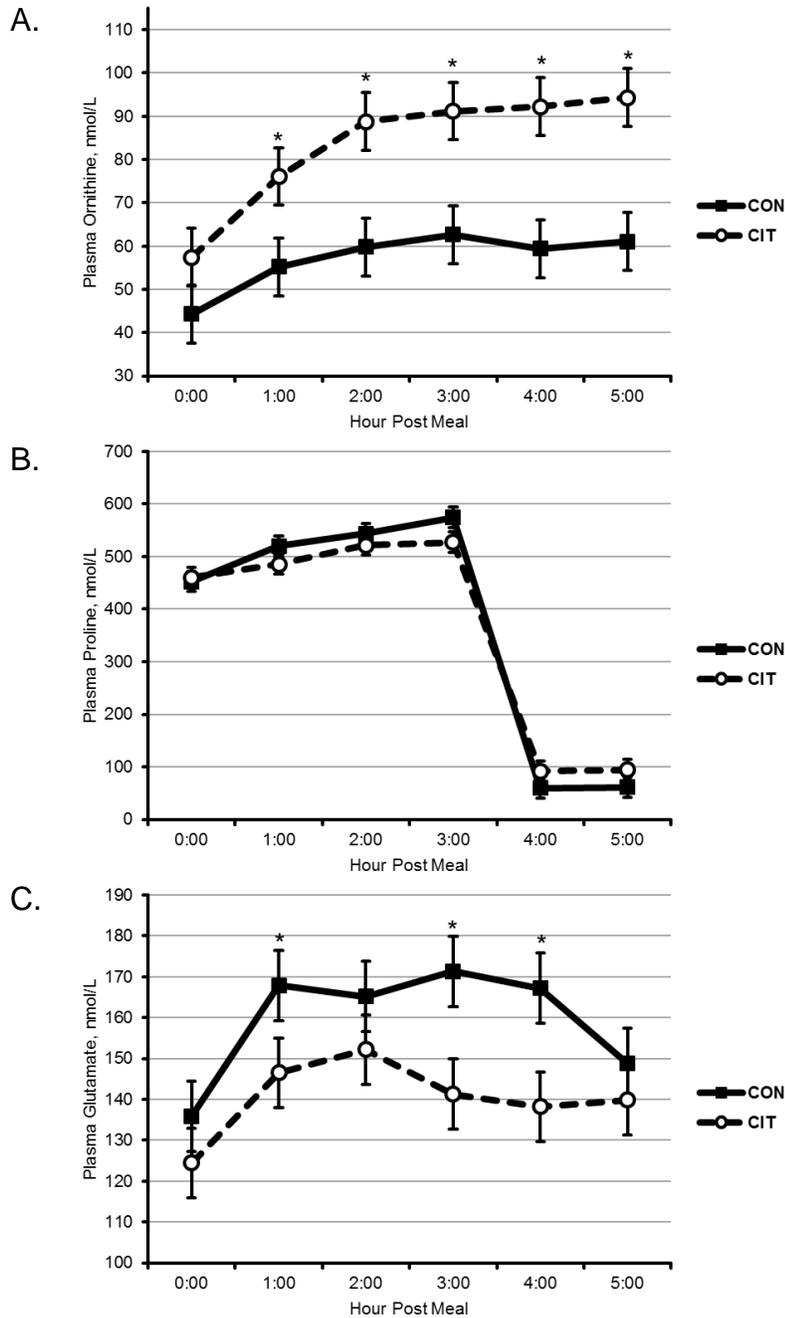


Figure 4-3. Plasma ornithine, proline, and glutamate concentrations before (0:00 h) and after horses consumed a grain meal containing citrulline-malate (CIT) or an isonitrogenous amount of urea (CON). A) Plasma ornithine: overall effect of time ($P < 0.0001$), treatment ($P = 0.0119$), and time*treatment ($P = 0.0659$). B) Plasma proline: overall effect of time ($P < 0.0001$), treatment ($P = 0.7686$), and time*treatment ($P = 0.1012$). C) Plasma glutamate: overall effect of time ($P < 0.0037$), treatment ($P = 0.0290$), and time*treatment ($P = 0.4991$). An asterisk (*) indicates a difference between treatments at a specific time point ($P < 0.01$).

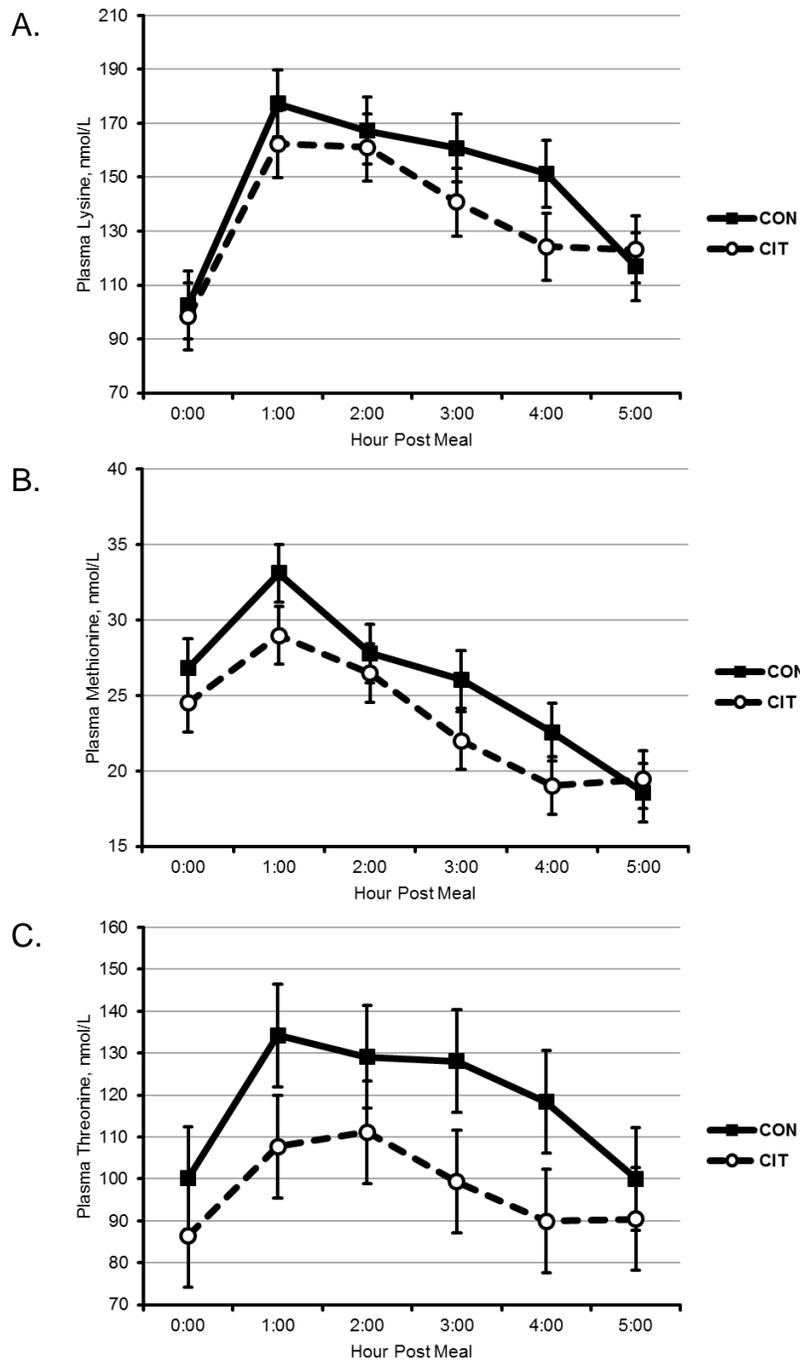
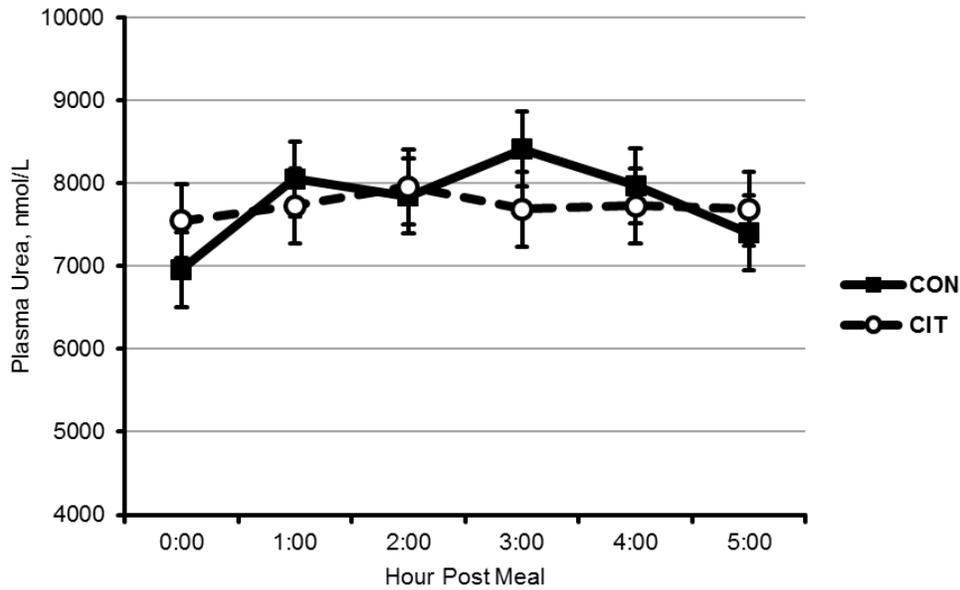


Figure 4-4. Plasma lysine, methionine, and threonine concentrations before (0:00 h) and after horses consumed a grain meal containing citrulline-malate (CIT) or an isonitrogenous amount of urea (CON). A) Plasma lysine: overall effect of time ($P < 0.0001$), treatment ($P = 0.3607$), and time*treatment ($P = 0.5834$). B) Plasma methionine: overall effect of time ($P < 0.0001$), treatment ($P = 0.2855$), and time*treatment ($P = 0.3234$). C) Plasma threonine: overall effect of time ($P < 0.0001$), treatment ($P = 0.2200$), and time*treatment ($P = 0.3327$).

A.



B.

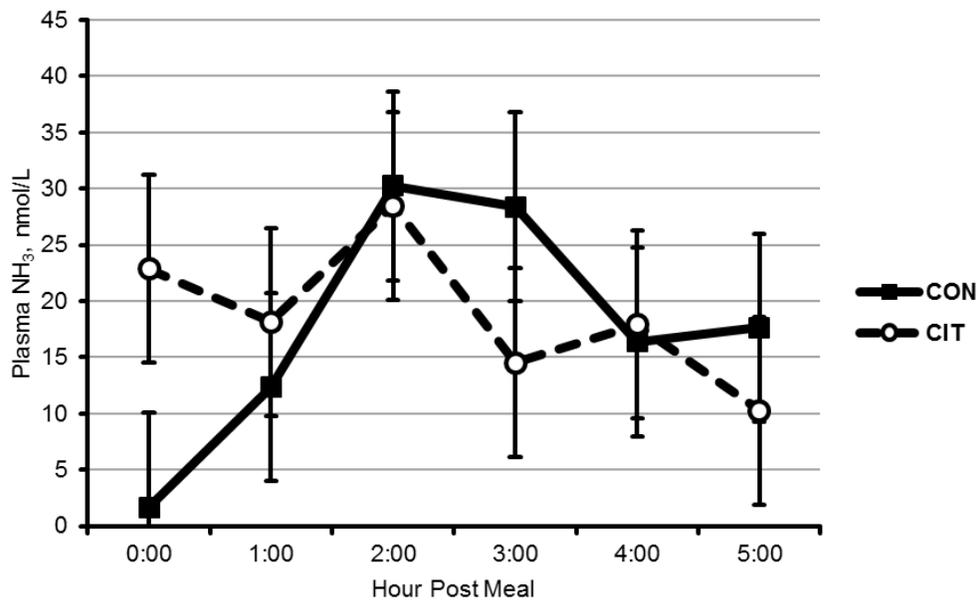


Figure 4-5. Plasma urea and NH₃ concentrations before (0:00 h) and after horses consumed a grain meal containing citrulline-malate (CIT) or an isonitrogenous amount of urea (CON). A) Plasma urea: overall effect of time ($P = 0.1224$), treatment ($P = 0.9175$), and time*treatment ($P = 0.1500$). B) Plasma NH₃: overall effect of time ($P = 0.3348$), treatment ($P = 0.8803$), and time*treatment ($P = 0.4040$).

CHAPTER 5 DISCUSSION

The key findings of this study were: 1) CIT supplementation increased the pool of citrulline, arginine and ornithine in plasma; 2) CIT supplementation did not appear to interfere with availability of lysine and other potentially limiting amino acids in equine diets; 3) glycemic control in response to a grain meal was accomplished in CIT-supplemented horses with a lower circulating insulin concentration; and 4) short-term supplementation with CIT had no effect on insulin sensitivity, as estimated by RISQI and MIRG.

With the notable exception of watermelon, which is not commonly fed to horses, there is typically little to no citrulline present in the diet (Rimando and Perkins-Veazie, 2005; Curtis et al., 2007; Marini et al., 2010a). Thus, circulating citrulline is believed to be almost entirely of endogenous origin (Curtis et al., 2007; Marini et al., 2010a). Hepatic uptake of citrulline from portal circulation has been shown to be minimal (Wu et al., 2009), although the extent of citrulline's exclusion from the liver has been recently questioned (van de Poll et al., 2007). In the current study, plasma citrulline was elevated 3 to 5-fold by CIT supplementation. Although synthesis of citrulline from ornithine, proline, and glutamine can occur in enterocytes and other tissues (Marini, 2010), the dramatic increase in plasma citrulline was likely attributed to the direct absorption of supplemented CIT, rather than a high level of *de novo* synthesis. This is further supported by the observation that plasma citrulline in the urea-fed horses remained unchanged during the 5 h period following the grain meal.

In the current study, plasma citrulline remained elevated through the 5 h observation period in horses supplemented with CIT. By comparison, plasma citrulline

appears to be cleared fairly rapidly from plasma in humans (Barr et al., 2007; Moinard et al., 2008). The half-life of citrulline was calculated to be approximately 60 minutes in humans (Barr et al., 2007). The prolonged elevation of plasma citrulline in horses in the current study compared to humans and other species supplemented with CIT could be due to differences in digestion and rate of passage between species. Studies have shown that diabetic patients have slower gastric emptying when compared to non-diabetic controls (Horowitz et al., 1991). Additionally, the oral vectors in which citrulline is supplemented to subjects are likely to influence rate of passage and subsequently citrulline absorption. For example, several rat and mice studies used water as a means of oral citrulline supplementation, whereas CIT was included in a meal matrix in the current study. It has been shown that liquid meals empty the stomach quicker than solid meals (Collins et al., 1991).

Metabolism of citrulline generally follows one of three pathways: 1) arginine biosynthesis; 2) nitric oxide (NO) cycle; or 3) urea cycle in the liver (Curis et al., 2005). Citrulline is converted to arginine mainly in the kidney via a partial urea cycle involving argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (Curis et al., 2005). The newly made arginine is released into general blood circulation where it can be used for protein synthesis, NO production, and ureagenesis, among other functions. In the current study, CIT supplementation resulted in a sustained increase in plasma arginine that superseded that observed in horses fed urea, in both concentration and duration. Similar increases to the arginine pool were reported with intravenous citrulline infusion (Lassala et al., 2009), oral citrulline supplementation (Bendahan et al., 2002; Osowska et al., 2006; Schwedheim et al., 2007; Sureda et al., 2009), and watermelon

consumption (Mandel et al., 2005; Collins et al., 2007; Wu et al., 2007c), which likely reflects the conversion of citrulline to arginine in the kidney and other extrahepatic tissues. Thus, CIT supplementation appears effective at increasing arginine availability in horses. In other species, intravenous or oral administration of citrulline has been shown to be at least, if not more effective at increasing arginine supply to the body than direct supplementation with arginine (Schwedhelm et al., 2007; Lassala et al., 2009). When sheep received an intravenous bolus dose of citrulline (155 $\mu\text{mol/kg BW}$) or the same dose of arginine-HCl on d 135 of gestation, citrulline was more effective in achieving and sustaining a prolonged increase in arginine concentration in the fetal circulation (Lassala et al., 2009). Lassala and coworkers (2009) also found that the $T_{1/2}$ of citrulline in maternal plasma was twice that of arginine in pregnant ewes. This may be explained by a higher arginase activity, which breaks down arginine compared to the activities of argininosuccinate synthase and argininosuccinate lyase, which metabolize citrulline (Wu and Morris, 1998). Additionally, the lower rate of transport of citrulline by animal cells (e.g. endothelial cells and macrophages) and the faster rate of transport of arginine also reduces the $T_{1/2}$ of arginine when compared to citrulline (Wu and Flynn, 1993; Li et al., 2001). These factors help to explain why only half the amount of citrulline compared to arginine supplementation was needed to raise plasma arginine in humans (Schwedhelm et al., 2008).

Plasma ornithine was also increased in response to a meal with CIT in the present study. Ornithine is an immediate precursor for citrulline biosynthesis in the intestine and other tissues (Marini, 2010). Subsequent metabolism of arginine by arginase yields ornithine and urea (Wu et al., 2009). Ornithine then precedes citrulline in the urea

cycle, which is converted into arginine by ornithine transcarbamylase, ASS, and ASL. Thus, arginine is a major positive ureagenesis regulator (Shigesada and Tatibana, 1971). In the current study, the increase in arginine availability in CIT-supplemented horses may have resulted in greater conversion of arginine to ornithine, thereby raising circulating ornithine concentrations. However, this did not appear to reflect an increase in ureagenesis, as plasma urea and ammonia remained unchanged in response to a CIT meal when compared to horses fed a meal containing urea. Oral supplementation with L-citrulline in humans (Schwedhelm et al., 2007) or watermelon pumice in diabetic rats (Wu et al., 2007c) also resulted in increased plasma ornithine, but not to the same magnitude as that seen with arginine supplementation. Thus, although CIT supplementation elevated circulating arginine and ornithine concentrations in the present study, it may not have been sufficient to trigger upregulation of the urea cycle in the liver. Increased ornithine availability may have benefits of its own, including serving as a substrate for production of polyamines and proline, which are important in cellular proliferation, tissue growth, and wound repair (Albina et al., 1988). Ornithine supplementation has also been evaluated for its ability to improve nitrogen balance in various acute and chronic malnutrition states (Cynober, 1991) and as a therapeutic treatment for hepatic encephalopathy, a serious complication of cirrhosis in humans (Ong et al., 2011).

Individual amino acid requirements, with the exception of lysine in growing horses, have not been established for the horse (NRC, 2007). Threonine is suggested to be the second limiting amino acid in growing horses (Graham et al., 1994). Because of reported antagonisms between arginine and other amino acids such as lysine (Ball et

al., 2007), the current study evaluated the impact of CIT supplementation on plasma lysine, threonine, and methionine. These amino acids are the most limiting for protein synthesis in equine diets (NRC, 2007). In pigs, lysine has been shown to compete with arginine for entry into cells and also inhibits arginase activity (Wu and Morris, 1998). Therefore, the dietary arginine:lysine ratio is a critical factor to consider when supplementing swine diets with arginine. In swine diets, it is recommended that dietary arginine should not exceed 150% than that of lysine (Wu et al., 2009). An older study evaluated arginine supplementation (0.94, 1.29, and 1.63%) in pigs to determine the effect of excess arginine on growth (Hagemeier et al., 1983). This study found no effects on plasma lysine levels, but found that plasma threonine and methionine levels were reduced by excess arginine. In the current study, inclusion of 86 mg/kg BW of citrulline malate in a grain meal had no effect on plasma lysine, threonine or methionine concentrations. These findings suggest that citrulline supplementation may provide a means to increase arginine supply while avoiding potential antagonisms with lysine and other key amino acids.

Emerging evidence, from both human and animal studies indicates that arginine supplementation may be a novel therapy for metabolic related disorders (McKnight et al., 2010). Arginine is well-known as a potent secretagogue for insulin, via NO production (Calver et al., 1992), or the accumulation of polyamines in the pancreatic islets cells (Sener et al., 1989). However, the effect of arginine supplementation on circulating insulin has been mixed, with studies finding an increase (Guoyao and Morris, 1998; Adeghate et al., 2001; Flynn et al., 2002; Kohli et al., 2004; Kohlhaas et al., 2011), a decrease (Lucotti et al., 2006), or no effect on serum insulin (Huynh and

Tayek, 2002; Fu et al., 2005; Wu et al., 2007c; Bertolo and Burrin., 2008; Jobgen et al., 2009). Presumably, citrulline supplementation would have similar effects on insulin via conversion to arginine, thereby increasing arginine availability for NO and polyamine production. Schwedheim et al. (2007) reported that oral L-citrulline supplementation augmented NO-dependent signaling in a dose-dependent manner and was at least as effective in this regard as L-arginine supplementation. In the current study, serum insulin was lower in response to a moderate-sized grain meal when horses were supplemented with 86 mg/kg BW citrulline-malate compared to a meal with an isonitrogenous amount of urea. These results are in contrast to Wu and coworkers (2007c), who observed no effect on serum insulin when 0.63% watermelon pomace (with 2.014 g/L citrulline) was provided in the drinking water of Zucker diabetic fatty rats for 4 wk. Osowska and coworkers (2006) reported a lower basal plasma insulin concentration after 1-wk of citrulline supplementation (5 g/(kg BW•d-1)) in malnourished rats. Reasons for the discrepancies between studies are likely multifactorial, including the level of arginine or citrulline supplementation, route of supplementation (e.g., intravenous vs. oral), length of supplementation, and the health status of the subjects it was evaluated in. Furthermore, most studies have evaluated basal (or fasting) insulin responses, whereas few studies have evaluated actual insulin secretion and removal in response to a glucose and/or insulin challenge. Although the lower insulinemic response observed in CIT-supplemented horses in the current study is difficult to explain, it may have useful application in the management of insulin resistant horses where hyperinsulinemia is thought to be responsible for a significant number of aberrant effects (Frank, 2008, 2009). The upper limit of resting serum insulin concentrations is >

20 $\mu\text{U}/\text{mL}$, which is suggestive of insulin resistance in horses (Frank, 2008). Pain and stress with laminitis markedly elevate resting serum insulin concentrations in EMS patients (Frank, 2008). Resting serum insulin concentrations can often range from 100 to 400 $\mu\text{U}/\text{mL}$ in horses and ponies with clinical laminitis (Frank, 2008). In regards to the noted upper limit concentrations, horses in the present study showed no signs of insulin resistance or laminitis according to these suggestive concentrations.

The malate portion of the citrulline-malate used in this study could have affected energy metabolism (Bendahan et al., 2002), thereby contributing to the effects observed on serum insulin. Malic acid supplementation was shown to increase both plasma glucose and serum insulin in dairy cattle (Wang et al., 2009); however, ruminants exhibit different glycemic control than monogastrics such as the horse. Because a malate-only supplemented group of horses was not included in the current study, the potential impact of malate (instead of citrulline) on serum insulin cannot be excluded.

A hallmark of insulin resistance is a disturbance in glucose homeostasis. Upper resting glucose concentrations suggestive of an insulin resistant horse are $> 100 \text{ mg/dL}$ or 5.5 mmol/L (Frank, 2008). Glucose homeostasis is primarily influenced by pancreatic β -cell response to glucose and the sensitivity of the body tissues to insulin. In the current study, although insulinemic response to a meal was reduced, short-term supplementation with CIT had no effect on insulin sensitivity or β -cell responsiveness, as estimated by RISQI, MIRG, HOMA, and glucose-to-insulin ratio. These proxies are calculated from fasting plasma glucose and serum insulin values, as opposed to dynamic responses to glucose and insulin evaluated with minimal model analyses of a frequently sampled glucose insulin tolerance test (FSGIT) or evaluation of insulin

sensitivity and pancreatic responsiveness to glucose using the euglycemic-hyperinsulinemic clamp technique. Nonetheless, the RISQI and MIRG proxies were developed as predictors for minimal model outcomes from an FSGIT (Treiber et al., 2005b). Together, they identify apparently healthy individuals that are compensating for low insulin sensitivity with increased β -cell activity. Further, these proxies provide a means to determine changes in glucose tolerance that may occur between plasma insulin and its target action at the level of the tissue or whether changes resulted from altered β -cell responsiveness. However, care should be taken when using RISQI and MIRG to determine insulin sensitivity and glucose tolerance as large daily variation and poor repeatability has been demonstrated in ponies and horses (Pratt et al., 2009). It should be noted that all horses in the present study were clinically normal and none had a history of laminitis. Additionally, all horses exhibited fasting serum insulin concentrations < 20 uU/mL indicating they were not hyperinsulinemic, as well as RISQI values ≥ 0.33 mIU/L^{-0.5} indicating they were not insulin resistant (Pratt et al., 2009). Further, MIRG was ≤ 5.5 (mU_{insulin}²/[10•L•mg_{glucose}]) in all horses, indicate adequate glucose tolerance (Pratt et al., 2009). Because many of the studies noting improvements in insulin sensitivity with arginine supplementation were performed in subjects with glycemic or pancreatic dysfunction, results with citrulline may be different in diabetic or insulin resistant subjects from those observed here.

Elevated levels of NO have been shown to reduce hepatic glucose production and increase glycogen disposal (e.g., via increased glycogen synthesis in skeletal muscle, liver and adipose tissues), as well as increase blood flow into tissues (Piatti et al., 2001; Kingwell et al., 2002; Wu et al., 2007c; Jobgen et al., 2009; Clemmensen et al., 2011;

Monti et al., 2012). In diabetic rats, arginine supplementation was suggested to increase insulin sensitivity by increasing the secretory response of the remaining pancreatic β -cells via enhanced production of NO, resulting in a concurrent reduction in plasma glucose (Kohli et al., 2004). Similar to findings for insulin, the effects of arginine on glucose have varied with some studies showing a decrease in plasma glucose (Wu et al., 2007c; Jobgen et al., 2009; Clemmensen et al., 2011; Monti et al., 2012) or no change in plasma glucose (Huynh and Tayek, 2002). Oral ingestion of citrulline is also known to increase NO production (Hayashi et al., 2005); thus, we hypothesized citrulline-malate supplementation would behave similarly to arginine in regards to glycemic control. Supplementation with a citrulline-rich watermelon pomace resulted in lower fasting blood glucose concentrations in Zucker diabetic fatty rats (Wu et al., 2007c). In the current study, there was a trend for a slight reduction in peak glucose concentration when horses were fed a meal containing CIT compared to urea. However, the overall glycemic response to a meal was not different between CIT- and urea-supplemented horses, despite reductions in circulating insulin concentrations.

In conclusion, CIT supplementation appeared to modify the insulinemic response to a high-starch meal, while maintaining glycemic control. These data should be confirmed with a more stringent evaluation of insulin sensitivity and pancreatic responsiveness, such as minimal model analysis with a FSGIT or euglycemic-hyperinsulinemic clamp. Insulin resistant horses usually have hyperinsulinemia, particularly in response to starch and sugar consumption as the pancreas tries to compensate for a decrease in tissue cell effectiveness (Zimmel and McFarlane, 2009).

Citrulline supplementation could be used as a potential therapeutic agent to reduce excessive secretion of insulin while maintaining glycemic control.

APPENDIX A
PLASMA GLUCOSE ASSAY

Materials

- Timer
- Gloves
- Microcentrifuge rack
- 56 Microcentrifuge vials (per assay plate)
- Four 20 mL scintillation vials (per kit; used for reconstituting kit enzyme mixture)
- 50 mL sterile centrifuge conical (for creating internal “pooled” control)
- Kimwipes
- Parafilm
- Bucket of ice to keep supplies at 4°C
- UltraPure water (approximately 5 mL)
- Incubator (set at 37°C)
- Plate reader
- Transfer pipettes
- Two 1 mL syringes for UltraPure water
- One 12 mL syringe
- Six small gauge needles (20g works well)
- 20 μ L Pipetteman
- 100 μ L Pipetteman
- 200 μ L Pipetteman
- 1000 μ L Pipetteman
- Pipette tips for each individual pipetteman size
- Glucose Assay Kit (Cayman Chemical Company, Ann Arbor, MI), containing:
 - One bottle of Glucose Assay Standard
 - One vial of Glucose Assay Buffer
 - Four vials of lyophilized Glucose Enzyme Mixture
 - Two 96-well plates
 - NOTE: one 96-well plate can analyze 39 samples, 1 internal control, and 8 standards (all run in duplicate). Thus, one Glucose Assay Kit can run 78 study samples. However, the company sometimes skimps on the amount of enzyme provided, reducing the number of samples per kit to ~56.

Before Beginning the Assay (the day before):

- Thaw samples and glucose kit in the refrigerator (4°C) overnight before using.
- Turn on the incubator and make sure it is set for 37°C.
- All reagents, samples and UltraPure water should be equilibrated to 4°C before the start of the assay.
- Take 96-well plates from glucose kit and let sit at room temperature overnight.
- ALWAYS vortex samples immediately prior to pipetting to make sure they are mixed well.

Preparation of the Internal Control

★NOTE: Inclusion of an internal control on each plate provides quality control for the assay, ensuring you/machine/reagents are all operating properly and consistently each time the assay is completed. It will also be used to calculate interassay variation.

1. To make a pooled internal control sample, pipette 250 μL from several study plasma samples into a 50 mL sterile conical vial.
2. Vortex the vial for 10 seconds to mix.
3. Dispense desired amount into microcentrifuge vials (*0.250 mL in each*) for storage. These internal control samples can be stored at -20°C if not needed on the day of preparation (similar to how plasma samples are stored long-term).

Preparation of the Glucose Standards

1. To prepare the **concentrated glucose standards**, begin by labeling **8** microcentrifuge vials **A** through **H** on the top and place vials in a rack.
2. Mix the “Glucose Assay Standard” bottle from the kit by gentle inversion to mix well prior to pipetting.
3. Using a 20 μL pipette, pipette the appropriate amount of “Glucose Assay Standard” from the kit and place in the appropriate microcentrifuge vial (according to the table below). Note the unique pipetting needed for vials **F**, **G**, and **H**; check off the boxes provided in the table to maintain accuracy.
4. Add the appropriate amount of “Glucose Assay Buffer” from the kit to each vial, according to the table below.

Table A-1. Preparation of glucose standards.

Vial	Glucose Assay Standard (μL)		Assay Buffer (μL)	FINAL Glucose Concentration (mg/dL)
	★ Use 20 μL Pipette		★ Use 200 μL Pipette	
A	0		200	0
B	2.5		197.5	12.5
C	5		195	25
D	10		190	50
E	20		180	100
F	30	★ pipette 20 μL <input type="checkbox"/> + 10 μL <input type="checkbox"/>	170	150
G	40	★ pipette 20 μL <input type="checkbox"/> + 20 μL <input type="checkbox"/>	160	200
H	50	★ pipette 20 μL <input type="checkbox"/> + 20 μL <input type="checkbox"/> + 10 μL <input type="checkbox"/>	150	250

Assay Part I – Pipetting Standards and Samples

1. Label **8** new microcentrifuge vials **A** through **H** for your **working standards**.
2. Label **39** microcentrifuge vials **1** through **39** for each plasma sample.
3. Label **1** microcentrifuge vial for your internal control (pooled) sample.

4. ★ Immediately prior to pipetting any standard or sample, vortex the sample to ensure it is mixed well.
5. Using the 20- μ L Pipetteman, pipette **5 μ L** of each **concentrated standard** to its respective, newly labeled microcentrifuge vial (created in step #1).
6. Using the 20- μ L Pipetteman, pipette **5 μ L** of **plasma sample** to microcentrifuge vials 1-39 (created in step #2). Make sure you have created a “key” to determine the actual sample’s identity when they are renamed by numbers 1-39.
7. Using the 20- μ L Pipetteman, pipette **5 μ L** of the **internal control** sample to microcentrifuge vial #40 from the pooled plasma prepared earlier.

Assay Part II – Enzyme Mixture Preparation

- ★ NOTE: The following steps #1 – 5 should be performed for each of the 4 “Glucose Enzyme Mixture” vials in the kit.
8. Add 500 μ L of 4°C UltraPure water to enzyme vial. Mix well by gentle inversion.
9. Transfer the reconstituted enzyme solution to a scintillation vial using a transfer pipette.
10. Add 6 mL of the kit’s “Assay Buffer” to the reconstituted enzyme solution in the scintillation vial.
11. Take an additional 6 mL of the “Assay Buffer” and rinse any residual solution from the original Enzyme vial, and then transfer this solution to the scintillation vial containing the reconstituted enzyme solution using a transfer pipette. In total, this means 12 mL of “Assay Buffer” were added to the enzyme mixture.
12. Cover top of the scintillation vial with Parafilm and invert 9 times to make sure mixture is completely dissolved and mixed.
- ★ NOTE: The reconstituted enzyme solution is stable for at least one hour when stored at 4°C.
- ★ NOTE: Two vials of reconstituted enzyme solution will be needed for every plate. However, sometimes the company fails to provide adequate enzyme to run two full plates/kit.

Assay Part III – Incubation and Plating

13. Add 500 μ L of the enzyme mixture forcefully down the side of your standard, internal control and plasma sample microcentrifuge vials.
14. Vortex vials to mix thoroughly.
15. Place vials in a 37°C incubator for **10 minutes**.
16. Using the 200 μ L Pipetteman, pipette 150 μ L from each microcentrifuge vial to the 96 well plate. Pipette standards, samples and the internal control into duplicate wells.
17. **Check for bubbles in the plate.** Pop bubbles with a needle when present. Make sure you wipe the needle with a Kimwipe after EACH bubble is popped to avoid cross-contamination of samples.
18. Read the absorbance at 500-520 nm using a plate reader.

APPENDIX B SERUM INSULIN ASSAY

Day 1 Procedures:

Preparation of Radioactive Tracer and Insulin Standards

- ★ NOTE: Make sure radioactive material (^{125}I tracer; also called “Hotstuff”) is in the correct location within the laboratory (HAZARDOUS MATERIAL).
- 1. Dilute the ^{125}I tracer with **100 mL of distilled or deionized water**, and mix by gentle inversion (ALWAYS WEAR GLOVES).
 - a. Set tracer in radioactive tray until needed.
 - b. This will be stable at 2-8°C for 30 days after preparation or until expiration date. Once finished with it, place it back in the refrigerator.
- 2. Do not place anything that is NOT labeled radioactive within the radioactive tray.
- 3. Take serum samples and insulin standards out of the refrigerator and allow them to warm to room temperature. You will need 200 μL of each sample.
- 4. Reconstitute insulin standards (lyophilized; processed in nonhuman serum).
 - a. Fill a 250-mL beaker with distilled or deionized water.
 - b. Locate a 10-mL volumetric pipette (preferred) or serological pipette for reconstituting insulin standards.
 - c. Add **6.0 mL** of distilled or deionized water to **Standard A** (zero calibrator A), mix by gentle swirling.
 - d. Add **3.0 mL** of distilled or deionized water to standards **B through G**. Mix by gentle swirling.
 - e. Standards will be stable at -20°C for 30 days after reconstitution.
 - f. Aliquot, if necessary to avoid repeated freezing and thawing.

Tube Labeling

- ★ NOTE: Make sure all components are at room temperature before use (15-20°C)
- 5. Set up tubes (always use **two** for each (duplicates) unless otherwise specified)
 - a. **Four tubes** – use clear, uncoated polypropylene plastic tubes (5 mL, 12 x 75 mm); note that these do not come with the insulin kit.
 - **Two**, Total Count (TC) tubes: label tubes **1 & 2**
 - **Two**, None Specific Binding (NSB) tubes: label tubes **3 & 4**
 - b. **24 Standard and Control tubes** – use green, Insulin Antibody-Coated Tubes (come with kit).
 - **Two**, Reference (**REF**) tubes: label tubes **5 & 6**.
 - **Two**, **A Standard-0**: label tubes **7 & 8**.
 - **Two**, **A1 Standard-1.525 $\mu\text{IU/mL}$** : label tubes **9 & 10**.

- **Two, A2 Standard**-3.05 $\mu\text{U/mL}$: label tubes **11 & 12**.
 - **Two, B Standard**-6.1 $\mu\text{U/mL}$: label tubes **13 & 14**.
 - **Two, C Standard**-16.5 $\mu\text{U/mL}$: label tubes **15 & 16**.
 - **Two, D Standard**-55 $\mu\text{U/mL}$: label tubes **17 & 18**.
 - **Two, E Standard**-103 $\mu\text{U/mL}$: label tubes **19 & 20**.
 - **Two, F Standard**-200 $\mu\text{U/mL}$: label tubes **21 & 22**.
 - **Two, G Standard**-371 $\mu\text{U/mL}$: label tubes **23 & 24**.
 - **Two**, known low sample Quality Control (**QC-Low**): label tubes **25 & 26**.
 - **Two**, known high sample Quality Control (**QC-High**): label tubes **27 & 28**.
- c. Double check your tube labeling with Table B-1; they SHOULD match!
- d. **Sample tubes:** These are the horse serum samples.
- Use green, Insulin Antibody-Coated Tubes (come with kit).
 - Label two tubes per sample (ie, in duplicate); continue the numbering system used with the standards above (ie, the first horse sample would be tubes **29 & 30**, etc)
 - Be sure to create a “key” to determine the actual sample’s identity when they are renamed by numbers.

Radioimmunoassay Procedure

6. Step 1:

- a. Gently swirl each standard before use
- b. Add standards to appropriate tubes according to **Step 1** in Table B-1.
 - EACH tube should have a total volume **200 μL** when you are done.
 - Use 200 μL pipette for this process.
 - Always pipette directly into the bottom of the tube.
 - Change pipette tips between pipetting samples!
 - ALWAYS check labels before pipetting into them!

7. Step 2:

- a. Add **1000 μL** (1.0 mL) of insulin- ^{125}I tracer (Hotstuff) to each tube (Table B-1).
 - Use Eppendorf repeating pipette with 12.5 mL total volume and 250 μL increments. Set dial to 4 (4 x 250 μL = 1000 μL)
 - This is radioactive! Use gloves and be in the correct radioactive area of your lab.

★★IMPORTANT: Do not let more than **40 minutes** elapse between the addition of the first sample and the completion of the tracer!!! Further, do not forget to keep track of how many tubes you use per bottle of tracer!

8. Once all samples have the 1000 μL of insulin- ^{125}I , make sure everything is labeled with your last name and today's date.
9. Cover tubes with saran wrap and let incubate at room temperature for **18-24 hours**.
 - a. Always note the time in which you completed process.
 - b. Always note which kit you used if you have more than one and how many tubes were used.
10. **★RECORD HOW MUCH RADIOACTIVE MATERIAL WAS USED!!**
 - a. Each bottle of ^{125}I has 3.0 uci of radioactive material. Make sure you label how much was used from each kit's bottle, EACH day, in the log book within the lab.
 - b. Record:
 - 1) Date
 - 2) Activity removed (3 uci/100 tubes = 0.03 * _____tubes used for day)
 - 3) Remaining activity amount in bottle (3.0 uci – number from #2)
 - 4) The use: "RIA"
 - 5) Final disposal: "WASTE"
 - 6) Provide the initials of your name for the day.

Table B-1. Preparation of standards and samples for the serum insulin radioimmunoassay.

Contents	Tube Label	# of tubes	Tube Type	Step 1 ^b			Step 2 ^b		
Total	1,2	2	Clear Plastic	Nothing			1000 µL insulin I ¹²⁵		
NSB	3,4	2	Clear Plastic	Nothing			1000 µL insulin I ¹²⁵		
Ref	5,6	2	Green Coat-a-count	200 µL A standard			1000 µL insulin I ¹²⁵		
A standard 0 µIU/mL	7,8	2	Green Coat-a-count	200 µL A standard			1000 µL insulin I ¹²⁵		
A1 Standard 1.525 µIU/mL (extra standard)	9,10	2	Green Coat-a-count	150 µL A standard			1000 µL insulin I ¹²⁵		
				50 µL B standard					
A2 Standard 3.05 µIU/mL (extra standard)	11,12	2	Green Coat-a-count	100 µL A standard			1000 µL insulin I ¹²⁵		
				100 µL B standard					
B standard 6.1 µIU/mL	13,14	2	Green Coat-a-count	200 µL B standard			1000 µL insulin I ¹²⁵		
C standard 16.5 µIU/mL	15,16	2	Green Coat-a-count	200 µL C standard			1000 µL insulin I ¹²⁵		
D standard 55 µIU/mL	17,18	2	Green Coat-a-count	200 µL D standard			1000 µL insulin I ¹²⁵		
E standard 103 µIU/mL	19,20	2	Green Coat-a-count	200 µL E standard			1000 µL insulin I ¹²⁵		
F standard 200 µIU/mL	21,22	2	Green Coat-a-count	200 µL F standard			1000 µL insulin I ¹²⁵		
G standard 371 µIU/mL	23,24	2	Green Coat-a-count	200 µL G standard			1000 µL insulin I ¹²⁵		
Sample QC- Low	25,26	2 ^a	Green Coat-a-count	200 µL sample			1000 µL insulin I ¹²⁵		
Sample QC – High	27,28	2 ^a	Green Coat-a-count	200 µL sample			1000 µL insulin I ¹²⁵		
All other samples (horse serum)	29,30 ..etc.	2 ^a	Green Coat-a-count	200 µL sample			1000 µL insulin I ¹²⁵		

^a Depends on pipetting skills. Duplicates were run in this study, but with good pipetting skills a single sample can be run.

^b Empty boxes in Step 1 and Step 2 can be used as check-boxes during pipetting, to ensure completion of those steps.

^c Calculations for insulin concentration in standards were based on 1 IU of insulin = 455 ng, so 1 µIU should = 0.455 ng. (e.g., if you had 10 µIU it would be 4.55 ng).

Day 2 Procedures:

Decant Samples

1. Cut a piece of absorbent paper big enough to fold in half to turn your tubes upside-down on and place in radioactive tray.
2. Transfer all tubes **EXCEPT** the **Total Count (TC)** tubes to a green foam rack. Make sure they are inserted firmly in the green foam rack.
3. Decant (dump liquid) into specified radioactive tub located under the fume hood. Then place the tubes inside the foam rack upside down on the absorbent paper you cut in step one, within the radioactive area and strike the tubes a few times to remove all visible moisture and residual droplets. This will enhance precision. Allow them to dry for 2 – 3 minutes.
4. Transfer tubes (including the **Total Count (TC)** tubes, which should still have liquid in them) to plastic racks in order to carry them to the Auto-Gamma Counter.
5. Place racks with tubes on radioactive tray and grab extra gloves. Make sure when carrying radioactive material to another lab you are only wearing **ONE** glove to carry the tray, and the other hand is bare to open doors as necessary. Carry Tray to the gamma counter.

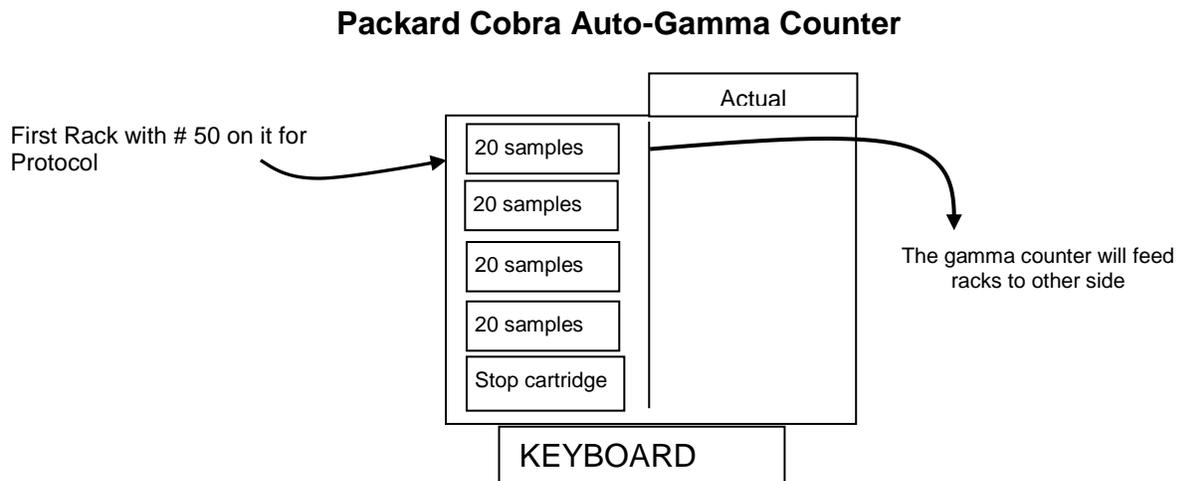


Figure B-1. Packard Cobra Auto-Gamma Counter

1. Insert a floppy disk (yes, it is very old school!) into the machine so your work will be saved upon completion of a run.
2. Record your name, date, time, isotope, and number of tubes in the gamma counter log book.
3. Grab the correct ID for the Equine Insulin Protocol (which is **50**) from drawer.
 - This goes on the side of the black cassettes that hold 20 samples (machine will automatically read this ID strip).
 - Be sure to put all tubes in the cassettes from the right (spot 1) to the left (spot 20).

- Keep your order of the tubes the same!
 - Your first cassette should be the one that has your TC, NSB, Ref., and all standards within it labeled **50**.
4. Place tube racks into the gamma counter in order (**see diagram above**)
 - The TC tubes should be on the right near the actual gamma counter, at the front in spots 1 and 2 (labeled on cartridge)
 - Then load the other racks in order (from “front” to “back” of the counter)
 - Put the “**STOP**” rack at the end (towards the “back” of the counter), this will tell the machine that your samples have all been run
 5. On the counter/keyboard: press any key to start
 - Hit **F1** until all of the protocols show up on the screen (you’ll eventually see a screen that shows different protocols for things like insulin, cortisol, estrogen, P4, etc.)
 - Hit **F3** (Protocol for Edit) and enter **50** for the *Equine Insulin Protocol*.
 - Hit **F2** (to get to Commands)
 - Hit **F6** – Next protocol, which will then **START** the assay

“Swipe” Protocol

★ **A swipe MUST be made within seven days of an assay being run, according to the Division of Environmental Health and Safety to ensure no radioactive material has been spread.**

1. Obtain 10 clear plastic tubes (5 mL, 75 x 12 mm tubes).
2. Obtain a radioactive swipe sheet from the front pocket of the binder to fill out (binder is labeled “RALGO”).
3. Put the clear tubes in a rack and go to the radioactive (hot) room.
4. In the top drawer to the left is the filter paper needed to make swipes (in a little green box with the # 41 on it).
5. Follow the instructions on the sheet regarding the particular swiping areas.
 - Each area is labeled 1 through 10.
 - Put gloves on, take a piece of the filter paper and swirl it around on the surface of each numbered area. Once completed, fold filter paper up and put it into the tube according to the correct number 1 through 10.
 - To prepare to take it to the gamma counter, remove and dispose of one glove in order to open doors. It’s a good idea to put extra gloves in your lab coat.

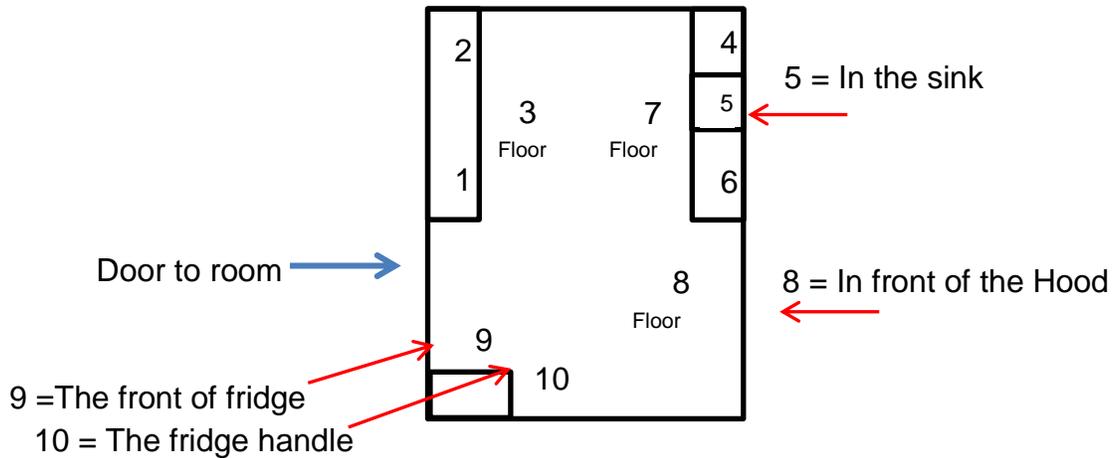


Figure B-2. Swipe protocol.

6. Grab the plastic background cassette with the plastic tubes already in it from the top, left drawer by the gamma counter. These are the tubes for measuring the environment and should have nothing in them. This should be labeled with the clip number **30**
7. Grab the clip number **29** and load your swipe samples in the black cassette from right to left
8. Always make sure you place the plastic tube holder with the **STOP** clip on it, behind your samples.
9. Press **F2** to initiate the protocol to run
10. Press **F6** to RUN the protocol (says "next protocol")
11. Once you have printed results you need to log this in the binder you initially got the paper for the swipes out of (RALGO binder).
12. Completely fill out the paper and put it in the binder with results you printed
13. Calculate the background first by **averaging your CPM** for the background (first tube holder labeled **30**), the first print out with 5 tubes.
14. Calculate the swipes by taking the number found in 13, and subtracting all A:CPM from your swipe
15. Take your number from 14 and multiply it by 0.70 for the 70% efficiency for each swipe, 1 through 10. Your resulting numbers should NEVER be above 100. If so, notify someone immediately.

APPENDIX C AMINO ACID, UREA, AND AMMONIA ANALYSIS

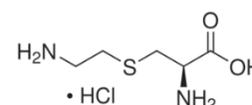
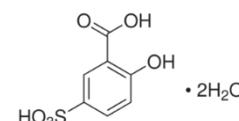
Materials Needed

- 2-mL microcentrifuge vials
 - Eppendorf safe-lock, 2 mL; hinged lid; clear vials
 - Fisherbrand: 500 pk (Part number: 05-402-7)
- Syringe and needle
 - 3cc, 20g with 1 inch needles
 - Fisherbrand: 100 pk (Part number: 1484061)
- Syringe filters
 - Nylon membrane; pore size: 0.22 μm , Diameter: 25mm, sterile
 - Fisherbrand: 50 per box (Part number: 09-719C)
 - Low hold-up volume helps you recover virtually your entire sample after filtering. Tested for 100% membrane and housing integrity, eliminating concerns about fluid loss.
- Glass vials "Snap-Its"
 - Vial, autosampler; Snap Cap, wide-opening, 12 mm dia. X 32 mm, 2 mL Clear glass vial
 - Fisherbrand: 100 pk (Part number: 03-395C or C4011-5)
- Closures for Glass Vials
 - Pre-slit blue PTFE/Silicone Septa; Clear
 - Fisherbrand: 100 pk (Part number: 03-396X or C4011-55)
- 100 or 200 μL pipette tips
- 1000 μL pipette tips



Reagents for Physiologic Fluid Analysis of Amino Acids

- 35% (w/v) sulfosalicylic acid (SSA): 5-Sulfosalicylic Acid Dihydrate
 - SIGMA-ALDRICH: 100G (Part number: S2130-100G)
- Aminoethyl cystine (AEC): S-(2-Aminoethyl)-L-cysteine hydrochloride $\geq 98\%$ (TLC)
 - SIGMA-ALDRICH: 250MG (Part number: A2636-250MG)
- 0.02 N HCL – hydrochloric acid
 - F.W.: 36.46, 1 hydrogen; $(36.46\text{g} * 0.02 = 0.7292\text{g/L})$



Recipes for SSA, HCL, AEC

35% (w/v) SSA Recipe

1. Measure 350 g of sulfosalicylic acid (% = (g/mL) * 100))
2. Fill to 1 L with ddH₂O

0.02 N HCL Recipe

1. Calculate equivalent mass ($E_{\text{mass}} = \text{FM}/\# \text{H}$) (FW = formula weight, #H = number of hydrogen donors)
2. Grams = (N desired)(E_{mass}) (Volume in L)
Or for liquids use: (Volume₁) (Concentration₁) = (Volume₂)(Concentration₂)

AEC/SSA Solution

1. 126 mg AEC/ 250 mL 35% SSA.
2. Use 50 μL of this AEC/SSA solution per 0.5 mL of plasma, with usual preparation of samples for analysis.
3. Used to provide a percent recovery of amino acids (a measure of extraction efficiency).
4. Recommended by Dr. Thomas P. Mawhinney, Director of the Agricultural Extension Service Chemistry Laboratory at the University of Missouri (MawhinneyT@missouri.edu, 573-882-2608).

Calculating Percent Recovery of AEC

- Need 1 part AEC/SSA solution to 10 parts plasma
- Thus, 1/11 part AEC/SSA which is mixed into 1 part what? to 1 part 0.02 N HCL
- Therefore, (1/11) * (1/2) = 1/22 is used to account for dilutions

$$\frac{0.126 \text{ g AEC}}{200.69 \text{ MW of AEC}} = 0.000627834 \text{ moles of AEC}$$

$$\frac{0.000627834 \text{ moles of AEC}}{.25 \text{ L of 35\% SSA solution}} = 0.002511 \frac{\text{mole}}{\text{L}} \text{ of AEC}$$

$$\frac{0.002511 \frac{\text{mole}}{\text{L}} \text{ of AEC}}{22 \text{ dilution}} = 0.000114136 \frac{\text{moles}}{\text{L}} \text{ of AEC for dilution}$$

$$0.000114136 \frac{\text{moles}}{\text{L}} \times 1000 = .114136 \text{ mmoles/mL}$$

$$0.114136 \frac{\text{mmoles}}{\text{L}} \times 1000 = 114.136 \text{ nmoles/mL}$$

$$1.14 \times 10^{-4} = 1.14 \times 10^{-1} \frac{\text{mmoles}}{\text{L}} = .114 \frac{\text{mmoles}}{\text{mL}}$$

$$= 114 \frac{\text{nmoles}}{\text{mL}} \text{ of AEC per sample upon preparation}$$

- ★ NOTE: There is a 2.2 multiplier factor on the amino acid analyzer. Thus, $114 \times 2.2 = \mathbf{250 \text{ nmol/mL}}$
- ★ NOTE: Molecular Weight (MW) of AEC = 200.69
- ★ NOTE: For this study, which had 72 samples that used 50 μL of the AEC/SSA solution per sample, a total of 3.6 mL of AEC/SSA solution was needed.

Preparation of Plasma Samples for Amino Acid Analysis

1. Thaw plasma samples in the refrigerator prior to amino acid extraction.
2. Keep samples on ice at **ALL TIMES** once you take them out of the fridge.
3. Vortex each plasma sample prior to pipetting.
4. Pipette 0.5 mL (500 μL) of plasma into a 2-mL microcentrifuge vial.
5. Add 50 μL of AEC/35% SSA solution to each microcentrifuge vial containing plasma
6. Vortex each vial to mix well.
7. Incubate the vortexed plasma and AEC/SSA solution at 4°C for 20 minutes.
8. Centrifuge the microcentrifuge vials at 4°C for 10 minutes at 11,000 g.
9. Draw up 0.5 mL of supernatant with syringe from centrifuged samples. Do not disturb the precipitated pellet on the bottom of the microcentrifuge vial.
 - ★ **NOTE:** Record the amount of supernatant recovered. Sometimes it may only be 0.2 or 0.3 mL. If it is less than 0.5 mL, you will need to adjust the amount of HCl added in step #11 below to ensure a 1:1 ratio between supernatant and HCl.
 - E.g., if you only have 0.3 mL of supernatant, then you need 300 μL of HCl.
10. Filter the recovered supernatant through a 0.2 μm pore filter into a glass Snap-It vial.
11. Add 0.5 mL (500 μL) of 0.02 N HCl into the same Snap-It vial and place the cap on the vial.
 - Make sure you have adjusted for your supernatant. See **NOTE** in step #9.
12. Vortex the Snap-It vial to mix well.
13. Keep samples on ice!
14. Samples are now ready for the amino acid analyzer.
15. Samples can be run immediately or stored at 4°C for no longer than 3 days.
16. Refer to the manual “How to Run Amino Acid Analyzer” (located in the lab) for the how to utilize the Hitachi L-8900 Amino Acid Analyzer.

Table C-1. Composition of buffers used in Hitachi L-8900 Amino Acid Analyzer

Name	PF-1	PF-2	PF-3	PF-4	PF-RG
Vessel (buffer)	B1	B2	B3	B4	B5
Lithium concentration(N)	0.09	0.255	0.721	1.00	0.20
Distilled water (approx.)	700 mL	700 mL	700 mL	700 mL	700 mL
Lithium citrate (4H ₂ O)	5.73 g	9.80 g	8.79 g	9.80 g	-----
Lithium chloride	1.24	6.36	26.62	38.15	-----
Citric acid (H ₂ O)	19.90 g	12.00 g	11.27 g	3.30 g	-----
Lithium hydroxide	-----	-----	-----	-----	8.40 g
Ethanol*	30.0 mL	30.0 mL	100.0 mL	-----	30.0 mL
Thiodiglycol	5.0 mL	5.0 mL	-----	-----	-----
Benzyl alcohol	-----	-----	3.0 mL	-----	-----
Brij-35	4.0 mL	4.0 mL	4.0 mL	4.0 mL	4.0 mL
pH (nominal)	2.8	3.7	3.6	4.1	-----
Total (adjust)	1.0 L	1.0 L	1.0 L	1.0 L	1.0 L
Caprylic acid	0.1 mL	0.1 mL	0.1 mL	0.1 mL	0.1 mL

(Note*) indicate solution that was made in lab, all other solutions were purchased.

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BIOGRAPHICAL SKETCH

Leigh Ann Skurupey was born in Williston, North Dakota, yet she spent most of her childhood in Parker, Colorado, and later graduated from Ponderosa High School in 2002. Immediately following graduation, she moved to Sterling, Colorado to attend Northeastern Junior College where she graduated in 2004 with president's honors and received an Associate of Applied Science Degree in equine management. After her hiatus from school and training horses for two years, she decided to continue her education in Cheyenne, Wyoming, at Laramie County Community College where she graduated in 2008 with honors distinction, and received Associate of Science Degree in agriculture. Immediately following, she transferred to Colorado State University in Fort Collins, Colorado, where she was awarded a Bachelor of Science Degree with a first major in equine science, a second major in animal science, and a minor in business administration. She graduated *cum laude* from Colorado State University in 2010. She is currently pursuing her Master of Science at the University of Florida in Gainesville, Florida, specializing in equine nutrition with the intention of graduation in summer 2012.

Her passion for horses has its root in working with her mother raising miniature horses, in addition to visiting her father in North Dakota where she would ride horses with her aunts every chance she could get. Leigh Ann, only three years of age, would be hoisted onto green ponies with her Papa's hand in the belt loop for of her pants for constant support in efforts to break the ponies to ride. Her love for sports dealing with horses had excited her to perform and test her talents. Her college accolades abounds most of her experiences and Leigh Ann hasn't stopped riding since. She has successfully competed in college on the rodeo team, the stock horse team, and made it to Intercollegiate Horse Show Association Nationals for her college equestrian team in

2007. She also successfully competed on the intercollegiate horse judging team at Colorado State University where she was sixth high individual overall at the All American Quarter Horse Congress competition, and finished on the reserve champion team. Further, she was ninth high individual overall at the American Quarter Horse World Show judging contest, and was also on the reserve champion team. Her passion for horse judging awarded her the opportunity to attend the University of Florida for her Master of Science on an assistantship for coaching the University of Florida's horse judging team.

Upon completion of her Master of Science program, she will begin her fall semester at the University of Florida as a doctoral candidate in the Department of Animal Sciences, with a specialization in equine nutrition and a minor in exercise physiology. She hopes to be able to use the information and experience gained through her education to continue researching ways for innovative therapies and interventions to improve the well-being and health of horses.