METABOLOMICS ALLOWS FOR INSIGHT INTO THE METABOLIC ADAPTATIONS AND PERTURBATIONS ASSOCIATED WITH DIETARY CARBOHYDRATE PROFILES, AGING, AND INSULIN DYSREGULATION IN HORSES

By

Sarah Ilyse Jacob

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ABSTRACT

METABOLOMICS ALLOWS FOR INSIGHT INTO THE METABOLIC ADAPTATIONS AND PERTURBATIONS ASSOCIATED WITH DIETARY CARBOHYDRATE PROFILES, AGING, AND INSULIN DYSREGULATION IN HORSES

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Understanding the relationship between age, diet, and glucose and insulin dynamics in horses is important given their role in equine metabolic disorders such as insulin dysregulation, equine metabolic syndrome (EMS), and pituitary pars intermedia dysfunction (PPID). However, our understanding of the pathophysiology of metabolic disorders is limited, which hampers the development of new treatment and management strategies, and identification of reliable clinical diagnostic tests. In human medicine, the advent of technologies for comprehensive metabolic analysis ("metabolomics") has opened new avenues for understanding metabolic diseases. To date, there has been minimal application of metabolomics for the study of metabolic disorders of horses.

Chapter 1 is a literature review that describes the interaction between diet and physiologic state on glucose and insulin dynamics in horses and explores the use of metabolomics to gain insight into the underlying physiology and pathophysiology of healthy and diseased individuals.

Chapter 2 describes the effect of age and dietary carbohydrate profiles on glucose and insulin dynamics in healthy horses. Sixteen horses, a combination of Thoroughbred and Standardbred mares and geldings, were divided into two groups by age. Using a balanced Latin square design, horses were fed four isocaloric diets: CONTROL (restricted-starch-and-sugar, fortified pellets), STARCH (control plus kibbled corn), FIBER (control plus unmolassed sugar beet pulp/soybean hull pellets), and SUGAR (control plus dextrose powder). Following dietary adaptation, horses underwent an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT), modified oral sugar test (OST), and a dietary meal challenge. Data were analyzed using a multivariable linear mixed regression model. Aged horses had higher insulin responses to both intravenous and oral glucose challenges. However, the effect of diet on glucose and insulin dynamics was variable depending on the method of assessment.

Chapter 3 describes the effect of dietary carbohydrate profiles and time of year on adrenocorticotropic hormone (ACTH) and cortisol concentrations in adult and aged horses. Following dietary adaptation, thyrotropin releasing hormone (TRH) stimulation tests and overnight dexamethasone suppression tests were performed in March, May, August, and October. Aged horses had higher baseline ACTH and post-dexamethasone cortisol adapted to the starch-rich diet. After controlling for age and diet, baseline ACTH concentrations were significantly increased in October compared to March, May, and August while post-TRH ACTH was higher in August and October compared to March and May. Post-dexamethasone cortisol was significantly higher in October compared to March, May, and August. Diet, age, and time of year are potential confounders on endocrine parameters.

Chapter 4 describes the use of untargeted metabolomics for insight into metabolic adaptations associated with age and dietary carbohydrate profiles. The metabolomic analysis was performed on plasma samples before (day 0) and after dietary adaptation (day 42) as well as during a modified oral sugar test (0 minutes and 75 minutes). The metabolomic profile revealed a large number of metabolite ion peaks (> 2000) were significantly different between age groups and diet groups demonstrating changes in cellular metabolism. On-going analysis and improved metabolite identification are needed to fully interpret this dataset.

Chapter 5 describes the use of metabolomic approaches for insight into metabolic perturbations in Welsh Ponies with insulin dysregulation, obesity, and history of laminitis. The metabolomic analysis was performed on serum samples obtained at 0 minutes (baseline) and 75 minutes during an oral sugar test (OST). Significant metabolite differences, primarily in the lipid and amino acid pathways, were detected between groups which provides new knowledge regarding the pathophysiology of metabolic perturbations.

Chapter 6 focuses on conclusions and future directions based on this research. The effect of age and dietary carbohydrate profiles on glucose and insulin dynamics, ACTH concentrations, and cortisol concentrations are important factors to consider when evaluating hormonal and biochemical parameters. In addition, metabolomics is a powerful tool for defining metabolic changes in different physiologic (age) and pathophysiologic states (insulin dysregulation) and in response to changes in diet.

This dissertation is dedicated to Mom and Dad.
Thank you for your endless support, encouragement, and love.

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CHAPTER 1

Literature Review

INTRODUCTION

Equine endocrine disorders are an ever-growing concern in the horse population. Laminitis, a debilitating and often life-threatening disease of the foot [1,2] affects 15% to 20% of horses over the course of their lifetime [3,4]. Although laminitis has several inciting causes, alterations in insulin dynamics likely play a causal role in the development of the disease. Insulin dysregulation is characterized by an abnormal insulin response to oral glucose and/or feeding which leads to hyperinsulinemia and tissue insulin resistance [5]. Insulin dysregulation is a major health concern as hyperinsulinemia is associated with pasture-associated laminitis and experimentally-induced hyperinsulinemia in healthy horses and ponies results in laminitis [6–8]. In addition, insulin resistance has been linked to equine metabolic syndrome (EMS) [2], pituitary pars intermedia dysfunction (PPID) [9], obesity, and aging. Tissue insulin resistance occurs when the ability of insulin to promote the uptake of glucose from the circulation (primarily tissues such as skeletal muscle and adipose) is diminished due to a decreased responsiveness of the insulin receptors and/or the resulting intracellular signaling [5]. This tissue insulin resistance, together with increased pancreatic sensitivity to glucose, causes the pancreas to produce excessive amounts of insulin in response to dietary nonstructural carbohydrates, leading to hyperinsulinemia.

Horses, non-ruminant herbivores, survive primarily on high roughage diets with varying amounts of protein, fat, and fiber; however, these roughage diets are often supplemented with a grain concentrate to meet the animal's daily energy demand. Previous work has shown that metabolic phenotypes vary due to *physiologic factors* such as age, sex, breed, and genetics [10–12]. In addition to these *innate risk factors*, several studies have linked environmental factors such as nutrition [13,14], forage nonstructural carbohydrate content [15], lack of physical activity [16,17], endocrine disrupting chemicals [18], and alterations in the gut microbiome [19] to insulin dysregulation, obesity, and/or laminitis. Obesity may also alter insulin dynamics, although its role is unclear. Of all the factors contributing to insulin dysregulation,

diet can be manipulated and has been the focus of multiple studies. Current dietary recommendations for horses at-risk for endocrinopathic laminitis include limiting dietary nonstructural carbohydrates thereby reducing the postprandial insulin response. Although these studies have provided insight into factors affecting insulin sensitivity and response in horses, we lack information on underlying physiologic/pathophysiologic mechanisms affecting this system.

In human medicine, the advent of technologies for comprehensive metabolic analysis ("metabolomics") has opened new avenues for understanding metabolic diseases. To this end, recent studies in humans have used metabolomic profiling to reveal characteristic 'metabolic signatures' of type-II diabetes mellitus, obesity, and fatty liver disease. Multiple studies have identified higher concentrations of branched-chain amino acids (and BCAA metabolites), sugar metabolites, and acylcarnitines in type-II diabetes mellitus [20–25]. Moreover, this metabolic signature of type-II diabetes mellitus emerges well in advance (>10 years) of disease onset, highlighting the diagnostic potential of metabolomics. To date, there has been minimal application of metabolomics for the study of metabolic disorders of horses. The *overarching goal* of this dissertation is to use metabolomic approaches to gain deeper insight into metabolic adaptations and perturbations associated with dietary carbohydrate profiles, aging, and insulin dysregulation. *It is hypothesized that changes in tissue metabolism are responsible for changes in tissue insulin sensitivity after dietary adaptation.* The following objectives are integral to the *long-term goals* to understand the physiology and pathophysiology of insulin dysregulation in horses hopefully leading to improved diagnostics, management, and treatment of clinical cases. The **objectives** of this dissertation are to **1)** determine the effect of diet and age on glucose and insulin dynamics; **2)** determine the effect of diet and age on the plasma metabolome, and **3)** determine the relationship between glucose and insulin dynamics and the plasma metabolome.

GLUCOSE AND INSULIN DYNAMICS

Each cell in the body requires glucose to function making it a necessary substrate for survival. Fortunately for horses, glucose can be found in plants in the form of a simple monosaccharide in addition

to being obtained from carbohydrate metabolism. Serum or plasma measurements of glucose from the peripheral blood reflect the net effect of absorption, liver utilization, and peripheral tissue uptake. However, too much glucose can have a negative effect and must be regulated. Insulin, a polypeptide hormone produced by beta cells in the islets of Langarhans in the pancreas, regulates blood glucose levels by increasing the uptake of glucose into tissues (liver, muscle, adipose) and storage as glycogen or lipid. Insulin consists of a α chain (21 amino acids) and a β chain (30 amino acids) joined by two disulfide bonds [26] and is important in the regulation of carbohydrate and lipid metabolism. Serum or plasma measurements of insulin from the peripheral blood reflect the net effect of pancreatic secretion and insulin clearance. In the liver, insulin increases glucose uptake and formation of glucose-6-phosphate. It also activates a phosphatase enzyme that dephosphorylates and activates glycogen synthetase. The role of insulin in muscle may be the most extensive. Insulin stimulates the uptake of glucose and amino acids, stimulates glycogen and protein synthesis, and increases blood flow and nutrient supply to the muscles through direct vasodilatory mechanisms. The muscles enhanced sensitivity to insulin leads to rapid growth and leanness; however, in the case of decreased insulin sensitivity, a decreased growth rate and increased amount of carcass fat will be present.

INSULIN AND CARBOHYDRATE METABOLISM

Following consumption of a carbohydrate meal, glucose is absorbed into the portal circulation via the small intestine. An increase in blood glucose triggers the release of insulin thereby causing glucose to enter the liver, muscle, and adipose tissue via the GLUT-4 transporters. In the liver, insulin causes glucose uptake and storage in the form of glycogen via the following mechanisms: inactivation of liver phosphorylase, increased activity of the enzyme glucokinase, and increased activity of glycogen synthase [27]. Liver phosphorylase is an enzyme that splits glycogen into glucose thereby preventing the breakdown of liver glycogen. Glucokinase causes phosphorylation of glucose thus trapping it inside the liver cells. Glycogen synthase promotes glycogen synthesis through polymerization of the monosaccharide units that

form glycogen molecules. In addition, in the liver, insulin promotes the conversion of excess glucose (not able to be stored as glycogen) into fatty acids, packaged into triglycerides, and transported to adipose tissue.

INSULIN AND LIPID METABOLISM

The presence of insulin increases the production and storage of triglycerides in adipose tissue and inhibits hydrolyzation of triglycerides to fatty acids. Insulin promotes fatty acid synthesis by increasing the transport of glucose into liver cells. Glucose is split into pyruvate and subsequently converted to acetyl coenzyme A (acetyl-CoA), the substrate necessary for synthesis of fatty acids. The fatty acids are used to form triglycerides, which is the usual form of storage fat in adipose tissue. Insulin also promotes glucose transport into fat cells where the glucose is synthesized into α-glycerol phosphate. This substance supplies the glycerol, which combined with fatty acids forms triglycerides. A decrease in circulating insulin promotes hydrolyzation of triglycerides and release of glycerol and non-esterified fatty acids (NEFAs) into circulation to be used as energy via gluconeogenesis and β-oxidation.

DIETARY DETERMINANTS OF POSTPRANDIAL GLUCOSE AND INSULIN

The horse is well-adapted to a high structural carbohydrate (cellulose and hemicellulose) diet but supplementation with nonstructural carbohydrates (starch, sugar, fructans) often occurs. The glycemic index, influenced by the type of carbohydrate, of a feed characterizes the postprandial glycemic response to a measured amount of feed [28]. Hay is often classified as having a low glycemic index while grains have a high glycemic index. Starch and sugar concentrates have a higher glycemic index compared to primarily fat and fiber concentrates [29]. Many factors including feed type, feed amount, and feed processing technique may influence postprandial glycemic and insulinemic responses.

Feed Type and Processing

Determining the most appropriate concentrate feed for horses to meet daily energy requirements without adverse effects remains a challenge. Several studies have linked dietary components such as meal

size, amount of starch, type of grain and method of grain processing to metabolic adaptations and perturbations. Based on meal size alone, when all other components are equal, consumption of a small meal empties the stomach faster than a large meal [30]. In addition, feeds with an increased starch content had a slower gastric emptying rate compared to feeds with a low-starch content. An increase in glucose and insulin responses of horses was seen with an increase in starch intake most likely due to glucose absorption in the small intestine [31,32]. Several studies have evaluated the processing techniques with variable results. Consumption of corn [33] or oats [33] yielded an increase in glucose and insulin concentrations; however, the processing technique did not influence these parameters. In contrast, feeding steam-processed corn resulted in a greater glycemic response compared to cracked or ground corn [34,35]. In addition, the processing technique of barley did play in role in the horses glycemic and insulinemic response; extruded barley yielded the highest concentrations and rolled barley yielded the lowest concentrations [36].

Forage Carbohydrates

A forage analysis can be a worthwhile tool when evaluating and designing a nutrition regimen as carbohydrate (starch, sugar, cellulose) concentrations can influence glucose and insulin responses. The nonstructural carbohydrate (NSC) content of pasture and hay, the forages most commonly consumed by horses, are variable. Nonstructural carbohydrate, the percentage of starch plus percentage of water-soluble carbohydrate, concentration greater than 10% (dry matter basis) may have adverse effects on glucose and insulin dynamics, especially in horses with metabolic dysregulation [2].

Addition of Fiber, Oil, Simple Sugars

There are several different components that may be added to an equine ration. The addition of fiber to a high starch meal did not alter glycemic and insulinemic responses [37]. However, diets higher in fat and/or fiber and lower in starch may decrease insulin concentrations but findings are inconsistent across studies [38–40]. Feeding glucose and fructose to healthy ponies resulted in higher glucose and insulin concentrations compared to control ponies [41].

INNATE AND ENVIRONMENTAL DETERMINANTS OF GLUCOSE AND INSULIN

Alterations in glucose and insulin dynamics may vary due to innate (physiologic) risk factors such as age, sex, breed, pregnancy, and genetics [10–12] and environmental risk factors such as such as nutrition [13,14], forage nonstructural carbohydrate content [15], lack of physical activity [16,17], and endocrine disrupting chemicals [18]. In humans, aging is associated with the development of glucose intolerance and insulin resistance with exaggerated glucose and insulin responses to a carbohydrate challenge [42,43]. Following an oral glucose challenge, aged horses (27 ± 0.4 years) had a greater insulin response compared to middle-aged horses (15.2 ± 0.4 years) and young horses (6.8 ± 0.4 years) [44]. Further, mature horses (14.2 ± 0.5 years) had reduced insulin sensitivity compared to young horses (2.0 ± 0.1 years) [35]. A newborn foal can display transient insulin resistance in the first 48-hours of life as the pancreatic β-cells are maturing [45].

Breed is another factor that may influence glucose and insulin dynamics. Previous studies have shown that ponies have reduced insulin sensitivity compared to horses [46]. Further, donkeys have reduced insulin sensitivity compared to ponies and horses [47]. Certain breeds such as Morgans, Arabians, Warmbloods, and Welsh Ponies are at greater risk for development insulin dysregulation [10–12,48]. Overall, there appears to be a genetic predisposition to alterations in glucose and insulin dynamics.

Pregnancy can also influence glucose and insulin parameters. Fowden et al. [49] showed that there are significant changes in carbohydrate metabolism during pregnancy which includes hyperinsulinemia, enhanced β cell secretion to endogenous and exogenous glucose, and exaggerated responses to fasting and feeding. In a cohort of Thoroughbred mares, insulin sensitivity in pregnant animals (25 to 31 weeks gestation) was significantly lower compared to nonpregnant mares [50]. In addition, Hoffman et al. [51] demonstrated that reproductive status influenced glucose and insulin metabolism in grazing mares consuming varying carbohydrate diets.

Physical activity may improve glucose and insulin parameters. Previous studies, in a cohort of Standardbred horses, have shown that exercise training resulted in improvement of insulin sensitivity [52,53]. Further, exercise training resulted in a decrease in acute insulin response to glucose (AIRg) and

improved insulin sensitivity in both obese and non-obese horses [54]. These findings are consistent with findings in other species as studies in humans and rats demonstrated improved insulin sensitivity following short periods of exercise [55,56].

MEASUREMENT OF GLUCOSE AND INSULIN DYNAMICS

Determining insulin sensitivity of an individual has been a focus in both human and equine biomedical research. Collection of a single basal (fasting) blood sample is the simplest non-specific measurement of circulating glucose and insulin levels, but the diagnostic interpretation is difficult. The finding of basal hyperglycemia (> 110 mg/dL), decrease in tissue glucose uptake, does not distinguish between a decrease in tissue insulin sensitivity versus an increase in insulin secretion. These values can fluctuate substantially due to environmental factors (stress, feeding, diurnal and seasonal variation). A basal insulin concentration of > 20 mU/L has been suggested as a cut-off to indicate an increase in insulin secretion due to insulin insensitivity [5]. False-negatives may arise as hyperinsulinemia may be persistent or be present for only a few hours postprandially. These basal values may also be used as proxy estimates for insulin sensitivity and secretion by evaluating the glucose-to-insulin ratio (positive correlation to insulin sensitivity) and the insulin-to-glucose ratio (positive correlation to insulin secretion) [57].

Glucose and insulin concentrations may also be measured via response to an intravenous or oral glucose or feed challenge. These challenges, also known as dynamic tests, approximate glycemic and insulinemic challenges to exogenous glucose and/or insulin. These dynamic tests are generally used to quantify tissue insulin sensitivity, pancreatic islet cell responses, or both. The euglycemic-hyperinsulinemic clamp (EHC), considered to be the reference method (“gold standard”) for measurement of tissue insulin sensitivity, looks to maintain a physiologic glucose concentration by adjusting the intravenous glucose concentration in response to an intravenous constant rate infusion of insulin simulating glucose disposal [57]. The insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT) aims to assess glucose and insulin dynamics under physiologic insulin concentrations [58]. Minimal model analysis of glucose and insulin measurements during the FSIGTT yields estimates of tissue insulin sensitivity (SI;

insulin-mediated glucose disposal), acute insulin response to glucose (AIRg; a measure of the degree of insulin secretory response to glucose), glucose-mediated glucose disposal (Sg), and disposition index (DI = SI x AIRg; describes the pancreatic beta-cell response). The glucose tolerance test involves serial measurement of blood glucose concentrations following intravenous administration of dextrose. Evaluation of the time it takes for the glucose concentration to return to baseline demonstrates the individual's ability to absorb, utilize, and store glucose. The insulin tolerance test measures serial glucose concentrations following intravenous administration of insulin. In healthy individuals, blood glucose concentration should decline to 50% below baseline within thirty minutes of insulin administration. Exaggerated glucose responses may suggest impaired pancreatic insulin secretion or impaired glucose disposal [57]. The combined glucose insulin tolerance test (CGIT) assesses the effects of simultaneous administration of dextrose and insulin via serial measurement of blood glucose concentrations [59]; however, repeatability of this test is poor [60].

Unlike intravenous challenges, oral challenges approximate postprandial glucose and insulin responses. The oral glucose tolerance test (OGTT) measures glucose and insulin concentrations following administration of a measured amount of oral glucose [61]. The oral sugar test (OST) [62], a modification of an oral glucose tolerance test, measures glucose and insulin concentrations after oral administration of commercially available corn syrup (Karo® light). An in-feed challenge or standardized meal challenge measures an animal's glucose and insulin dynamics to a predetermined amount of a cereal grain-based meal (such as sweet feed). In addition, a dietary meal challenge may be performed to determine the glycemic and insulinemic response to a particular diet to which the horse is adapted.

WHAT IS METABOLOMICS?

Applications

Metabolomics, the study of molecules involved in cellular metabolism, refers to a global interrogation of the biochemical components in a biological sample (serum, plasma, urine, saliva, cerebrospinal fluid). Metabolite profiles are powerful tools for defining metabolic changes in physiologic

and pathophysiologic states and may aid in understanding the mechanism of disease. Small molecule metabolites such as lipids, amino acids, peptides, nucleic acids, organic acids, fatty acids, vitamins, carbohydrates, hormones, and steroids are the end products of cellular regulatory processes. As the ultimate response of biological systems to genetic or environmental changes, the comprehensive measurement of metabolites reflects perturbations in metabolism thus providing insight into biological mechanisms and pathogenesis of disease through an understanding of molecular pathways.

Used as a diagnostic tool, metabolomics can detect disease prior to the onset of disease, which allows for earlier intervention into prevention and treatment. In addition, metabolomics gives us the ability to better understand the mechanisms of disease occurrence in both the physiological and pathological states. On the most basic level, metabolomics can differentiate between healthy individuals and diseased individuals [63–65]. However, taking it one step further, it can provide sub-classification of disease types by separating individuals based on the underlying pathophysiology within the same disease category. For example, are all individuals with type-II diabetes the same or are there metabolite differences that detect variation in the underlying pathophysiology and disease mechanisms. This technique allows for exploration of the in-depth interaction between compounds in a biological sample and their role in complex biological systems [66]. It reflects changes downstream of genomic, transcriptomic, and proteomic fluctuations representing an organism in health and disease.

Metabolomics involves two complementary approaches, untargeted and targeted analyses. Untargeted metabolomics examines as many metabolites as possible whereas a targeted analysis quantifies discrete groups of chemically related metabolites. Studies in humans have used metabolomic profiling to reveal characteristic metabolic signatures of type-II diabetes, obesity, and fatty liver disease [20–25]. More than 4,000 metabolites have been identified in human serum [67] by high-throughput mass spectrometry and chromatography. The use of metabolomics has the potential to provide information to understand physiology and pathophysiology and can be a useful tool for understanding the impact of genetic and environmental factors.

Sample Preparation

Analysis of biofluids (serum, plasma, urine, saliva, cerebrospinal fluid) reflects organism-level perturbations in metabolism [63]. Biofluids are often the favored sample type for analysis as they are minimally invasive, representative of organism metabolism, and possess an extensive array of high-throughput experimental protocols for metabolite extraction [68,69]. Protein precipitation and extraction of metabolites from high-molecular-weight macromolecules is required. Three solvent systems (methanol/water, methanol/acetonitrile/water, methanol/chloroform/water) have been shown to extract a wide range of hydrophilic compounds with variable efficiency for mass spectrometry and nuclear magnetic resonance (NMR) [70–73]. The extraction protocol is influenced by study design and sample type.

Separation and Detection of Analytes

Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are two powerful approaches for the analysis of small molecules in targeted and untargeted experimental designs. The dominance of NMR and MS in metabolomics is facilitated by their capacity to identify metabolites and quantify their relative abundance via high-throughput sampling without significant reductions in sensitivity or resolution [74]. However, these single platform approaches are unable to adequately quantify all metabolites in a sample thus the combination of multiple analytical platforms provides greater coverage of metabolism.

Nuclear Magnetic Resonance (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is a technique that uses the magnetic properties of atomic nuclei to determine the physical and chemical properties of atoms within a mixture. This technique allows for a high-resolution, rapid analysis; however, it has a low sensitivity with more than one peak per component [75]. A simplified NMR spectrometer is comprised of four components: a sample tube, a superconducting magnet, a radio frequency transmitter, and a radio frequency transmitter and amplifier [76]. A deprotonated sample is pipetted into an NMR tube and placed in the machine. A super conducted magnet creates a magnetic field that orients magnetic atomic nuclei parallel to the field. Nuclei aligned with

the magnetic field are in a low-energy state while those aligned against the magnetic field are in a high-energy state. The most common nuclei analyzed are ^{1}H and ^{13}C, whose unique chemical shifts in NMR analysis is representative of the backbone of most biological molecules.

Mass Spectrometry (MS)

Mass spectrometry is an analytical technique that measures the characteristics of individual molecules by sorting ions based on their mass to charge ratio. A mass spectrometer has three associated components: an ion source, a mass analyzer, and a detector. Ionization is required to induce vaporization of metabolites prior to analysis, converting each compound into a charged ion to be detected in the instrument. Mass analyzers differentiate ions by their mass to charge ratio, selecting predefined masses for analysis or providing a full-scan. Detectors measure the abundance of ions sampled. Mass spectrometry coupled with separation techniques such as liquid or gas chromatography enhance the analysis of complex mixtures while improving the resolving power of the compounds. Tandem spectrometry (MS/MS) facilitates the structural elucidation of compounds through fragmentation. A combination of separation and fragmentation, accompanied with authentic standards, represents the gold standard for compound identification in metabolomics.

Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography-mass spectrometry allows for the analysis of volatile compounds such as eicosanoids, carotenoids, flavonoids, and lipids in complex samples. Gas chromatography is a separation technique in which the mobile phase is gas. It is a trusted and well-established technique in analytical chemistry as it is robust and sensitive with large commercial and public libraries available for compound identification. However, this technique is slow, often requires derivatization, and many analytes are too large and thermally-unstable for the analysis [75]. Vaporized compounds flow through a capillary column with an inert gas, separating compounds as they interact with the column packing based on biochemical properties. As compounds exit the column they are ionized, typically through electron impact (EI)

ionization. This form of ionization, categorized as hard ionization, often fragments the compound during vaporization allowing for compound identification without MS/MS. To avoid misidentification from poor fragmentation or co-eluting compounds, tandem GC-MS/MS or two-dimensional separation GC×GC/MS are utilized to produce the highest confidence identifications. Many consider GC-MS to be the "gold standard" for identification in analytical chemistry because of its sensitivity, specificity, and reproducibility of results.

Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid chromatography is a separation technique in which the mobile phase is liquid. This technique has many modes of separation available and can accommodate a large sample capacity; however, it is slow with a limited number of commercial libraries available for compound identification [75]. High-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) are techniques that utilize pressurized liquid solvents to separate, identify and quantify each component in a mixture. UHPLC-MS allows for the analysis of non-volatile compounds such as amino acids, bile acids, fatty acids, sterols and carboxylic acids in complex samples. Similar to gas chromatography, liquid chromatography flows compounds through a capillary column with an inert liquid, separating compounds as they interact with the column packing based on biochemical properties. The choice of packing material and mobile phase influences the class of compounds analyzed. Normal phase liquid chromatography utilizes a polar stationary phase and nonpolar mobile phases, separating compounds by hydrophilicity. Reverse phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) reverse the separation polarity, separating compounds by hydrophobicity. HILIC allows for identification of polar molecules such as carbohydrates and sugars in complex samples. Targeted and untargeted metabolomics by LC-MS is a very popular approach for metabolic profiling. A wide-breadth of chemical classes may be detected, and open-source and commercial software is available for analysis.

Analysis of Metabolomics Data

Analysis of metabolomics data requires specialized mathematical and statistical tools. Prior to tackling statistical modeling, the raw data must be sequentially processed in multiple phases: file conversion, feature detection, alignment, normalization, and metabolite identification [77]. Once a data matrix has been created, metabolomics data can be analyzed using standard statistical methods for identifying differences between experimental groups (i.e. ANOVA, linear regression) for individual metabolites. However, due to the complexity of metabolomic datasets, and the correlation between individual metabolites, multivariate analysis is often required to account for the conditions of the study design.

In addition to standard regression analyses, unsupervised and supervised statistical and machine-learning algorithms are typically employed to identify patterns across metabolites. Unsupervised methods group data without using information from predefined labels or classifiers. Unsupervised methods include clustering analysis [78] and principal components analysis (PCA) [79]. Unsupervised principal components analysis, often used as a starting point in the analysis, clusters samples based on the variance of signals in the metabolite profile [80]. It is a method to reduce the dimensionality of the data while retaining the maximum amount of information. The first principal component is the linear combination of the original variables that explain the greatest amount of variation, the second component is the linear combination of the original variables that account for the greatest amount of variation uncorrelated to the first component, the third component is the linear combination of the original variables that account for the greatest amount of variation uncorrelated to the first and second component, and so on. Clustering analysis divides the datasets into subclasses (clusters) using hierarchical or nonhierarchical algorithms. In both types of clustering, the similarity between pairs of objects is used to group them into subsets (clusters) that have meaning. However, in nonhierarchical clustering the relationship between clusters is undetermined, whereas in hierarchical clustering the relationship between clusters is determined. Hierarchical clustering shows large-scale differences by determining the similarity of two samples and pairing them together in the same cluster, and then by determining the relationship between two clusters and pairing them together, this

continues until all pairs of clusters are linked creating a structure (the hierarchy) that shows the relationships between all samples/clusters.

In contrast to unsupervised methods, supervised methods rely on predefined groups or classes of data (e.g., case vs. control). Supervised methods, including partial least squares discriminant analysis (PLS-DA) and random forest analysis, are used to identify the metabolite(s) most useful for classification of experimental samples (e.g. horse) into groups (i.e., case vs. control). Partial least squares discriminant analysis constructs predictive models based on the regression of class information [81]. It is a method used to sharpen the separation between groups. Random forest is a technique that distinguishes two groups by assembling a decision tree without dimensional reduction [82]. Finally, interpretation of metabolomics data utilizes heatmaps or pathways to visualize changes and responses of metabolites within the study.

Use of Metabolomics for Understanding Metabolism and Disease

The measurement of small cellular molecules within a tissue or biofluid provides a footprint of the whole body's metabolic processes. The quantification of cellular compounds from a biological sample provides information about the disruption of metabolic processes in endogenous and exogenous pathways and insight into physiology and pathophysiology of an individual. Further, these compounds (i.e. metabolites) can serve as biomarkers for early detection of disease thus leading to early treatment intervention.

Metabolomics in Human Metabolic Disease

Human metabolic diseases are heterogeneous in nature as both innate and environmental factors influence disease development and progression. The use of metabolomics to understand the link between obesity, insulin resistance, and type-II diabetes mellitus has provided valuable information regarding disease onset and pathophysiology. Obesity is at the forefront of human medicine as it predisposes individuals to potentially life-threatening diseases such as type-II diabetes mellitus and cardiovascular disease. Obese individuals also suffer from metabolic alterations characterized by hyperglycemia,

hyperlipidemia, and insulin resistance. Type-II diabetes mellitus develops once insulin secretion cannot compensate for insulin resistance. Several studies in humans have identified plasma metabolites associated with obesity, insulin resistance, glucose intolerance and type-II diabetes mellitus. Further, these metabolites indicate that primary pathway disruption occurs in carbohydrate metabolism, tricarboxylic acid cycle, lipid metabolism, and amino acid metabolism.

Several metabolites have been identified as potential biomarkers for obesity. Amino acids (tyrosine, phenylalanine, alanine, proline), branched-chain amino acids (leucine, isoleucine, valine), and phospholipids were positively correlated with body mass index [83] and hyperlipidemia [84]. Specifically, branched-chain amino acid concentrations are increased in obese individuals [85–88]. Further, acylcarnitines [85,86,89,90] and lipids such as phospholipids [91] and non-esterified fatty acids [92] are altered in obese individuals. These individuals have an increased availability of lipids; however, free fatty acid oxidation is blunted as evident by increased acylcarnitine concentrations [93].

Metabolites have also been identified as potential biomarkers for insulin resistance and type-II diabetes mellitus. Branched-chain amino acids, amino acids, and acylcarnitines are markers of early insulin resistance [65,94–96]. Further, a consistent pattern of reduced glycine and acylcarnitines [25,97,98] with increased concentrations of valine, isoleucine and α-hydroxybutyrate [99,100] has been associated with both basal and dynamic measures of insulin resistance and higher concentrations of branched-chain amino acids, sugar metabolites, and acylcarnitines are noted in type-II diabetes mellitus [21–25,100]. More specifically, alterations in α-hydroxybutyrate and linoleoylglyceraphosphocholine have been identified as joint markers of insulin resistance in type-II diabetes mellitus individuals [96]. In addition, alterations in carbohydrate metabolism and the tricarboxylic acid cycle, including increases in pyruvate, lactate, and citrate as well as decreases in malate, fumarate, and succinate are seen with type-II diabetes mellitus [101,102]. Importantly, metabolic perturbations characterizing and contributing to type-II diabetes mellitus are evident years before disease onset and comprehensive metabolomic profiling has been used to elucidate alterations in novel metabolic pathways implicated in disease development.

Metabolomics in Veterinary Medicine

A limited number of studies in domestic animals (horses, cows, dogs, cats) have identified metabolites associated with individual variability and dietary profiles. The metabolite profile is truly unique to an individual as Colyer et al. [103] illustrated the variance in metabolite profiles of dogs and cats fed the same diet. While dogs and cats are different species there are metabolite commonalities; however, the majority of the variability occurred within lipid metabolism [103]. Further, within the same species, variability across breeds exists. Breed-specific dietary metabolism can be detected by examining metabolite profiles using urine [104] and plasma [105]. Breed and gender are the main drivers of variance.

The understanding of the complex interaction between the individual and the environment, particularly diet, remains elusive; however, nutritional metabolomic studies in dogs and cats begin to provide worthwhile information. In human medicine, obesity is a known risk factor for the development of insulin resistance and diabetes, and metabolomics has been used for identification of biomarkers for dietary assessment. The ability to apply this technique to veterinary medicine may allow for better nutritional management of healthy and diseased animals, but the measurement of metabolites is costly. Obesity in domestic cats is a common nutritional disorder. Overweight cats are at risk for medical abnormalities such as insulin resistance, diabetes, hepatic lipidosis, and reduced lifespan. Obesity most commonly results from an imbalance between energy intake and its expenditure but also may result from hypothyroidism, insulinoma, and hyperadrenocorticism. In a study by Deng et al. [106] changes in the feline blood metabolome were evaluated in response to dietary macronutrient composition (high-fat, high-protein, high-carbohydrate). Examination of the metabolome detected distinct differences between diets with primary changes in amino acid and lipid metabolism. Cats on the high-protein diet had decreased nucleotide catabolism and increased amino acid metabolism and gut microbial metabolism. Cats on the high-fat diet had increased lipid metabolism. The following three potential biomarkers were identified to distinguish between diets: γ-glutamylleucine, 3-hydroxyisobutyrate, and 3-indoxyl sulfate [106].

Metabolomics has been used in dairy cattle for improving milk production which could have a significant impact on the agricultural industry. In a study that examined the effect of forage quality on milk

production, biofluids yielded metabolites and metabolic pathways that may serve as potential biomarkers for higher milk yield and higher quality milk protein [107]. Further, another study demonstrated an association between somatic cell counts and metabolite profiles in milk. Milk samples with high somatic cell counts (720,000 cells/mL) had increased concentrations of lactate, butyrate, isoleucine, acetate, and β-hydroxybutyrate compared to milk samples with low somatic cell counts (14,000 cells/mL) [108].

Equine studies have used metabolomics to gain insight into various aspects of equine medicine and disease such as the bacterial community and volatile organic compounds of healthy Thoroughbred horses [109], detection of steroidal aromatase inhibitors in performance horses [110], impact of exercise [111,112], sepsis [113], and breed differences [114].

CONCLUSION

Understanding the effect of age and dietary carbohydrate profiles on glucose and insulin dynamics and the plasma metabolome will improve the welfare of horses. The application of metabolomics has the potential to improve the health and well-being of animals and to provide information about physiology and pathophysiology of aging and insulin dysregulation. Identification of differing metabolites between the healthy and diseased phenotypes has the potential to serve as a diagnostic tool. In addition, the ability to define a metabolomic signature may reveal specific biomarkers that predict and/or diagnose metabolic abnormalities leading to a better understanding of disease processes that may help identify new therapeutic targets. Overall, metabolomic profiling will be a relevant approach for further defining equine metabolic alterations and perturbations.

CHAPTER 2

Effect of Age and Dietary Carbohydrate Profiles on Glucose and Insulin Dynamics in Horses

SUMMARY

Background: Glucose and insulin dynamics may be different in adult and aged horses.

Objectives: To determine the effect of age and dietary carbohydrates on glucose and insulin dynamics in healthy horses.

Study Design: Balanced Latin square design with four isocaloric diets: *control* (restricted-starch-and-sugar, fortified pellets), *starch* (control plus kibbled corn), *fiber* (control plus unmolassed sugar beet pulp/soybean hull pellets), and *sugar* (control plus dextrose powder).

Methods: Sixteen healthy Thoroughbred and Standardbred horses were divided into two groups by age: *adult* (8.8 ± 2.9 years; n = 8) and *aged* (20.6 ± 2.1 years; n = 8). Following dietary adaptation, horses underwent an insulin-modified intravenous glucose tolerance test (FSIGTT), modified oral sugar test (OST), and a dietary meal challenge. Outcome variables included: insulin sensitivity (SI), disposition index (DI), glucose effectiveness (Sg), and acute insulin response to glucose (AIRg) from the FSIGTT; peak glucose, peak insulin, time to peak, area under the curve for glucose (AUCg) and insulin (AUCi) from the OST and dietary meal challenge. Data were analyzed using a multivariable linear mixed regression model.

Results: AIRg was higher in *aged* (582.0 ± 59.1) compared to *adult* (358.0 ± 62.2; $P = 0.03$) horses. *Adult* and *aged* horses had a higher SI on the *sugar* (adult: 3.4 ± 0.4; aged: 4.0 ± 0.4) diet compared to the *control* (*adult*: 2.0 ± 0.4, $P = 0.009$; *aged*: 1.4 ± 0.4, $P \leq 0.001$) and *fiber* (*adult*: 2.0 ± 0.5, $P = 0.014$; *aged*: 2.4 ± 0.4, $P = 0.004$) diets. Feeding a single *starch* (*adult*: 21581.0 ± 3273.0; *aged*: 35205.0 ± 2996.0) or *sugar* (*adult*: 26050.0 ± 3072.0; *aged*: 25720.0 ± 2963.0) meal resulted in postprandial hyperinsulinemia (AUCi).

Main Limitations: The study cohort contained two different insulin-sensitive breeds.

Conclusions: Age and diet should both be considered when evaluating glucose and insulin dynamics.

INTRODUCTION

Understanding the relationship between glucose and insulin dynamics, age, and dietary adaptation in horses is important due to their association with metabolic diseases such as equine metabolic syndrome and/or pituitary pars intermedia dysfunction. However, a number of other innate (breed, sex, adiposity, genetics) and environmental (diet, exercise) factors affect insulin dynamics in equids [10,16,48,52,58,115]. Previous studies have demonstrated that insulin responses are higher in aged horses than young horses. These differences have been demonstrated in studies evaluating insulin responses to intravenous glucose challenge (aged: 22.0 ± 0.7 years; young: 7.3 ± 0.6 years) [54], oral glucose challenge (aged: 14.2 ± 0.5 years; young: 6.8 ± 0.4 years) [44] and to feeding (aged: 14.2 ± 0.5 years; young: 2.0 ± 0.1 years) [35].

Insulin sensitivity and insulin response to feeding are also impacted by dietary composition. In animals kept at pasture, exacerbation of hyperinsulinemia and incident laminitis often coincides with an increase in forage nonstructural carbohydrate content [14]. Other studies have reported that the feeding of a starch-rich diet results in a decrease in insulin sensitivity when compared to feeding low-starch diets that contain higher fat (oil) and/or fiber content [52,58,116,117]. However, there have been few reports on the effect of diet on glucose and insulin dynamics in older horses. Nielson et al. [35] reported that age was positively correlated with the magnitude of glycemic and insulinemic responses to feeding, while Rapson [118] reported higher tissue insulin sensitivity in aged horses adapted to a hay and grain diet when compared to a hay only diet. The latter is a surprising finding in light of earlier studies in adult horses that demonstrated reduced insulin sensitivity after adaptation to starch-rich feeds.

Given the association between aging and abnormal response to oral glucose or feeding leading to hyperinsulinemia and/or tissue insulin resistance (insulin dysregulation) [5], and evidence that dietary effects on glucose and insulin regulation may be different in adult and aged horses, it is clear that the impact of diet on glucose and insulin dynamics in aged horses needs further evaluation. Our objectives were to 1) evaluate the effect of adaptation to diets containing varying amounts of starch, sugar, and fiber on glucose and insulin dynamics in healthy adult and aged horses as assessed by minimal model analysis of an insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT) as well as glucose and insulin

concentrations during a modified oral sugar test (OST); and 2) to determine the postprandial glycemic and insulinemic responses to meal consumption for each diet. We hypothesized that adaptation to differing carbohydrate diets would alter glucose and insulin dynamics, and aged horses would have an exaggerated insulin response to intravenous and oral glucose as well as to feeding starch and sugar rich diets when compared to adult horses.

MATERIALS AND METHODS

Horses and Groups

Sixteen healthy Thoroughbred (TB) and Standardbred (STB) mares and geldings were divided into two groups by age: *adult* (5 to 13 years old; 8.8 ± 2.9 years; n = 9; 4 TB mares, 1 STB mare, 3 TB geldings, 1 STB gelding) and *aged* (18 to 24 years old; 20.6 ± 2.1 years; n = 9; 3 TB mares, 6 STB mares). Horses originated from a single source. Sixteen horses were used in each dietary period (8 per age group); however, one aged horse and one adult horse had to be replaced during the study due to failure to eat the diet (n = 1) and a colonic torsion, not considered to be associated with diet, that resulted in euthanasia (n = 1). Replacements were the same sex and similar in age. Prior to the study, horses were maintained on pasture for an acclimation period of at least one month. One week prior to the study, horses were acclimated to the dry lot conditions used throughout the study. All animals received routine anthelmintic, vaccination, dental, and farrier treatment as appropriate.

Study Design and Diets

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University. The study was performed from February to October. Horses were randomly assigned to groups of four, blocked for age, and fed four isocaloric diets using a balanced Latin square design. The *control* diet consisted of restricted-starch-and-sugar, fortified pellets[a]. For the three remaining diets, a portion of the *control* diet was removed, and the appropriate energy substrate rich complementary feed added: *starch* (control plus kibbled corn[a]), *fiber* (control plus unmolassed sugar beet pulp/soybean hull pellets[a]), and *sugar*

(control plus dextrose powder[b]). All horses received four 7-week dietary treatments, with the total ration being fed at a daily rate of 2.0% to 2.2% of bodyweight (hay: 1.2% and concentrate: 0.8% - 1.0%). During each dietary period, horses were group fed the same batch of grass hay (mean of three determinations during the study nonstructural carbohydrate (dry matter basis) = 13.1% ± 0.4%) once daily and individually fed two meals of one of the above diets at 7:00 and 17:00. Quantity was pre-determined based on the horse's weight tape[c] estimated weight and delivered at approximately 0.18 megajoules per kilogram bodyweight digestible energy (DE). For a 500-kg horse this equates to: *control* (4.0-kg), *starch* (control: 2.3-kg and kibbled corn: 2.0-kg), *fiber* (control: 2.0-kg and unmolassed sugar beet pulp/soybean hull pellets: 2.8-kg), and *sugar* (control: 3.1-kg and dextrose powder: 1.0-kg) per day. Further details of the dietary composition are described in **Table 2.1**. Horses were gradually introduced to each diet during the first five days of each period. For each meal, horses were given sixty minutes to consume the diet, after which any remaining feed was removed, weighed (orts) and recorded. Dietary periods were separated by a two-week washout period during which all horses received the control diet and free access to the same hay and pasture.

Prior to, and following each dietary period, bodyweight of the horses was estimated by use of a weight tape[c] and body condition score (BCS) determined (1-9; Henneke scale) [119] by two evaluators. Basal insulin measurements (adult 13.3 ± 0.5 mU/L and aged: 13.6 ± 0.6 mU/L) were obtained prior to each dietary period as a crude estimate of insulin status. During the 6th week of each dietary period, the glycemic and insulinemic responses to the diets were assessed. In the 7th (final) week of each dietary period, each horse underwent an insulin-modified FSIGTT and a modified OST with at least 48-hours between each test (randomized, blocked by age and diet). An overnight dexamethasone suppression test [120–122] was performed to evaluate pituitary function at least 24-hours after dynamic testing.

Minimal Model

An insulin-modified FSIGTT [58] was administered after 10-hours of feed withholding (overnight). An intravenous catheter was placed in the jugular vein one hour prior to the start of testing. A baseline blood sample was taken at -10 and -1 minutes prior to the rapid intravenous administration of 300 mg/kg

bodyweight glucose administered as a 50% solution followed, at 20 minutes' post dextrose, by administration of an intravenous bolus of insulin (20 mU/kg bodyweight) (Humulin R[d]). Blood samples for glucose and insulin measurement were collected at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, 19, 22, 23, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, 180, 210 and 240 minutes' post glucose administration.

Minimal model analysis [123,124] (MINMOD Millennium, version 6.02[e] and WinSAAM[f]) of the glucose and insulin data yielded estimates of insulin sensitivity (SI ($L \cdot min^{-1} \cdot mU^{-1}$); insulin-mediated glucose disposal), acute insulin response to glucose (AIRg ($mU/L \cdot min^{-1}$); the endogenous insulin secretory response to glucose), glucose effectiveness (Sg (min^{-1}); glucose-mediated glucose disposal), and disposition index (DI = SI x AIRg).

The following parameters were calculated to explore the magnitude of the deflection in blood glucose below baseline (hypos): baseline glucose (Gb), lowest glucose concentration below baseline (Gmin), time point at lowest glucose concentration (Tmin), glucose concentration at the sampling endpoint (Ge), percent deflection of glucose below baseline (dGb = ([Gmin – Gb] x 100/Gb), percent deflection of glucose below the sampling endpoint (dGe = [Ge – Gmin] x 100/Ge), area under the curve below baseline glucose (HAUC), and the time point where glucose deflection returned to baseline (Ttmax).

Oral Sugar Test

A modified OST [62] was performed after 10-hours of feed withholding (overnight), as a standardized challenge to compare the horses' responses after dietary adaptation. After placement of a catheter in the jugular vein, one-hour prior to the commencement of testing, and collection of baseline blood samples (-10 and -1 minutes' relative to dosing), a commercially-available corn syrup (Karo® light[g]) was administered orally by use of a dose syringe (0.25 mL/kg bodyweight). Additional blood samples were collected at 15, 30, 60, 75, 90, 120, 150, and 180 minutes. The following parameters were calculated (GraphPad Prism, version 6.07[h]): peak glucose, peak insulin, time to peak, and area under the curve [AUC] for glucose and insulin.

A higher dose of Karo® syrup was used, 0.25 mL/kg bodyweight instead of 0.15mL/kg bodyweight [64], because our previous work has demonstrated that the higher dose is needed to elicit a consistent insulinemic response in some light breed horses [12].

Dietary Meal Challenge

A dietary meal challenge was performed to evaluate the effect of feeding a single meal on postprandial glucose and insulin dynamics for a particular diet (i.e. *control*, *starch*, *fiber*, or *sugar*). Following a 10-hour (overnight) fast, horses were given a meal of the diet to which the horse was currently adapted. Blood samples were collected, via an indwelling jugular catheter, at 0, 30, 60, 90, 120, 180, 240, and 300 minutes' relative to the start of the meal for evaluation of glucose and insulin responses. The hay portion was withheld until completion of sample collection.

Sample Collection

Blood was collected in lithium heparin tubes for glucose analysis and serum tubes for insulin analysis. Plasma tubes were immediately placed on ice while serum tubes were allowed to clot at room temperature for one hour. Tubes were centrifuged (2000 x g for 15 minutes at 22°C) within two hours of collection, supernatants were collected, aliquoted, and stored at -80°C for future analysis.

Determination of Glucose and Insulin Concentrations

Glucose concentrations were determined in duplicate by a membrane based glucose oxidase method (YSI 2300 STAT Plus™ Glucose & Lactate Analyzer[i]). Insulin concentrations were determined in duplicate by radioimmunoassay (ImmuChem™ Coated Tube Insulin 125-I RIA[j]), validated for horse serum by our group. Intra- and inter- assay coefficients of variability were calculated for low and high equine serum controls using four replicates per assay (Intra: 8.36 (low) and 6.99 (high); Inter: 10.69 (low) and 9.14 (high)).

Insulin Assay Validation

The in-house validation of the ImmuChem™ Coated Tube 125-I RIA[j] consisted of three steps: precision, recovery on addition, and dilutional parallelism. Precision was determined by calculating the intra- and inter- assay coefficients of variability (CV) using equine serum samples with low (mean = 24.1 mU/L), medium (mean = 113.5 mU/L), and high (mean = 203.5 mU/L) insulin concentrations. Each sample was run six times in a total of fourteen assays. Intra-assay CVs for the different insulin concentrations were as follows: low (7.6%), medium (5.7%), and high (5.2%). Inter-assay CVs for the different insulin concentrations were as follows: low (8.7%), medium (3.0%), and high (3.0%). Second, recovery on addition, an equine serum sample with an extremely low (7.9 mU/L) insulin concentration was spiked with an equal volume of each of the porcine insulin standards (range: 5.5 – 310 mU/L) provided by the manufacturer. Percent recovery was calculated as the observed (measured) concentration divided by the expected concentration multiplied by 100. The average percent recovery across all samples was 98.2% ± 8.0%. Lastly, dilutional parallelism, six equine serum samples were diluted from 2 to 64 times with the zero standards supplied by the manufacturer. The percent recovery of insulin in the diluted samples was calculated following previously published guidelines [125]. The calculated mean percent recovery was not significantly different from 100%.

Endocrine Testing

Overnight Dexamethasone Suppression Test (ODST): A baseline blood sample (EDTA plasma) was collected via jugular venipuncture in the early evening followed by administration of dexamethasone (0.04 mg/kg bodyweight) intramuscularly. An additional blood sample was collected at 19-hours post dexamethasone injection. Samples were immediately placed on ice, centrifuged (2000 x g at 4°C for 15 minutes) within one hour of collection, and plasma collected and frozen at -80°C for subsequent analysis. Cortisol concentrations were determined via chemiluminescent technology (Immulite®[k]), validated for horses, at the Animal Health Diagnostic Center at Cornell University [126].

Statistical Analysis

Data were analyzed using a multivariable linear mixed regression model in the statistical program R[l]. The model included fixed effects (age group, diet, breed, period), interaction term(s) (age group*diet and breed*diet), and a random effect (horse). Model selection was performed using Akaike information criteria (AIC). All data are reported as least squares means estimates and pairwise differences ($P \leq 0.05$). Pairwise differences were determined using the *lmerTest* package *difflsmeans* function which gives differences of the least squares means table with p-values and confidence intervals using Satterthwaite's approximation for the degrees of freedom.

RESULTS

Animals and Diets

Aged and *adult* horses did not show clinical signs of pituitary pars intermedia dysfunction [9] during the study period. All horses, except one aged Thoroughbred mare during the final period (collected in October), had cortisol suppression following an ODST (**Table 2.2**). Horses tolerated all diets and refusals were negligible. The mean amount of remaining feed constituted less than 2% of the total concentrate fed to each horse (76.2 ± 0.6 grams/horse/day). No significant differences in weight or body condition score were noted between age groups, diets, or time points (**Table 2.3**).

Minimal Model Analysis

Overall, mean tissue insulin sensitivity (SI) did not differ between *aged* and *adult* horses across all diets. AIRg least squares means estimates were significantly higher in *aged* compared to *adult* horses after controlling for diet (**Table 2.4**); and AIRg was significantly higher in *aged* than *adult* horses after adaptation to the *fiber* and *sugar* diets. Basal insulin was higher in *aged* than *adult* horses adapted to the *starch* and *sugar* diets (**Table 2.5)**. DI least squares means estimates were significantly higher in *aged* compared to *adult* horses after controlling for diet; and DI was significantly higher in *aged* versus *adult* horses after

adaptation to the *fiber* and *sugar* diets. Sg was higher in *aged* compared to *adult* horses after adaptation to the *sugar* diet.

Within *adult* horses, SI and DI were higher following adaptation to the *starch* and *sugar* diets than the *control* and *fiber* diets. In addition, AIRg was higher after adaptation to *starch* compared to the *control* and *fiber* diets. *Adult* horses had a higher Sg on *starch* compared to *fiber* and *sugar*. Within *aged* horses, SI, DI, AIRg, and Sg were higher after adaptation to *sugar* compared to the *control*, *starch*, and *fiber* diets (**Table 2.5**).

Assessment of Glucose Deflection Below Baseline

All horses regardless of diet, except one *aged* horse on the *fiber* diet, had a deflection of blood glucose below baseline concentrations (hypos). Nineteen of the 64 observations did not return to baseline glucose levels by 240 minutes. Clinical signs of hypoglycemia were not detected as values did not fall below a clinically abnormal level.

No significant difference was detected in outcome measures used to assess glucose deflection below baseline between age groups independent of diet (**Table 2.6**); however, an effect of diet was noted (**Table 2.7**). *Aged* horses had a greater area under the curve below baseline glucose (HAUC) than *adult* horses following adaptation to the *sugar* diet. No other differences were detected between *aged* and *adult* horses on the same diet.

Aged horses adapted to the *sugar* diet needed less time to reach the lowest glucose concentration (Tmin) and a significantly greater HAUC and percent glucose deflection below baseline compared to the *control* and *fiber* diet. In addition, *aged* horses had a larger deflection of glucose below end point (dGe) on the *sugar* diet compared to the *control* diet. *Adult* horses had a higher endpoint glucose (Ge) on the *sugar* diet compared to the *fiber* diet.

Oral Sugar Test Analysis

Age had a significant influence on peak insulin with least squares means estimates higher in *aged* compared to *adult* horses after controlling for diet (**Table 2.4**). Further, peak insulin was significantly higher in *aged* horses on *fiber* compared to *adult* horses adapted to the same diet (**Table 2.8**). Age had a significant influence on AUCi, with least squares means estimates higher in *aged* versus *adult* horses, after controlling for diet. *Aged* horses had a greater AUCi on the *control* and *fiber* diets compared to *adult* horses adapted to the same diets. Glucose parameters did not differ between age groups.

Within age group, *adult* horses had significantly higher basal insulin following adaptation to the *sugar* diet compared to the *starch* diet. Within *aged* horses, AUCg was significantly greater on the *control* and *fiber* diets compared to the *sugar* diet (**Table 2.8**).

Dietary Meal Challenge Analysis

Aged horses had significantly higher basal insulin and lower peak glucose compared to *adult* horses after feeding *sugar*. In addition, peak insulin and AUCi were significantly higher in *aged* horses compared to *adult* horses after feeding the *starch* diet.

Adult horses had significantly higher basal insulin after feeding *starch* compared to the *sugar* diet. *Adult* horses also had significantly higher peak insulin and AUCi after feeding *sugar* relative to the other diets. After feeding *starch*, *aged* horses had significantly higher peak insulin and AUCi compared to feeding the *control*, *fiber*, and *sugar* diets. *Aged* horses also had significantly higher AUCi after feeding the *sugar* diet compared to the *control*, *starch*, and *fiber* diets (**Table 2.9**).

Breed Differences

The FSIGTT demonstrated that breed, after controlling for diet, had an influence on basal insulin and DI (**Table 2.10**). Following adaptation to the *fiber* diet, Standardbreds had a significantly lower DI and Sg compared to Thoroughbreds. SI and AIRg did not differ between groups (**Table 2.11**).

The OST revealed that Standardbreds had significantly lower AUCi, basal glucose, and AUCg compared to Thoroughbreds after controlling for diet (**Table 2.10**). Standardbreds also had significantly lower peak insulin, AUCi, basal glucose, peak glucose, and AUCg following adaptation to the *fiber* diet compared to Thoroughbreds on the same diet (**Table 2.12**).

Assessment of the glycemic and insulinemic responses to the diet profile did not differ between breeds after controlling for diet. Pairwise comparisons demonstrated that Standardbreds had significantly lower basal insulin after feeding the *sugar* diet compared to Thoroughbreds on the same diet. Standardbreds had lower basal glucose on the *starch* and *fiber* diets compared to Thoroughbreds. After feeding the *sugar* diet, Standardbreds had higher peak glucose compared to Thoroughbreds. Within each breed, there was an effect of diet (**Table 2.13**).

DISCUSSION

The effect of age, breed, and diet on glucose and insulin dynamics in healthy non-obese horses is variable depending on the assessment. The responses at the tissue level (FSIGTT) reveal that age influences AIRg, regardless of diet, while adaptation to sugar improves SI in both adult and aged horses. However, at the enteral level (OST), minimal changes in glucose and insulin parameters due to dietary adaptation were detected. In contrast, the dietary meal challenge demonstrated enhanced postprandial hyperinsulinemia in both adult and aged horses, following adaptation to both starch and sugar rich diets.

Aged horses appear to have higher insulin secretory responses evidenced by higher AIRg, peak insulin, and AUCi. However, it is difficult to discern whether this occurs due to increased pancreatic secretion or decreased clearance. Aged horses may demonstrate an increased insulin response due to decreased glucose uptake by the liver, skeletal muscle, and adipose tissue, due to reduced tissue insulin sensitivity. In humans, aging results in various processes being disturbed such as cellular senescence, mitochondrial dysfunction, altered intercellular communication, genomic instability, and deregulated nutrient sensing [127].

In this study, feeding sugar (2.0 g/kg bodyweight) twice daily to adult and aged horses resulted in improved tissue insulin sensitivity; however, the biological significance of this finding is unknown. Previous work has demonstrated that feeding sugar (1.5 g/kg bodyweight) once daily improves insulin sensitivity [128]. Other studies, looking at younger horses or that included obesity as a factor, have reported that the feeding of a starch-rich diet results in a decrease in insulin sensitivity when compared to a low-starch diet that contains higher fat (oil) and/or fiber content [52,58,116,117]. In contrast, our findings demonstrate that feeding starch to healthy non-obese adult horses results in improved insulin sensitivity. Healthy, non-obese aged horses had improved insulin sensitivity following adaptation to starch and sugar rich diets.

Within the age groups, adaptation to diet influenced all minimal model (SI, DI, Sg, AIRg) parameters; however, diet did not influence the OST glucose and insulin variables, suggesting an apparent disconnect between the two tests. Specifically, adaptation to starch or sugar diets was associated with an increase in SI but no change in insulin dynamics during the OST. The lack of agreement between the FSIGTT and OST may raise questions about the most appropriate assessment of metabolic/endocrine status. However, the two tests are assessing different aspects of glucose and insulin dynamics; the FSIGTT measures tissue level responses, while the OST evaluates postprandial (enteral) responses. Alternate explanations for this finding are that an OST may not be sensitive enough to detect differences in glucose and insulin dynamics in overtly healthy horses, or that dietary adaptation did not result in changes in postprandial glucose and insulin dynamics that were of large enough magnitude to be detected by the OST.

Feeding a sugar-rich diet improved tissue insulin sensitivity (SI) but did not alter OST responses suggesting that a sugar-rich diet could be beneficial to horses. However, both tests ignore the glycemic and (importantly) the insulinemic effects of consuming such diets. Following dietary adaptation and without a dynamic challenge, postprandial response to starch and sugar rich diets resulted in hyperinsulinemia. A causal role for insulin in the development of laminitis has been supported by studies that have documented disease in healthy horses/ponies subjected to experimental sustained hyperinsulinemia [6–8], therefore one should be cautioned away from feeding a diet that results in significant postprandial hyperinsulinemia.

Recent studies have demonstrated breed differences in relation to glucose and insulin dynamics. Specifically, Standardbreds were reported to have higher SI values compared to Andalusian horses and mixed-breed ponies [11,128]. Although the ideal study cohort would be restricted to a single breed, these data demonstrate that even insulin-sensitive breeds have important differences in glucose and insulin dynamics.

In summary, following dietary adaptation in healthy non-obese horses, when glucose and insulin responses were assessed with different tests, the outcomes were not equivalent. The FSIGTT identified that adaptation to sugar improves tissue level insulin sensitivity in both adult and aged horses, but this did not correlate to the OST responses. Glycemic and insulinemic responses to the starch and sugar rich diets showed relative postprandial hyperinsulinemia. In addition, aged horses appear to have a greater insulinemic response to intravenous and oral glucose and feeding; however, the exact mechanism is unknown. The impact of diet and the glucose and insulin responses to such a diet is an important factor to consider when choosing how to feed horses especially older animals and those with certain clinical conditions. Further research is required to better understand the influence of age and diet on glucose and insulin dynamics thus leading to improved nutritional management of aging and metabolically abnormal equids.

FOOTNOTES

[a]MARS Horsecare US Inc, Dalton, Ohio, USA.

[b]Sigma-Aldrich, Saint Louis, Missouri, USA.

[c]The Coburn Company, Whitewater, Wisconsin, USA.

[d]Eli Lilly and Company, Indianapolis, Indiana, USA.

[e]Bergman Laboratory, Los Angeles, California, USA.

[f]University of Pennsylvania, Kennett Square, Pennsylvania, USA.

[g]ACH Food Companies Inc., Cordova, Tennessee, USA.

[h]GraphPad Software Inc., La Jolla, California, USA.

[i]YSI Incorporated Life Sciences, Yellow Springs, Ohio, USA.

[j]MP Biomedicals LLC Santa Ana, California, USA.

[k]Siemens Medical Solutions USA, Inc., Malvern, Pennsylvania, USA.

[l]R Core Team, Vienna, AUSTRIA.

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APPENDIX

Table 2.1 *Key nutrients of the dietary carbohydrate profiles (grass hay + concentrate) on a dry matter basis* (Dairy One, Ithaca, New York, USA). The value in parentheses is the concentrate profile (without the grass hay).

	Components			
	% NDF[a]	**% Starch**	**% Sugar**	**% NSC[b]**
Control	54.8 (17.8)	5.1 (4.3)	9.4 (2.2)	14.4 (6.6)
Starch	48.1 (12.2)	15.7 (14.9)	8.8 (1.9)	24.5 (16.9)
Fiber	55.4 (21.2)	2.9 (2.2)	9.7 (3.0)	12.5 (5.2)
Sugar	50.3 (13.7)	4.0 (3.3)	18.7 (11.6)	22.7 (14.9)

[a]NDF (neutral detergent fiber) = hemicellulose, cellulose, lignin
[b]NSC (nonstructural carbohydrate) = starch + water soluble carbohydrates (monosaccharides, disaccharides, polysaccharides)

Table 2.2 *Least squares means estimates for cortisol concentrations (µg/dL) from the overnight dexamethasone suppression test in adult and aged horses following dietary adaptation.*

	Adult		Aged		
	least squares means	**95% confidence interval**	**least squares means**	**95% confidence interval**	**p-value**
Baseline	3.2	(2.7 – 3.6)	3.4	(3.0 – 3.9)	0.5
Post-Dexamethasone	0.4	(0.2 – 0.5)	0.4	(0.2 – 0.6)	0.9

Table 2.3 *Least squares means estimates for weight (kg) and body condition score (BCS) before and after dietary adaptation.*

	Adult				Aged			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Weight (Before)	493.8	483.9	486.9	491.2	490.0	487.5	482.1	482.5
Weight (After)	483.0	487.4	481.0	482.3	487.1	479.4	480.2	487.7
BCS (Before)	5.3	5.0	5.2	5.0	5.1	5.0	5.2	5.1
BCS (After)	5.0	5.1	4.9	4.9	5.1	5.2	4.9	5.0

Table 2.4 *Least squares means estimates and pairwise significant differences ($P \leq 0.05$) for glucose and insulin parameters during the frequently sampled intravenous glucose tolerance test (FSIGTT) and oral sugar test (OST) after controlling for diet.* SI = insulin sensitivity, DI = disposition index, Sg = glucose effectiveness, AIRg = acute insulin response to glucose, AUCi = area under the curve for insulin, AUCg = area under the curve for glucose.

	Adult		Aged		
	least squares means	**95% confidence interval**	**least squares means**	**95% confidence interval**	**p-value**
FSIGTT Parameters:					
Basal Insulin (mU/L)	16.1	(12.7 – 19.4)	22.3	(19.1 – 25.5)	0.02
SI ($L \cdot min^{-1} \cdot mU^{-1}$)	2.7	(2.0 – 3.3)	2.6	(2.0 – 3.3)	0.9
DI	909.0	(577.0 – 1241.0)	1467.0	(1151.0 – 1783.0)	0.03
Sg (min^{-1})	2.0	(1.8 – 2.3)	2.2	(2.0 – 2.5)	0.3
AIRg ($mU/L \cdot min^{-1}$)	358.0	(224.0 – 491.0)	582.0	(455.0 – 709.0)	0.03
OST Parameters:					
Basal Insulin (mU/L)	20.1	(13.8 – 26.4)	24.8	(18.8 – 30.9)	0.3
Peak Insulin (mU/L)	50.2	(40.5 – 59.8)	65.0	(55.7 – 74.2)	0.04
Time to Peak Insulin (minutes)	69.4	(59.0 – 79.8)	74.4	(64.7 – 84.2)	0.5
AUCi ($[mU \cdot L^{-1}] \cdot min$)	5330.0	(4287.0 – 6372.0)	7652.0	(6655.0 – 8648.0)	0.006
Basal Glucose (mmol/L)	4.8	(4.6 – 5.0)	4.9	(4.8 – 5.1)	0.2
Peak Glucose (mmol/L)	6.7	(6.4 – 6.9)	6.8	(6.6 – 7.1)	0.4
Time to Peak Glucose (minutes)	71.8	(59.4 – 84.2)	76.1	(64.3 – 87.9)	0.6
AUCg ($[mmol \cdot L^{-1}] \cdot min$)	997.8	(958.1 – 1037.5)	1038.5	(1001.0 – 1076.0)	0.2

Table 2.5 *Least squares means estimates for glucose and insulin parameters during the frequently sampled intravenous glucose tolerance test (FSIGTT).* Pairwise significant differences ($P \leq 0.05$) within age groups indicated by lowercase letters. Differences between age groups for each diet indicated by *. SI = insulin sensitivity, DI = disposition index, Sg = glucose effectiveness, AIRg = acute insulin response to glucose.

	Adult				Aged			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Basal Insulin (mU/L)	16.9	15.5^{*}	14.7	17.1^{*}	20.9^{a}	23.2^{*}	19.6^{a}	25.5^{b*}
SI ($L \cdot min^{-1} \cdot mU^{-1}$)	2.0^{a}	3.3^{b}	2.0^{a}	3.4^{b}	1.4^{a}	2.8^{b}	2.4^{ab}	4.0^{c}
DI	590.0^{a}	1445.0^{b}	396.0^{a*}	1205.0^{b*}	659.0^{a}	1503.0^{b}	1233.0^{ab*}	2473.0^{c*}
Sg (min^{-1})	2.0	2.4^{a}	1.8^{b}	2.0^{b*}	2.2^{a}	2.2^{a}	1.9^{a}	2.7^{b*}
AIRg ($mU/L \cdot min^{-1}$)	307.0^{a}	452.0^{b}	299.0^{a*}	371.0^{*}	504.0^{a}	608.0^{b}	524.0^{ab*}	691.0^{c*}

Table 2.6 *Least squares means estimates and pairwise differences for outcome measures used to assess glucose deflection below baseline during the frequently sampled intravenous glucose tolerance test (FSIGTT).* Gmin = lowest glucose concentration below baseline, Tmin = time point at lowest glucose concentration, Ge = glucose concentration at sampling endpoint, dGb = percent deflection of glucose below baseline, dGe = percent deflection of glucose below the sampling endpoint, HAUC = area under the curve below baseline glucose, Ttmax = time point where glucose deflection returned to baseline.

	Adult		Aged		
	least squares means	**95% confidence interval**	**least squares means**	**95% confidence interval**	**p-value**
Gmin	61.2	(56.0 – 66.4)	60.5	(55.5 – 65.5)	0.8
Tmin	144.9	(115.2 – 175.0)	121.2	(92.5 – 150.0)	0.3
Ge	80.3	(76.0 – 84.6)	84.3	(80.2 – 88.4)	0.2
dGb	-23.5	(-30.9 – [-16.2])	-26.3	(-33.4 – [-19.3])	0.6
dGe	21.8	(13.3 – 30.2)	28.0	(19.8 – 36.1)	0.3
HAUC	9991.0	(7875.0 – 12108.0)	11663.0	(9616.0 – 13710.0)	0.3
Ttmax	211.3	(195.0 – 227.0)	212.7	(199.0 – 226.0)	0.9

Table 2.7 *Least squares means estimates in adult and aged horses adapted to each diet (control, starch, fiber, sugar) for outcome measures used to assess glucose deflection below baseline during the frequently sampled intravenous glucose tolerance test (FSIGTT).* Pairwise significant differences ($P \leq 0.05$) within age groups indicated by different lowercase letters for each variable. Differences between age groups for each diet indicated by *. Gmin = lowest glucose concentration below baseline, Tmin = time point at lowest glucose concentration, Ge = glucose concentration at sampling endpoint, dGb = percent deflection of glucose below baseline, dGe = percent deflection of glucose below the sampling endpoint, HAUC = area under the curve below baseline glucose, Ttmax = time point where glucose deflection returned to baseline.

	Adult				Aged			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Gmin	62.1	58.8	61.2	62.8	63.4	59.3	63.8	55.4
Tmin	144.3	118.4	178.3	138.8	150.4[a]	125.6	133.8[a]	75.0[b]
Ge	81.3	81.8	73.6[a]	84.6[b]	80.8	87.8	81.2	87.8
dGb	-24.8	-24.8	-21.6	-22.9	-20.1[a]	-28.0	-21.4[a]	-35.9[b]
dGe	22.1	26.4	13.4	25.2	21.0[a]	31.5	22.7	36.7[b]
HAUC	9947.0	11268.0	8327.0	10424.0[*]	9703.0[a]	11467.0	10418.0[a]	15064.0[b*]
Ttmax	227.8	211.9	188.0	217.5	210.2	200.2	230.0	210.6

Table 2.8 *Least squares means estimates for glucose and insulin parameters during the oral sugar test (OST).* Pairwise significant differences ($P \leq 0.05$) within age groups indicated by lowercase letters. Differences between age groups for each diet indicated by *. AUCi = area under the curve for insulin, AUCg = area under the curve for glucose.

	Adult				Aged			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Basal Insulin (mU/L)	21.3	18.9[a]	14.1	26.1[b*]	25.1	22.4	23.2	28.6[*]
Peak Insulin (mU/L)	53.2	52.3	41.2[*]	53.9	69.3	61.6	64.3[*]	64.6
Time to Peak Insulin (minutes)	69.8	74.8	79.9	52.9	79.5	67.0	79.8	71.4
AUCi ([mU·L^{-1}]·min)	6004.0[*]	4995.0	4901.0[*]	5418.0	8329.0[*]	6982.0	7783.0[*]	7513.0
Basal Glucose (mmol/L)	4.8	4.9	4.7	4.8	5.0	4.9	5.0	4.8
Peak Glucose (mmol/L)	6.8	6.6	6.6	6.7	7.1	6.7	7.1	6.4
Time to Peak Glucose (minutes)	70.0	70.3	83.5	63.3	71.8	80.6	83.7	68.3
AUCg ([mmol·L^{-1}]·min)	1023.3	981.4	1007.9	978.5	1087.0[a]	1003.3	1096.6[a]	967.1[b]

Table 2.9 *Least squares means estimates for glucose and insulin responses during the dietary meal challenge.* Pairwise significant differences ($P \leq 0.05$) within age groups indicated by lowercase letters. Differences between age groups for each diet indicated by *. AUCi = area under the curve for insulin, AUCg = area under the curve for glucose.

	Adult				Aged			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Basal Insulin (mU/L)	17.8	22.2[a]	19.6	14.8[b*]	22.7	22.9	23.9	23.3[*]
Peak Insulin (mU/L)	76.4[ac]	105.7[ab*]	57.7[c]	130.2[b]	87.1[ac]	174.8[b*]	77.1[a]	121.3[c]
Time to Peak Insulin (minutes)	105.8	149.4	110.3	144.6	82.7[a]	103.8	91.1[a]	147.6[b]
AUCi ([mU·L^{-1}]·min)	15100.0[ac]	21581.0[bc*]	11921.0[a]	26050.0[b]	15639.0[a]	35205.0[b*]	13010.0[ac]	25720.0[d]
Basal Glucose (mmol/L)	4.6	4.7	4.8	4.7	4.8	4.8	4.8	4.8
Peak Glucose (mmol/L)	6.8[ac]	7.8[bc]	6.1[a]	8.7[b*]	6.4[a]	7.9[b]	6.1[a]	6.9[*]
Time to Peak Glucose (minutes)	126.8[a]	130.6[a]	122.8	93.1[b]	97.8	99.2	100.7	108.4
AUCg ([mmol·L^{-1}]·min)	1820.4	1859.2	1670.5[a]	1925.8[b]	1742.7	1864.8	1687.2	1753.8

Table 2.10 *Least squares means estimates and pairwise significant differences (P ≤ 0.05) for glucose and insulin responses during the frequently sampled intravenous glucose tolerance test (FSIGTT) and oral sugar test (OST) after controlling for diet.* SI = insulin sensitivity, DI = disposition index, Sg = glucose effectiveness, AIRg = acute insulin response to glucose, AUCi = area under the curve for insulin, AUCg = area under the curve for glucose.

	Standardbred		Thoroughbred		p-value
	least squares means	95% confidence interval	least squares means	95% confidence interval	
FSIGTT Parameters:					
Basal Insulin (mU/L)	16.0	(12.5 – 19.4)	22.3	(19.2 – 25.5)	0.02
SI ($L \cdot min^{-1} \cdot mU^{-1}$)	2.2	(1.5 – 2.9)	3.0	(2.4 – 3.7)	0.1
DI	913.0	(571.0 – 1256.0)	1463.0	(1154.0 – 1772.0)	0.03
Sg (min^{-1})	2.0	(1.7 – 2.3)	2.3	(2.0 – 2.5)	0.2
AIRg ($mU/L \cdot min^{-1}$)	419.0	(281.0 – 558.0)	520.0	(397.0 – 643.0)	0.3
OST Parameters:					
Basal Insulin (mU/L)	18.9	(12.4 – 25.5)	25.9	(20.1 – 31.8)	0.1
Peak Insulin (mU/L)	51.2	(41.2 – 61.2)	63.9	(54.9 – 72.9)	0.08
Time to Peak Insulin (minutes)	68.0	(57.3 – 78.7)	75.8	(66.2 – 85.4)	0.3
AUCi ($[mU \cdot L^{-1}] \cdot min$)	5510.0	(4433.0 – 6587.0)	7472.0	(6500.0 – 8443.0)	0.02
Basal Glucose (mmol/L)	4.7	(4.5 – 4.9)	5.0	(4.9 – 5.2)	0.01
Peak Glucose (mmol/L)	6.6	(6.3 – 6.9)	6.9	(6.6 – 7.2)	0.2
Time to Peak Glucose (minutes)	73.1	(60.3 – 85.8)	74.8	(63.3 – 86.3)	0.8
AUCg ($[mmol \cdot L^{-1}] \cdot min$)	982.2	(941.4 – 1023.1)	1054.1	(1017.3 – 1090.8)	0.02

Table 2.11 *Least squares means estimates for glucose and insulin parameters during the frequently sampled intravenous glucose tolerance test (FSIGTT).* Pairwise significant differences ($P \leq 0.05$) within breed groups indicated by lowercase letters. Differences between breed groups for each diet indicated by *. SI = insulin sensitivity, DI = disposition index, Sg = glucose effectiveness, AIRg = acute insulin response to glucose.

	Standardbred				Thoroughbred			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Basal Insulin (mU/L)	17.3	15.1^{*}	14.3	17.2^{*}	20.4^{a}	23.6^{*}	20.0^{a}	25.4^{b*}
SI ($L \cdot min^{-1} \cdot mU^{-1}$)	1.5^{b}	2.6	1.4^{b}	3.4^{a}	1.9^{a}	3.5^{bc}	2.9^{b}	4.0^{c}
DI	613.0^{ac}	1152.0^{c}	348.0^{a*}	1540.0^{b}	636.0^{a}	1796.0^{bc}	1281.0^{b*}	2139.0^{c}
Sg (min^{-1})	2.1^{a}	2.0^{a}	1.5^{b*}	2.3^{a}	2.2	2.5^{b}	2.1^{a*}	2.4
AIRg ($mU/L \cdot min^{-1}$)	373.0^{a}	483.0^{b}	338.0^{a}	484.0^{b}	439.0^{a}	577.0^{b}	486.0^{a}	578.0^{b}

Table 2.12 *Least squares means estimates for glucose and insulin responses during the oral sugar test (OST).* Pairwise significant differences ($P \leq 0.05$) within breed groups indicated by lowercase letters. Differences between breed groups for each diet indicated by *. AUCi = area under the curve for insulin, AUCg = area under the curve for glucose.

	Standardbred				Thoroughbred			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Basal Insulin (mU/L)	21.4	17.3	14.8	22.3*	24.9	24.0	22.5	32.3*
Peak Insulin (mU/L)	52.5	58.1	42.0*	52.4	70.0	55.9	63.5*	66.2
Time to Peak Insulin (minutes)	71.8	75.7	58.9*	65.6	77.5	66.1[a]	100.8[b*]	58.8[a]
AUCi ([mU·L^{-1}]·min)	5916.0*	5846.0	4905.0*	5371.0*	8418.0[a*]	6131.0[b]	7778.0*	7560.0*
Basal Glucose (mmol/L)	4.8	4.7	4.5*	4.7	5.0	5.1	5.1*	5.0
Peak Glucose (mmol/L)	6.9	6.8	6.2*	6.5	7.1	6.5[b]	7.5[a*]	6.6[b]
Time to Peak Glucose (minutes)	75.1	79.2	77.5	60.4	66.7	71.7	89.7	71.3
AUCg ([mmol·L^{-1}]·min)	1021.8	998.1	984.2*	946.7	1088.5[ac]	986.6[bc]	1143.2[a*]	999.0[c]

Table 2.13 *Least squares means estimates for glucose and insulin responses during the dietary meal challenge.* Pairwise significant differences ($P \leq 0.05$) within breed groups indicated by lowercase letters. Differences between breed groups for each diet indicated by *. AUCi = area under the curve for insulin, AUCg = area under the curve for glucose.

	Standardbred				Thoroughbred			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Basal Insulin (mU/L)	18.1	20.7	20.1	14.7*	22.3	24.4	23.4	23.4*
Peak Insulin (mU/L)	85.8[a]	137.3[b]	53.6[a]	130.4[b]	77.7[a]	143.2[b]	81.1[a]	121.1[b]
Time to Peak Insulin (minutes)	96.1[a]	166.5[b*]	128.4	128.3	92.4[a]	86.7[a*]	72.9[a]	163.9[b]
AUCi ([mU·L^{-1}]·min)	16073.0[a]	29315.0[b]	11152.0[a]	26293.0[b]	14666.0[a]	27471.0[b]	13780.0[a]	25478.0[b]
Basal Glucose (mmol/L)	4.6	4.6*	4.6*	4.7	4.8	4.9*	5.0*	4.8
Peak Glucose (mmol/L)	6.7[a]	8.2[b]	5.9[a]	8.5[b*]	6.6[b]	7.6[a]	6.2[b]	7.1*
Time to Peak Glucose (minutes)	111.0	119.0	120.4[a]	86.4[b]	113.7	110.8	103.0	115.1
AUCg ([mmol·L^{-1}]·min)	1748.2	1953.6[b]	1603.9[a]	1964.7[b]	1814.8	1770.4	1753.8	1714.9

CHAPTER 3

Effect of Dietary Carbohydrates and Time of Year on Adrenocorticotropic Hormone (ACTH) and Cortisol Concentrations in Adult and Aged Horses

SUMMARY

Background: Diagnosis of equine pituitary pars intermedia dysfunction (PPID) remains a challenge as multiple factors (stress, exercise, time of year) influence adrenocorticotropic hormone (ACTH) and cortisol concentrations.

Objectives: To assess endocrine status in a study designed to evaluate the effects of age and diet on glucose and insulin dynamics.

Study Design: Sixteen healthy Thoroughbred and Standardbred horses were grouped by age: *adult* (mean ± SD; 8.8 ± 2.9 years; n = 8) and *aged* (20.6 ± 2.1 years; n = 8) and fed grass hay plus four isocaloric concentrate diets (*control*, *starch*, *fiber*, *sugar*) using a balanced Latin square design.

Methods: Thyrotropin releasing hormone (TRH) stimulation tests and overnight dexamethasone suppression tests were performed in March, May, August, and October. Data were analyzed using a multivariable linear mixed regression model.

Results: None of the horses showed clinical signs (hypertrichosis, regional adiposity, skeletal muscle atrophy, lethargy) of pituitary pars intermedia dysfunction. Baseline ACTH was significantly higher in *aged* horses (mean ± SEM; 60.0 ± 10.7 pg/mL) adapted to the *starch* diet compared to *adult* horses (15.7 ± 12.0 pg/mL) on the same diet ($P = 0.017$). After controlling for age and diet, baseline ACTH concentrations were significantly increased in October (57.7 ± 7.1 pg/mL) compared to March (13.2 ± 7.1 pg/mL; $P < 0.001$), May (12.4 ± 7.1 pg/mL; $P < 0.001$) and August (24.2 ± 7.1 pg/mL; $P < 0.001$) while post-TRH ACTH was higher in August (376.6 ± 57.6 pg/mL) and October (370.9 ± 57.5 pg/mL) compared to March (101.9 ± 57.3 pg/mL; $P < 0.001$) and May (74.5 ± 57.1 pg/mL; $P < 0.001$). *Aged* horses had significantly higher post-dexamethasone cortisol on the *starch* diet (0.6 ± 0.1µg/dL) compared to the *sugar* diet (0.2 ± 0.1 µg/dL; $P = 0.021$). Post-dexamethasone cortisol was significantly higher in October (0.6 ± 0.1 µg/dL)

compared to March (0.3 ± 0.1 µg/dL; $P = 0.005$), May (0.2 ± 0.1 µg/dL; $P < 0.001$), and August (0.3 ± 0.1 µg/dL; $P = 0.004$). Breed did not influence ACTH or cortisol measurements.

Main Limitations: ACTH and cortisol concentrations were not measured prior to dietary adaptation.

Conclusions: In addition to age and time of year, diet is a potential confounder as animals on a starch diet may be incorrectly diagnosed with pituitary pars intermedia dysfunction.

INTRODUCTION

Pituitary pars intermedia dysfunction (PPID) is the most common endocrine disorder of older horses, yet definitive diagnosis remains a challenge. Measurement of plasma adrenocorticotropic hormone (ACTH) concentrations is a commonly used diagnostic test; however, ACTH concentrations are influenced by multiple factors such as stress [129], feeding status (fasted versus fed) [130,131], exercise [132,133], and time of year [121,134–136], therefore several dynamic tests have been proposed to evaluate endocrine responses in older horses. Measurement of ACTH concentration at baseline (9 – 35 pg/mL)[a] and at 10 minutes (< 110 pg/mL)[a], following administration of thyrotropin releasing hormone (TRH), is widely used for the diagnosis of PPID. In cases where clinicians suspect early PPID the TRH stimulation test is recommended [137]. However, from July to October clinically normal horses demonstrate increased ACTH concentrations following administration of TRH [134,135,138]. Further, there is variability in ACTH laboratory reference ranges complicating interpretation of the test results. Adequate reference ranges, especially in clinically normal animals, need to be established.

Similar to the TRH stimulation test, cortisol measurement at baseline (2 – 6 µg/dL)[a] and following an overnight dexamethasone suppression test (< 1 µg/dL)[a], suffers from variability due to the time of year [121,138]. The overnight dexamethasone suppression test is also looked at less favorably due to concern about the development of laminitis following corticosteroid administration although evidence is limited [139,140] and that a minimal number of PPID animals may demonstrate hyperadrenocorticism. Like ACTH concentrations, it is recommended that cortisol concentrations be interpreted with caution and in conjunction with the animal's clinical signs.

As part of a study to evaluate the effects of dietary carbohydrates on glucose and insulin dynamics in healthy adult and aged horses, we performed TRH stimulation tests and overnight dexamethasone suppression tests in a group of sixteen healthy horses consuming four different diets at four different times of the year. Our data demonstrate that ACTH and cortisol concentrations in these horses vary due to both diet and time of year. To the authors' knowledge, although a small sample size, this is the first study to demonstrate an effect of diet on ACTH and cortisol concentrations as well as TRH stimulation and dexamethasone suppression tests.

MATERIALS AND METHODS

Data presented in this manuscript were collected as part of a larger study designed to evaluate the effects of age and dietary adaptation to diets with varying carbohydrate composition [141]. The study was conducted from February to October. Endocrine testing was performed in March, May, August, and October. The Institutional Animal Care and Use Committee (IACUC) at Michigan State University approved all methods.

Horses and Groups

Sixteen healthy Thoroughbred (TB) and Standardbred (STB) mares and geldings were divided into two groups by age: *adult* (5 to 13 years old; 8.8 ± 2.9 years; n = 9; 4 TB mares, 1 STB mare, 3 TB geldings, 1 STB gelding) and *aged* (18 to 24 years old; 20.6 ± 2.1 years; n = 9; 3 TB mares, 6 STB mares). One horse had to be replaced in each age group due to failure to eat the diet (n = 1, aged Thoroughbred mare replaced by an aged Standardbred mare) and severe colic (unrelated to diet or age) resulting in euthanasia (n = 1, adult Thoroughbred mare replaced by an adult Standardbred mare). Body condition score was determined (1 – 9 Henneke scale) [123] by two evaluators at the start and end of each dietary period (mean ± SEM; *adult*: 5.2 ± 0.2 (start), 5.0 ± 0.1 (end) and *aged*: 5.1 ± 0.2 (start), 5.0 ± 0.1 (end)). Baseline (day 0) insulin concentrations were obtained prior to each dietary period; however, no significant difference was noted due

to age (mean ± SEM; *adult*: 11.9 ± 1.0 mU/L and *aged*: 14.7 ± 0.9 mU/L). All animals received routine anthelmintic, vaccination, dental, and farrier treatment as appropriate.

Study Design

Dietary periods occurred during the following times: Period 1 (February 9th – March 29th), Period 2 (April 13th – May 31st), Period 3 (June 15th – August 2nd), and Period 4 (August 17th – October 4th). Horses were randomly assigned to groups of four, blocked for age, and fed grass hay plus four isocaloric concentrate diets using a balanced Latin square design. The *control* diet consisted of restricted-starch-and-sugar, fortified pellets[b]. For the three remaining diets, a portion of the *control* diet was removed, and the appropriate energy substrate rich feed added: *starch* (control plus kibbled corn[b]), *fiber* (control plus unmolassed sugar beet pulp/soybean hull pellets[b]), and *sugar* (control plus dextrose powder[c]). **Table 3.1** shows the key nutrients of each diet. Horses were housed on a dry lot with no access to pasture or forced exercise during each dietary period. All horses received four 7-week dietary treatments, with the total ration being fed at a daily rate of 2.0% to 2.2% of bodyweight (hay: 1.2% and concentrate: 0.8% - 1.0%) on a dry matter basis. During each dietary period, horses were group fed the same batch of grass hay (nonstructural carbohydrate (dry matter basis): 13.1% ± 0.4%) once daily and individually fed two meals of one of the above diets at 7:00 and 17:00. Quantity was pre-determined based on the horse's estimated weight using a weight tape[d]. Dietary periods were separated by a two-week washout period during which all horses received the *control* diet and free access to the same hay and pasture. In the 7th (final) week of each dietary period, each horse underwent an overnight dexamethasone suppression test followed 36-hours later by a thyrotropin releasing hormone (TRH) stimulation test to evaluate adrenal cortical and pituitary function. Therefore, endocrine tests occurred in early spring (March), mid-to-late spring (May), mid-to-late summer (August), and fall (October).

Endocrine Testing

Thyrotropin Releasing Hormone (TRH) Stimulation Test**:** Following a 10-hour (overnight) feed withdrawal, a baseline blood sample (EDTA plasma) was collected via jugular venipuncture followed by intravenous administration of 1 milligram of TRH[c]. An additional blood sample was collected at 10 minutes post-TRH injection [post-TRH]. Samples were immediately placed on ice, centrifuged (2000 x g at 4°C for 15 minutes) within one hour of collection, and plasma separated and stored at -80°C for subsequent analysis. ACTH concentrations were determined via chemiluminescent technology (Immulite®[e]) validated for horses at the Animal Health Diagnostic Center at Cornell University [126].

Overnight Dexamethasone Suppression Test**:** A baseline blood sample (EDTA plasma) was collected via jugular venipuncture, after consumption of the evening meal, followed by administration of dexamethasone (0.04 mg/kg bodyweight) intramuscularly. An additional blood sample was collected at 19-hours post-dexamethasone injection. Samples were immediately placed on ice, centrifuged (2000 x g at 4°C for 15 minutes) within one hour of collection, and plasma collected and frozen at -80°C for subsequent analysis. Cortisol concentrations were determined via chemiluminescent technology (Immulite®), validated for horses, at the Animal Health Diagnostic Center at Cornell University [126].

Statistical Analysis

Data were analyzed using a multivariable linear mixed regression model in the statistical program R[f]. The model included fixed effects (age, diet, breed, time of year), interaction term(s) (age*time of year, age*diet, breed*diet), and a random effect (horse). Model selection was performed using Akaike information criteria (AIC). The full model, including interaction terms, was used for ACTH at baseline, ACTH after TRH stimulation, and cortisol at baseline. The post-dexamethasone cortisol model included the fixed effects (diet, age, time of year) and the random effect (horse) without the interaction terms. Data are reported as least squares means estimates in the tables. Pairwise significant differences ($P \leq 0.05$) were determined using the *lmerTest* package *difflsmeans* function, which gives differences of the least squares means table with p-values and confidence intervals using Satterthwaite's approximation for the degrees of

freedom. However, figures represent the raw data values, as would be used as a clinical diagnostic test, to illustrate the variability of diet and time of year, and the raw data in relation to currently suggested cut-off values for diagnosis of PPID.

RESULTS

Animals

Adult and *aged* horses did not show typical clinical signs (hypertrichosis, regional adiposity, skeletal muscle atrophy, lethargy) of PPID [9] during the study period. All diets were well tolerated and refusals (measured by weighing any remaining concentrate at the end of 60 minutes) were insignificant (mean ± SD; 76.2 ± 0.6 grams/horse/day).

ACTH Concentrations

Effect of Age and Time of Year

As expected, ACTH concentrations increased following administration of TRH in both age groups. Baseline ACTH concentrations were significantly higher in *aged* horses compared to *adult* horses after controlling for diet, time of year, and breed (**Table 3.2**). Further, baseline ACTH concentrations were significantly higher in October compared to March, May, and August after controlling for age, diet, and breed (**Table 3.3**). However, these main effects are qualified by an interaction between age and time of year where *aged* horses had a significantly greater increase in baseline ACTH concentrations in October compared to *adult* horses (**Table 3.4**).

No significant difference was detected in post-TRH ACTH concentrations in *aged* horses compared to *adult* horses after controlling for diet, time of year, and breed (**Table 3.2**). Further, post-TRH ACTH concentrations were significantly higher in August and October compared to March and May after controlling for age, diet, and breed (**Table 3.3**). However, these main effects are qualified by an interaction between age and time of year where *aged* horses had a significantly greater increase in post-TRH ACTH concentrations in August compared to *adult* horses (**Table 3.4**).

Variability in clinical laboratory ACTH concentrations at different times of year may lead to false positives. One *aged* horse (12.5%) in March and May, and two *aged* horses (25%) in August had baseline ACTH concentrations above the suggested cut-off (< 35 pg/mL). In October, three *adult* animals (37.5%) and four *aged* animals (50%) had elevated baseline ACTH concentrations (**Figure 3.1**). Following administration of TRH, in March, four *aged* horses (50%) had increased ACTH concentrations above the suggested cut-off (< 110 pg/mL). In May, two *adult* horses (25%) and four *aged* horses (50%) would be considered positive for PPID. In addition, in August and October, six *adult* horses (75%) and all *aged* horses (100%) had increased ACTH concentrations (**Figure 3.2**).

Effect of Diet

Baseline ACTH concentrations were significantly higher after adaptation to the *starch* diet compared to the *control* diet after controlling for age, time of year, and breed (**Table 3.5**). Further, baseline ACTH concentrations were significantly higher in *aged* horses compared to *adult* horses after controlling for diet, time of year, and breed (**Table 3.2**). However, these main effects are qualified by an interaction between age and diet where *aged* horses had a significantly greater increase in baseline ACTH concentrations after adaptation to the *starch* diet compared to *adult* horses adapted to the same diet (**Figure 3.3**, **Table 3.6**).

Following TRH administration, ACTH concentrations were not different between diets after controlling for age, time of year, and breed (**Table 3.5**). However, an age*diet interaction showed that *aged* horses had higher post-TRH ACTH concentrations following adaptation to the *control* diet compared to *adult* horses adapted to the same diet (**Table 3.6**).

Effect of Breed

Breed did not significantly influence ACTH concentrations in this study.

Cortisol Concentrations

Effect of Age and Time of Year

Age did not influence cortisol concentrations at either time point after controlling for diet, time of year, and breed (**Table 3.7**). However, while cortisol suppression occurred in both age groups across all times of the year, post-dexamethasone cortisol concentrations were significantly higher in October compared to the other three months after controlling for age, diet, and breed (**Table 3.8**). An age*time of year interaction demonstrated that *aged* horses had significantly higher post-dexamethasone cortisol concentrations in October compared to *adult* horses (**Table 3.9**). *Aged* horses had significantly higher baseline cortisol concentrations in March and October compared to May. In addition, *aged* horses had significantly higher post-dexamethasone cortisol concentrations in October compared to the other three months (**Table 3.8**).

Effect of Diet

Diet did not influence baseline cortisol concentrations in *adul*t and *aged* horses. However, an age*diet interaction demonstrated that aged horses had significantly higher post-dexamethasone cortisol concentrations on the *starch* diet compared to the *sugar* diet. The *control* and *fiber* diet did not significantly influence post-dexamethasone cortisol concentrations (**Table 3.10**).

Effect of Breed

Breed did not significantly influence cortisol concentrations in this study.

DISCUSSION

These results show that the effect of diet on ACTH and cortisol should be considered when interpreting endocrine results, in addition to effects of age and time of year. As expected, an increase in ACTH occurs in adult and aged horses following administration of TRH [134,135,142,143]; however, the magnitude is greater in aged horses. These data also suggest there is a diet*age interaction where aged

animals adapted to a starch-rich diet may have higher resting endogenous (baseline) ACTH concentrations. In addition, regardless of age and diet, time of year variability exists in ACTH concentrations.

Diagnosis of pituitary pars intermedia dysfunction using the TRH stimulation test may be unreliable at certain times of the year [121,135,138]. In the present study, using the laboratory cut-off of 110 pg/mL, in March, all adult animals (100%) would be considered negative for PPID. However, during May, August, and October, adult horses demonstrated significantly increased ACTH concentrations in the absence of clinical signs. Aged horses had elevated post-TRH ACTH concentrations at all four times of the year in the absence of clinical signs (**Figure 3.1**). The exact reason for the time of year variability remains unknown but may be due to photoperiod [136] and/or climate temperature. The currently recommended laboratory cut-offs for the TRH stimulation test are difficult to interpret for late summer to late fall. In August, twenty-five percent (25%) of the aged animals had baseline ACTH concentrations above the suggested cut-off (< 35 pg/mL) (**Figure 3.1)**. In the present study, in October 37.5% of the adult animals and 50% of the aged animals had elevated baseline ACTH concentrations. Following administration of TRH, in August and October, 75% of the adult horses and 100% of the aged horses had increased ACTH concentrations above the suggested cut-off (< 110 pg/mL) (**Figure 3.2)**; however, an elevation in the fall is normal in herbivores. In agreement with the equine endocrinology group recommendation [137], these data provide additional support for adjusted seasonal cut-offs from July to November. It is important to note that in both the adult and aged groups, elevations in ACTH above the laboratory reference range may suggest subclinical disease and pituitary hyperplasia. None of the animals in this study were evaluated by post-mortem examination or histopathology, therefore a true determination of false positives cannot be made. In contrast to the TRH stimulation test, the overnight dexamethasone suppression test appears to identify fewer false positives. In this cohort, suppression of cortisol occurred in both age groups regardless of diet and time of year.

Diet may influence ACTH and cortisol concentrations, especially in aged individuals. Aged horses adapted to a starch-rich diet had significantly higher endogenous ACTH concentrations and decreased cortisol suppression compared to the other carbohydrate diets. The unusual finding that aged horses had increased post-TRH ACTH measurements on the control diet compared to adults is difficult to explain but

could be an age or diet effect. It would be expected that this change would also be seen on the fiber-rich diet given the similarities in nonstructural carbohydrate content. A possible explanation for the variability may have to do with the higher starch content in the control versus fiber-rich diet thus lending support to dietary starch being a driving mechanism in the manipulation of equine endocrine hormones, although it is appreciated that the difference is relatively small. These are only speculations and the exact mechanism for these differences is unknown. Another explanation to explore is the role of gastrointestinal microbes in the gut-brain communication pathway. Further, a number of studies demonstrate the role of diet in shaping the microbiota [144–146]. Studies in mice indicate that alterations in the gastrointestinal microbiome can affect the regulation of neuroendocrine hormones of the hypothalamic-pituitary-adrenocortical (HPA) axis [147–152]. The data presented here suggest that changes in the HPA axis and differences in ACTH concentrations in relation to diet in horses needs to be explored further. The data presented here suggest that changes in the HPA axis and differences in ACTH concentrations in relation to diet in horses needs to be explored further.

In conclusion, following dietary adaptation in healthy non-obese horses, when endocrine parameters were assessed, the outcomes were influenced by age, time of year, and diet. Aged horses had higher ACTH concentrations at both time points (baseline and 10 minutes [post-TRH]) compared to adult horses. Diet may be another factor affecting the endocrine parameters as animals on a starch-rich diet may be incorrectly diagnosed with pituitary pars intermedia dysfunction. Further research is required to better understand the influence of age, time of year, and diet on ACTH and cortisol concentrations to establish accurate reference ranges thus leading to improved diagnosis and management of equine endocrine disease. These findings suggest the need for reference ranges for different times of the year [136] and possibly the need for different reference ranges for aged horses. Finally, these findings further support the need for a more sensitive diagnostic test for PPID.

FOOTNOTES

[a]Animal Health Diagnostic Center at Cornell University

[b]MARS Horsecare US Inc, Dalton, Ohio, USA.

[c]Sigma-Aldrich, Saint Louis, Missouri, USA.

[d]The Coburn Company, Whitewater, Wisconsin, USA.

[e]Siemens Medical Solutions USA, Inc., Malvern, Pennsylvania, USA.

[f]R Core Team, Vienna, AUSTRIA.

***This chapter is an accepted manuscript in Domestic Animal Endocrinology. The manuscript in its published form may be found at* https://doi.org/10.1016/j.domaniend.2017.10.005**

APPENDIX

Table 3.1 *Key nutrients for each dietary profile (grass hay + concentrate) on a dry matter basis* (Dairy One, Ithaca, New York, USA).

	Components				
	% Crude Protein	**% NDF[a]**	**% Starch**	**% WSC**	**% NSC[b]**
Control	12.4	54.8	5.1	9.4	14.4
Starch	11.1	48.1	15.7	8.8	24.5
Fiber	11.0	55.4	2.9	9.7	12.5
Sugar	10.7	50.3	4.0	18.7	22.7

[a]NDF (neutral detergent fiber) = hemicellulose, cellulose, and lignin
[b]NSC (nonstructural carbohydrate) = starch + water soluble carbohydrates (WSC; monosaccharides, disaccharides, polysaccharides)

Table 3.*2* *Least squares means estimates and pairwise differences for adrenocorticotropic hormone (ACTH) concentrations (pg/mL) from the thyrotropin releasing hormone (TRH) stimulation test after controlling for diet, time of year, and breed.*

	Adult		Aged		
	least squares means	**95% confidence interval**	**least squares means**	**95% confidence interval**	**p-value**
Baseline	14.6	([-1.09] – 30.1)	39.6	(24.4 – 54.9)	0.04
Post-TRH	141.0	(10.4 – 271.0)	321.0	(197.2 – 446.0)	0.06

Table 3.3 *Least squares means estimates for adrenocorticotropic hormone (ACTH) concentrations (pg/mL) from the thyrotropin releasing hormone (TRH) stimulation test at different times of the year.* For each time point (baseline or post-TRH), pairwise significant differences ($P \leq 0.05$) between months indicated by lowercase letters.

	March		May		August		October	
	least squares means	**95% confidence interval**	**least squares means**	**95% confidence interval**	**least squares means**	**95% confidence interval**	**least squares means**	**95% confidence interval**
Baseline	13.2[a]	([-1.1] – 27.5)	12.4[a]	([-1.9] – 26.6)	24.2[a]	(9.9 – 38.6)	57.7[b]	(43.3 – 72.0)
Post-TRH	101.9[a]	([-13.9] – 218.0)	74.5[a]	(40.9 – 190.0)	376.6[b]	(260.2 – 493.0)	370.9[b]	(254.8 – 487.0)

Table 3.4 *Least squares means estimates for adrenocorticotropic hormone (ACTH) concentrations (pg/mL) from the thyrotropin releasing hormone (TRH) stimulation test in adult and aged horses at different times of the year*. For each time point (baseline or post-TRH), pairwise significant differences ($P \leq 0.05$) within age groups indicated by lowercase letters. Differences between age groups indicated by *.

	Adult				Aged			
	March	**May**	**August**	**October**	**March**	**May**	**August**	**October**
Baseline	7.8^{ab}	4.2^{a}	14.9^{ab}	29.7^{b*}	18.8^{a}	20.5^{a}	33.6^{a}	85.7^{b*}
Post-TRH	-0.9^{a}	28.6^{a}	157.9^{a*}	376.3^{b}	204.6^{a}	120.4^{ad}	595.2^{b*}	365.5^{ac}

Table 3.5. *Least squares means estimates for adrenocorticotropic hormone (ACTH) concentrations (pg/mL) from the thyrotropin releasing hormone (TRH) stimulation test for each diet (control, starch, fiber, sugar).* For each time point (baseline or post-TRH), pairwise significant differences ($P \leq 0.05$) between diets indicated by lowercase letters.

	Control		Starch		Fiber		Sugar	
	least squares means	**95% confidence interval**	**least squares means**	**95% confidence interval**	**least squares means**	**95% confidence interval**	**least squares means**	**95% confidence interval**
Baseline	20.4[a]	(6.3 – 34.6)	37.9[b]	(23.6 – 52.1)	21.8[ab]	(7.6 – 36.1)	27.4[ab]	(13.3 – 41.5)
Post-TRH	236.0	(121.4 – 351.0)	205.0	(89.6 – 321.0)	252.0	(136.7 – 368.0)	230.0	(116.3 – 344.0)

Table 3.6 *Least squares means estimates for adrenocorticotropic hormone (ACTH) concentrations (pg/mL) from the thyrotropin releasing hormone (TRH) stimulation test in adult and aged horses following adaptation to each diet (control, starch, fiber, sugar).* For each time point (baseline or post-TRH), pairwise significant differences ($P \leq 0.05$) within age groups indicated by lowercase letters. Differences between age groups indicated by *.

	Adult				Aged			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Baseline	11.8	15.7*	11.7	17.2	29.1[a]	60.0[b*]	31.9[a]	37.6[ab]
Post-TRH	54.0*	110.3	244.6	153.1	418.5*	299.9	259.6	307.7

Table 3.7 *Least squares means estimates and pairwise differences for cortisol concentrations (µg/dL) from the overnight dexamethasone suppression test (ODST) after controlling for diet, time of year, and breed.*

	Adult		Aged		
	least squares means	**95% confidence interval**	**least squares means**	**95% confidence interval**	**p-value**
Baseline	3.2	(2.7 – 3.6)	3.4	(3.0 – 3.9)	0.4
Post-dexamethasone	0.4	(0.2 – 0.5)	0.4	(0.2 – 0.6)	0.9

Table 3.8 *Least squares means estimates for cortisol concentrations (µg/dL) from the overnight dexamethasone suppression test (ODST) at different times of the year*. For each time point (baseline or post-dexamethasone), pairwise significant differences ($P \leq 0.05$) between months indicated by lowercase letters.

	March		May		August		October	
	least squares means	**95% confidence interval**	**least squares means**	**95% confidence interval**	**least squares means**	**95% confidence interval**	**least squares means**	**95% confidence interval**
Baseline	3.4	(2.8 – 3.9)	3.0	(2.4 – 3.5)	3.4	(2.8 – 4.0)	3.5	(3.0 – 4.1)
Post-dexamethasone	0.3[a]	(0.2 – 0.5)	0.2[a]	(0.1 – 0.4)	0.3[a]	(0.2 – 0.5)	0.6[b]	(0.5 – 0.8)

Table 3.9 *Least squares means estimates for cortisol concentrations (µg/dL) from the overnight dexamethasone suppression test (ODST) in adult and aged horses at different times of the year.* For each time point (baseline or post-dexamethasone), pairwise significant differences ($P \leq 0.05$) within age groups indicated by lowercase letters. Differences between age groups indicated by *.

	Adult				Aged			
	March	**May**	**August**	**October**	**March**	**May**	**August**	**October**
Baseline	3.0	3.3	3.6	2.9	3.8^{a}	2.6^{b}	3.2^{ab}	4.1^{a}
Post-dexamethasone	0.4	0.2	0.3	0.5^{*}	0.3^{a}	0.3^{a}	0.3^{a}	0.8^{b*}

Table 3.10 *Least squares means estimates for cortisol concentrations (µg/dL) from the overnight dexamethasone suppression test (ODST) in adult and aged horses following adaptation to each diet (control, starch, fiber, sugar).* For each time point (baseline or post-dexamethasone), pairwise significant differences ($P \leq 0.05$) within age groups indicated by lowercase letters.

	Adult				Aged			
	Control	**Starch**	**Fiber**	**Sugar**	**Control**	**Starch**	**Fiber**	**Sugar**
Baseline	2.9	2.8	3.3	3.7	3.7	3.3	3.4	3.4
Post-dexamethasone	0.4	0.4	0.4	0.4	0.4^{ab}	0.6^{a}	0.3^{ab}	0.2^{b}

Figure 3.1 *Box and whisker plots of clinical laboratory baseline adrenocorticotropic hormone (ACTH) concentrations in adult and aged horses at different times of the year.* The solid horizontal line represents the median, the box indicates interquartile range, and the bars indicate the range of values. The dashed horizontal line represents the laboratory cut-off (35 pg/mL) for diagnosis of pituitary pars intermedia dysfunction.

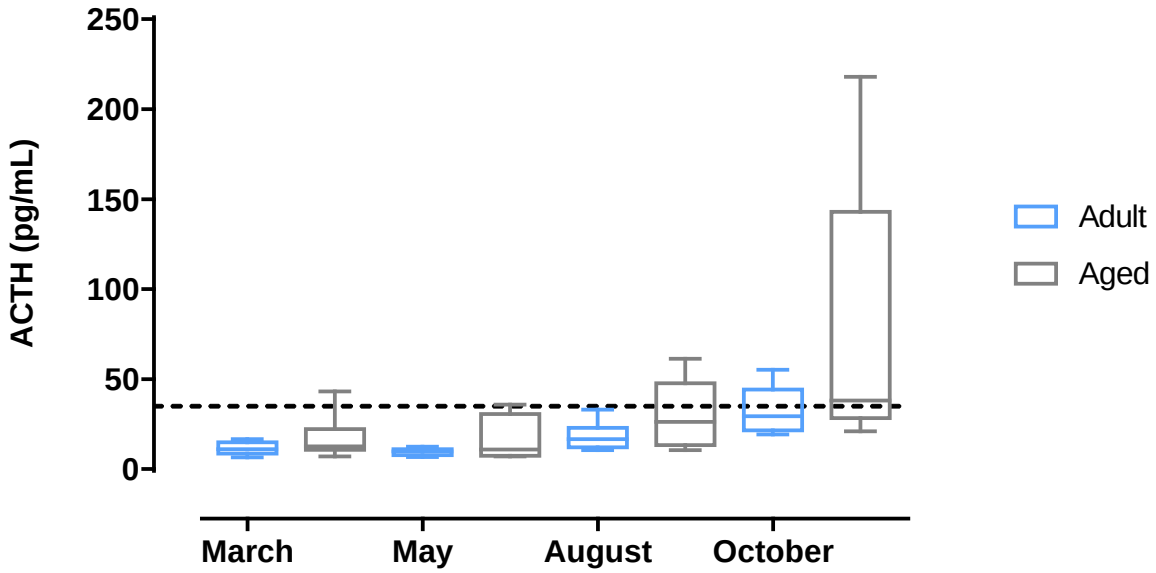


Figure 3.2 *Box and whisker plots of clinical laboratory adrenocorticotropic hormone (ACTH) concentrations, at 10 minutes, following administration of thyrotropin releasing hormone (TRH) in adult and aged horses at different times of the year.* The solid horizontal line represents the median, the box indicates interquartile range, and the bars indicate the range of values. The dashed horizontal line represents the laboratory cut-off (110 pg/mL) for diagnosis of pituitary pars intermedia dysfunction.

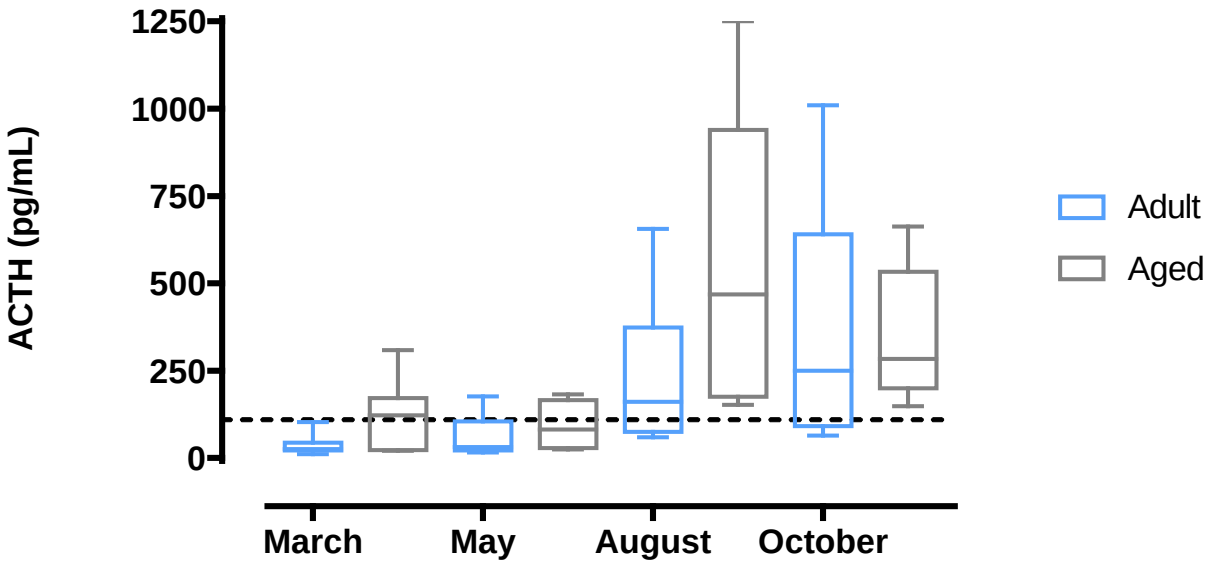
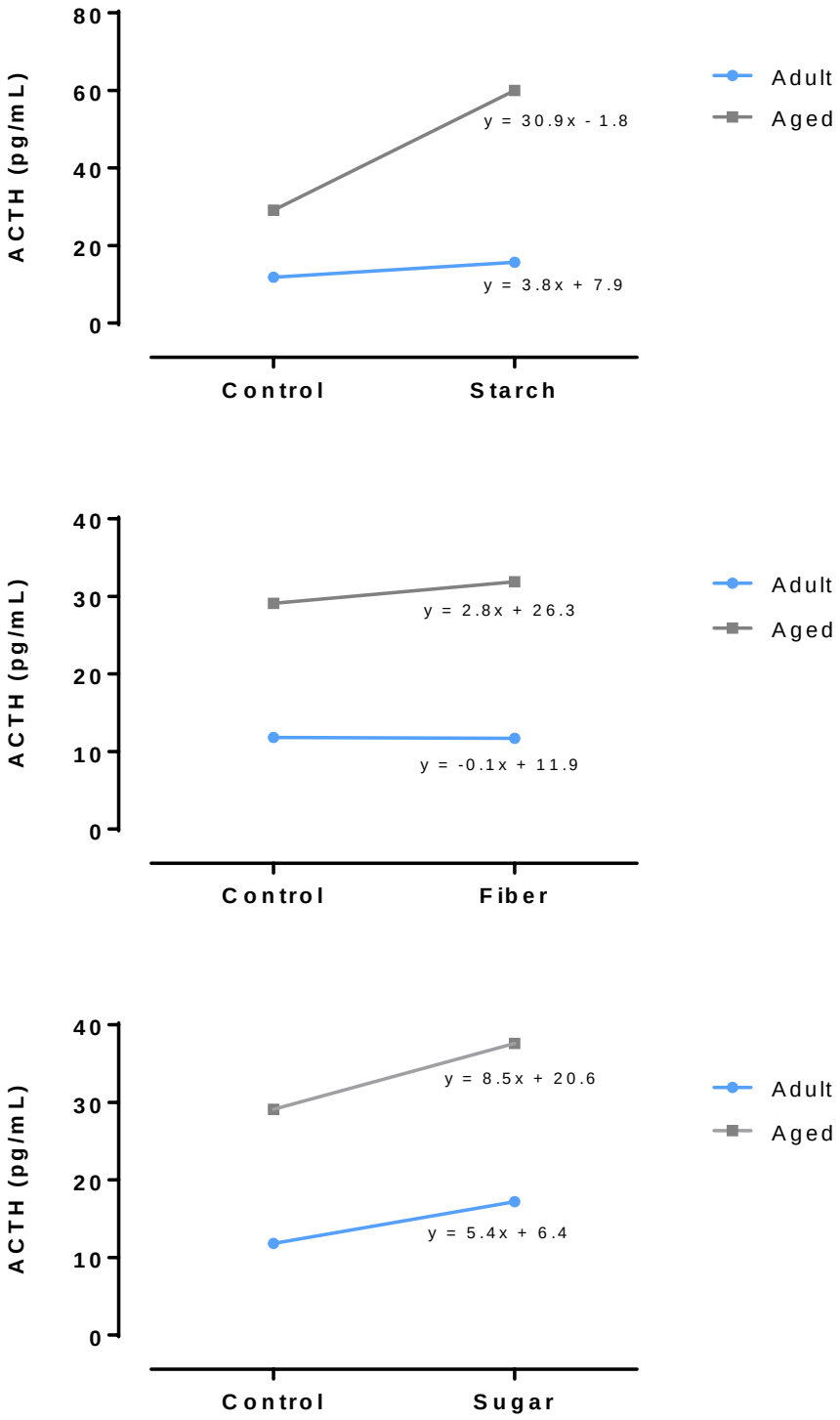


Figure 3.3 *Least squares means estimates of the age*diet interaction for baseline adrenocorticotropic hormone (ACTH) concentrations.* The linear equation $y = mx + b$ represents the relative magnitude of the main effects (age, diet) on the response where m represents the magnitude of the interaction (slope of the line). Each diet (*starch*, *fiber*, *sugar*) is compared to the *control* diet.



CHAPTER 4

Insight into Metabolic Alterations Associated with Aging and Dietary Carbohydrate Profiles

SUMMARY

Background: Metabolomics, the study of small-molecule metabolites, can provide information about changes in metabolic processes across the tissues.

Objectives: 1) To examine the plasma metabolome of horses before (day 0) and after (day 42) adaptation to dietary carbohydrate profiles. 2) To identify differences in metabolites in horses, following dietary adaptation, before (0 minutes) and during (75 minutes) a modified oral sugar test.

Study Design: Balanced Latin square with four isocaloric diets: *control* (restricted-starch-and-sugar fortified pellets), *starch* (control plus kibbled corn), *fiber* (control plus unmolassed sugar beet pulp/soybean hull pellets), and *sugar* (control plus dextrose powder).

Methods: Sixteen healthy Thoroughbred and Standardbred mares and geldings divided into two age groups: *adult* (8.8 ± 2.9 years; n = 8) and *aged* (20.6 ± 2.1 years; n = 8). The metabolomic analysis was performed on plasma samples collected before (day 0) and after (day 42) dietary adaptation as well as before (0 minutes) and during (75 minutes) a modified oral sugar test (OST). Data were analyzed using multivariable linear mixed regression modeling with significance set at $P \leq 0.05$.

Results: A large number of metabolite ion peaks were significantly different between age groups and diet groups; however, to date only 3.4% of the ion peaks have been identified.

Main Limitations: An equine-specific spectral library does not exist thus a number of significant metabolite ion peaks remain unknown. The application of stringent quality control parameters has identified several ultra-high-performance liquid chromatography-mass spectrometry (UHPLC-MS) runs that need to be repeated to assure data quality.

Conclusions: Data provides insight into physiologic differences between adult and aged horses as well as differences in dietary carbohydrate profiles following adaptation.

INTRODUCTION

Metabolomics provides a comprehensive analysis of all small molecules involved in cellular metabolism in a biological sample (serum, plasma, urine, saliva). Metabolomics involves two complementary approaches, untargeted and targeted analyses. With untargeted metabolomics, a qualitative approach examines as many metabolites as possible, whereas a targeted analysis quantifies discrete groups of chemically related metabolites (e.g. amino acids and products of amino acid metabolism). More than 4,000 metabolites have been identified in human serum by use of high-throughput mass spectrometry (gas chromatography [GC]-MS and liquid chromatography [LC]-MS) [67]. Both exogenous factors (diet, drugs, exercise, microbiome) and endogenous factors (age, genetics, body composition, reproductive status, diurnal cycle) likely influence the nutritional metabolome [153]. Understanding the role of nutrition in cellular metabolism is important due to the association with metabolic adaptation and perturbation. Manipulation of diet can have a negative impact on the transition from health to disease as well as serve as a positive treatment option in the transition from disease to health. In humans with metabolic abnormalities, dietary carbohydrate modification altered the serum metabolomic profile especially lipid metabolism and glucose and insulin metabolism [154]. Further, diet-induced weight loss altered lipid metabolites [155,156]. However, few studies have used metabolomics to gain insight into equine medicine, and to the author's knowledge no studies have used metabolomics to study dietary adaptation in horses [109,113,157].

Metabolomics is a powerful tool for defining metabolic changes in different physiologic and pathophysiologic states specifically the understanding of the effects of diet and physiologic state (age) on glucose and insulin dynamics and metabolic adaptation to diet. The objectives were to use untargeted metabolomics to explore the metabolic adaptation following consumption of various dietary carbohydrate profiles and to characterize the differences in the plasma metabolome before and during a modified oral sugar test (OST) in adult and aged horses.

MATERIALS AND METHODS

Study Design

Sixteen healthy Thoroughbred (TB) and Standardbred (STB) mares and geldings were divided into two groups by age: *adult* (5 to 13 years old; mean ± SD; 8.8 ± 2.9 years; n = 9; 4 TB mares, 1 STB mare, 3 TB geldings, 1 STB gelding) and *aged* (18 to 24 years old; 20.6 ± 2.1 years; n = 9; 3 TB mares, 6 STB mares). One aged horse and one adult horse had to be replaced during the study due to failure to eat the diet (n = 1) and a colonic torsion, not considered to be associated with diet, that resulted in euthanasia (n = 1). Replacements were the same sex and similar in age. All animals received routine anthelmintic, vaccination, dental, and farrier treatment as appropriate.

All methods were approved by the Institutional Animal Care and Use Committee at Michigan State University. Horses were randomly assigned to groups of four, blocked for age, and fed four isocaloric diets using a balanced Latin square design. The *control* diet consisted of restricted-starch-and-sugar, fortified pellets[a]. For the three remaining diets, a portion of the *control* diet was removed, and the appropriate energy substrate rich complementary feed added: *starch* (control plus kibbled corn[a]), *fiber* (control plus unmolassed sugar beet pulp/soybean hull pellets[a]), and *sugar* (control plus dextrose powder[b]). All horses received four 7-week dietary treatments, with the total ration being fed at a daily rate of 2.0% to 2.2% of bodyweight (hay: 1.2% and concentrate: 0.8% - 1.0%) on a dry matter basis. During each dietary period, horses were group fed the same batch of grass hay once daily and individually fed two meals of one of the above diets at 7:00 and 17:00. Quantity was pre-determined based on the horse's weight tape[c] estimated weight. In the 7th (final) week, all horses underwent a modified oral sugar test. Dietary periods were separated by a two-week washout period during which all horses received the *control* diet and free access to the same hay and pasture.

Sample Collection

Blood was collected in lithium heparin tubes via jugular venipuncture prior to the start of each dietary period (day 0) as well as following dietary adaptation (day 42) for metabolomic analysis. Plasma

tubes were immediately placed on ice and centrifuged (2000 x g for 15 minutes at 22°C) within thirty minutes of collection, supernatants were collected, aliquoted, and stored at -80°C for future analysis.

Following dietary adaptation, a modified oral sugar test (OST) was performed as previously described [62]. Briefly, administration of a commercially-available corn syrup (Karo® light[d]) was given orally by use of a dose syringe (0.25 mL/kg bodyweight) [12]. Blood was collected in lithium heparin tubes via an indwelling jugular catheter at 0 minutes (baseline) and 75 minutes for metabolomic analysis. Plasma tubes were immediately placed on ice and centrifuged (2000 x g for 15 minutes at 22°C) within thirty minutes of collection, supernatants were collected, aliquoted, and stored at -80°C for future analysis.

Metabolomics

A total of 256 samples were analyzed via an untargeted approach using a combination of chromatography and mass spectrometry following sample preparation. One-hundred-twenty-eight (128) samples represented the metabolome before (day 0) and after (day 42) adaptation to each dietary carbohydrate profile (*control*, *starch*, *fiber*, *sugar*). Further, one-hundred-twenty-eight (128) samples represented the metabolome at 0 minutes and 75 minutes during a modified oral sugar test following adaptation to each diet (*control*, *starch*, *fiber*, *sugar*).

Sample Preparation

Samples were divided into four fractions: analysis by ultra-high-performance liquid chromatography-mass spectrometry (reverse phase positive ionization), analysis by ultra-high-performance liquid chromatography-mass spectrometry (reverse phase negative ionization), analysis by hydrophilic interaction liquid chromatography (HILIC), and analysis by gas chromatography-mass spectrometry (GC-MS). However, only the liquid chromatography-mass spectrometry analysis will be discussed. Protein was removed from samples prior to analysis. Internal standards (phenylalanine, hippuric acid, cholic acid, glucose, palmitic acid) were added to the plasma sample followed by the addition of cold (-80ºC) 10% acetone/90% methanol. Samples were vortexed then incubated at -20ºC for 15 minutes. Samples were

centrifuged (13,000 x g x 10 minutes), supernatant transferred to a clean tube, and sample dried using vacuum centrifugation. A starting buffer (5% acetone, 95% water, 0.1% formic acid) was added to the dry sample.

Mass Spectrometry Analysis

The ultra-high-performance liquid chromatography (UHPLC) platform utilized a Thermo Scientific Ultimate 3000 UHPLC[f] and a Thermo Scientific Q Exactive™[e] Quadrupole Orbitrap mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source operated at 70,000 mass resolution. Three sample extracts were reconstituted in acidic or basic liquid chromatography compatible solvents. The first aliquot was analyzed using acidic, positive ion-optimized conditions, the second aliquot used basic, negative ion-optimized conditions, and the third aliquot was analyzed via negative ionization following elution from a hydrophilic liquid chromatography (HILIC) column (SeqQuant® ZIC®-pHILIC[f]). The gas chromatography-mass spectrometry platform utilized an Agilent 7200B Quadrupole Time-of-Flight GC/MS[g].

Samples were randomized prior to metabolomics analysis. Each sample batch consisted of a process blank (water with formic acid), experimental plasma samples, and a pooled sample. Blank samples were used to subtract background variation in metabolite ion peaks prior to statistical analysis. Pooled samples, used as quality control samples, were created by mixing equal volumes (20 µL) from each of the 256 experimental plasma samples. For the negative analysis, pooled samples were run after every ten experimental samples. For the positive analysis, pooled samples were run after every four experimental samples. For the GC-MS analysis, pooled samples were run after every third sample.

Compound Identification, Quantification, and Data Curation

Raw data files for each analysis method (LC-MS [positive], LC-MS [negative], HILIC, GC-MS) were uploaded separately into the Progenesis® QI software[h]. Retention times were aligned to the most suitable pooled sample based on total chromatogram similarity. Features were selected using absolute ion

sensitivity of 500,000 for reverse-phase liquid chromatography and 100,000 for HILIC and normalized to total ion abundance to correct for unwanted systematic variation. Multiple adduct ions were selected for each ion polarity for de-convolution. The positive adducts included: [M+H]+, [M+2H]2+, [M+H-H2O]+, [M+NH4]+, [M+Na]+, and [M+K]+. The negative and HILIC adducts included: [M-H]-, [M-H-H2O]- and [M+Cl]-. Following deconvolution, two-dimensional peak matrices of peak abundance were exported into the statistical program R[i] for further processing and statistical analyses. A multivariable mixed linear model was utilized to determine significant features ($P < 0.05$). The model included fixed effects (diet, age, breed, period), interaction terms (age*diet and breed*diet), and a random effect (horse).

MS/MS for Metabolite Identification

Inclusion lists were generated for features of statistical significance and subjected to fragmentation (MS/MS) to assist in identification of metabolites of interest. Three distinct inclusion lists were created following statistical analysis of the peak matrices. Missing values were imputed using feature means and log-transformed to assume normality. Predominant features in the blank samples were subtracted from each inclusion list. In addition, similar features within the chromatograph window of six seconds were excluded from the inclusion list. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was performed from the inclusion lists. Metabolites were identified by automated comparison of accurate mass and fragmentation pattern to an experimental spectral library (National Institute of Standards and Technology (NIST 2014)[j]) and theoretical spectral libraries (Human Metabolome Database (HMDB)[k] and LipidBlast[l]). Three tiers of compound identifications were utilized to discriminate quality and source of the match. For all searches, a liberal precursor and fragment mass tolerance window of 0.01 Da were used to reduce missed compound identifications. Identifications were manually validated. As needed, fragment spectra were compared to the METLIN[m] Metabolomics Database to assess the match quality.

Data Quality Control

All quality control (QC) analyses were performed in the statistical program R[i]. First, data were normalized to remove batch effects and correct for drift within a single batch (standard vector regression [158]). To evaluate if normalization was effective for pooled quality control samples and experimental samples thus removing drift and batch effects, a regression model was fit to the data and an R^2 value calculated. Metabolite ion peaks with an R^2 value greater than 0.8 in the pooled samples were removed. Metabolite ion peaks with an R^2 value greater than 0.5 in the experimental samples were removed. Normalized metabolite ion peaks were visually inspected and metabolites in which normalized sample quantities did not vary around the pooled samples were excluded. Second, a variant of the Dixon's outlier test was used to test for outliers. Outliers were removed if the metabolite failed the outlier test at a cutoff of 0.3; a maximum of 10% of outliers on each side was allowed. Third, metabolite ion peaks with > 10% missing values were excluded from the dataset and missing values imputed using random forest.

Statistical Analysis

Data were analyzed using a multivariable linear mixed model fit for each metabolite. All models were mixed regression models containing both fixed and random effects. Full model predictors included: *age* (adult, aged), *diet* (control, fiber, starch, sugar), *breed* (Thoroughbred, Standardbred), and *period (*first, second, third, fourth*)* and the interactions between *age* and *period (age*period), age* and *diet* (age*diet), and *breed* and *diet* (breed*diet) as fixed effects. A random effect for *horse* nested within *period* was included in all models to account for animal-to-animal (inter-animal) variability, to account for the variation and correlation in data due to repeated measures on the same observational unit over time (i.e. repeated measures for horse due to the Latin square design) and to account for any effect of diet sequence. The inclusion of horse as a random effect variable also accounts for missing data from the four horses that were not sampled in the entire study. To evaluate if there was an effect of the previous diet (despite a washout period) on the "Day 0" sample, multivariable linear mixed models were fit for each metabolite for the

second, third, and fourth period. The model included fixed effects (age, last diet, breed, period), interaction terms (age*last diet, breed*last diet, age*period), and a random effect (horse).

All data are reported as least squares means estimates and pairwise significant differences ($P \leq 0.05$) from the full model. Pairwise differences for all models were determined using the *lmerTest* package *difflsmeans* function which gives differences of the least squares means table with p-values and confidence intervals using Satterthwaite's approximation for degrees of freedom. For pairwise comparisons between variables with more than two levels, p-values and confidence intervals were corrected for multiple comparisons using the Tukey Honest Significant Difference (HSD) method.

RESULTS

Animals

Diets were well tolerated by the horses and refusals were insignificant (mean ± SD; 76.2 ± 0.6 grams/horse/day). No significant differences in weight or body condition score were noted between age groups, diets, or time points.

Metabolomics

Before normalization and removal of outliers, the negative phase metabolomic analysis had 304 samples (16 blank + 32 pooled + 256 experimental) and a total of 2,580 metabolite ion peaks of which 1,000 features were significantly different in initial statistical analyses and were subjected to MS/MS. Four experimental samples and 143 metabolite ion peaks were removed due to > 10% missingness. After MS/MS fragmentation, 73 metabolites were of known identity based on homology with human metabolites. The positive phase metabolomic analysis had 342 samples (16 blank + 70 pooled + 256 experimental) and 3,252 metabolite ion peaks of which 1,000 features were significantly different in initial statistical analyses and were subjected to MS/MS. Two experimental samples and 147 metabolite ion peaks were removed due to > 10% missingness. After MS/MS fragmentation, 100 metabolites were of known identity based on homology with human metabolites.

After normalization and removal of outliers, the negative phase metabolomic analysis had a total of 1,996 metabolite ion peaks and the positive phase metabolomic analysis had a total of 2,704 metabolite ion peaks. **Figure 4.1** shows a metabolite ion peak that improved following normalization. **Figure 4.2** demonstrates a metabolite ion peak in which normalization did not correct for batch effects. An additional 24 samples were excluded from both the positive and negative analyses due to inadequate correction for batch effects after normalization. Known metabolites were classified into eight metabolic pathways (lipid, amino acid, carbohydrate, cofactors and vitamins, energy, nucleotide, peptide, xenobiotics) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway classification system.

Metabolite Differences Before (day 0) and After (day 42) Dietary Adaptation

Overall, across both the fixed effects (diet, age, period, breed) and interaction terms (age*period, diet*age, diet*breed), 7,228 metabolite ion peaks (248 known peaks) were significant. Due to the exclusion of 24 experimental samples and issues with the quality control samples, the data presented here only includes the effects of age and diet, the two primary variables of interest. To determine if metabolic alterations were present for the main effects of age and diet, metabolite ion peaks were compared at baseline (day 0) and after adaptation (day 42). There was no effect of the previous diet on the baseline sample. For the main effect of age, a total of 2,177 metabolite ion peaks (71 known peaks) were significant with 982 metabolite ion peaks (23 known peaks) at baseline, 373 metabolite ion peaks (18 known peaks) after adaptation, and 822 metabolite ion peaks (30 known peaks) changed between the timepoints. For the main effect of diet, a total of 1,644 metabolite ion peaks (59 known peaks) were significant with 442 metabolite ion peaks (15 known peaks) at baseline, 742 metabolite ion peaks (28 known peaks) after adaptation, and 460 metabolite ion peaks (16 known peaks) changed between the timepoints.

When comparing *adult* horses versus *aged* horses, 982 metabolite ion peaks at baseline and 373 metabolite ion peaks following adaptation were statistically different. Known metabolites (n = 41) significantly different between age groups are listed based on their metabolic pathways and sub-pathways: lipid (n = 11), amino acid (n = 9), cofactors and vitamins (n = 1), nucleotide (n = 3), peptide (n = 4),

xenobiotics (n = 9), and unknown (n = 4) (**Table 4.1**). At baseline, 12 metabolites had increased concentrations while 10 metabolites had decreased concentrations in *aged* horses compared to *adult* horses. Following dietary adaptation, 18 metabolites had increased concentrations while 1 metabolite had a decreased concentration in *aged* horses compared to *adult* horses.

When comparing the *fiber*, *starch*, and *sugar* diets versus the *control* diet, 219 metabolite ion peaks at baseline and 275 metabolite ion peaks following adaptation were statistically different. Known metabolites (n = 39) significantly different between diets are listed based on their metabolic pathways and sub-pathways: lipid (n = 7), amino acid (n = 13), carbohydrate (n = 1), nucleotide (n = 2), peptide (n = 3), xenobiotics (n = 6), other (n = 4), and unknown (n = 3). Following dietary adaptation, compared to the *control* diet, 4 metabolites had increased concentrations while 3 metabolites had decreased concentrations on the *fiber* diet (**Table 4.2**), 5 metabolites had increased concentrations while 3 metabolites had decreased concentrations on the *starch* diet (**Table 4.3**), and 4 metabolites had increased concentrations while 5 metabolites had decreased concentrations on the *sugar* diet (**Table 4.4**).

Metabolite Changes During an Oral Sugar Test

A total of 7,325 metabolite ion peaks (248 known peaks) were significant for fixed effects (diet, age, period, breed) and interaction terms (age*period, age*diet, diet*breed) combined. Due to the exclusion of 24 experimental samples and issues with the quality control samples, the data presented here includes only the main effects of age and diet, the two main variables of interest. To determine if metabolic alterations were present for the main effects of age and diet, metabolite ion peaks were compared at 0 minutes (baseline) and 75 minutes. For the main effect of age, a total of 2,954 metabolite ion peaks (108 known peaks) were significant with 944 metabolite ion peaks (48 known peaks) at 0 minutes, 891 metabolite ion peaks (27 known peaks) at 75 minutes, and 1,119 metabolite ion peaks (33 known peaks) changed from 0 to 75 minutes. For the main effect of diet, a total of 1,851 metabolite ion peaks (59 known peaks) were significant with 753 metabolite ion peaks (25 known peaks) at 0 minutes, 833 metabolite ion

peaks (29 known peaks) at 75 minutes, and 265 metabolite ion peaks (5 known peaks) changed from 0 minutes to 75 minutes.

When comparing *adult* horses versus *aged* horses, 944 metabolite ion peaks at 0 minutes (baseline) and 891 metabolite ion peaks at 75 minutes were statistically different. Known metabolites (n = 51) significantly different between age groups are listed based on their metabolic pathways and sub-pathways: lipid (n = 13), amino acid (n = 11), carbohydrate (n = 3), cofactors and vitamins (n = 1), nucleotide (n = 2), peptide (n = 4), xenobiotics (n = 11), other (n = 1), and unknown (n = 5) (**Table 4.5**). At 0 minutes (baseline), 28 metabolites had increased concentrations while 6 metabolites had decreased concentrations in *aged* horses compared to *adult* horses. At 75 minutes, post administration of Karo® syrup, 15 metabolites had increased concentrations while 6 metabolites had decreased concentrations in *aged* horses compared to *adult* horses.

When comparing the *fiber*, *starch*, and *sugar* diets versus the *control* diet, 469 metabolite ion peaks at 0 minutes (baseline) and 421 metabolite ion peaks at 75 minutes were statistically different. Known metabolites (n = 39) significantly different between diets are listed based on their metabolic pathways and sub-pathways: lipid (n = 4), amino acid (n = 11), carbohydrate (n = 2), cofactors and vitamins (n = 1), nucleotide (n = 1), peptide (n = 5), xenobiotics (n = 10), other (n = 4), and unknown (n = 1). At 0 minutes (baseline), compared to the *control* diet, no metabolites had increased concentrations while 3 metabolites had decreased concentrations on the *fiber* diet (**Table 4.6**), 2 metabolites had increased concentrations while 14 metabolites had decreased concentrations on the *starch* diet (**Table 4.7**), and 4 metabolites had increased concentrations while 2 metabolites had decreased concentrations on the *sugar* diet (**Table 4.8**). At 75 minutes, post administration of Karo® syrup, compared to the *control* diet, 1 metabolite had an increased concentration while 5 metabolites had decreased concentrations on the *fiber* diet (**Table 4.6**), no metabolites had increased concentrations while 10 metabolites had decreased concentrations on the *starch* diet (**Table 4.7**), and 4 metabolites had increased concentrations while 11 metabolites had decreased concentrations on the *sugar* diet (**Table 4.8**).

DISCUSSION

Metabolomics remains a powerful approach for defining changes in cellular metabolism in relation to age and nutrition. In this study, untargeted metabolomics has identified thousands of significant metabolite ion peaks; however, the identity of a number of these peaks remains unknown. While substantial conclusions cannot be made at this point, this study has provided evidence that metabolomic profiling is a relevant approach for further defining metabolic alterations due to age and diet in horses. Examination of the plasma metabolome demonstrated significant differences in metabolites primarily derived from amino acids, lipids, and xenobiotics. However, these results are likely to change when additional metabolites are identified through additional MS/MS analyses.

Application of metabolomics to aging studies identifies metabolites and metabolic pathways that change during the aging process. Aging is a multifaceted process characterized by a general decline in cellular function and homeostasis. In humans, studies have identified changes in sphingolipid metabolism, arachidonic acid metabolism, and glycerophospholipid metabolism during the aging process. Specifically, sphingosine, oleamide, indolelactic acid, and lysophosphatidylcholines were strongly (positively) correlated with age [159]. Despite limitations in metabolite identification, we have identified increases in sphinganine, an arachidonic acid metabolite (15-HETE), and a lysophosphatidylcholine metabolite (phosphatidylethanolamine (20:1/0:0)) in aged horses when compared to adult horses. Further, citric acid cycle intermediates (isocitrate, α-ketoglutarate, malate), nucleotides (allantoin, xanthine, uridine), amino acids (lysine, L-kynurenine), and branched-chain amino acids (leucine, isoleucine, valine) were increased in aged humans [160]. Similarly, in aged horses in this study, we have identified increases in a lysine metabolite (N-epsilon-acetyl-L-lysine) and nucleotide metabolites (allantoic acid, uridine) in aged horses relative to adult horses. Interestingly, we observed a decrease in L-kynurenine in aged horses, which is opposite to the observations in humans. Identification of age-related biomarkers is relevant to understanding underlying metabolic changes to target preventative and therapeutic treatments to delay aging-associated pathological changes.

Application of metabolomics to nutrition studies shows that interactions between nutrients and metabolism lead to metabolic alterations. Dietary metabolites play a significant role in an individual's cellular metabolism by acting as building blocks of macromolecules and cellular membranes, signaling messengers, antioxidants, and sources of energy. For example, feeding a diet enriched with fatty acids alters neural membranes and concentrations of docosahexaenoic acid (DHA) leading to a change in neurotransmission and brain development [161–163]. In addition, dietary sugars enter glycolysis, which generates nucleotide adenosine triphosphate, an energy-rich metabolite; however, dietary sugars may also enter the fatty acid synthesis pathway. In humans, consumption of sugar-rich foods leads to fatty liver disease and diabetes [164]. In horses, consumption of a sugar-rich diet leads to improved tissue insulin sensitivity [128,165]; however, its role in the manifestation of disease is unknown. Understanding the effect of dietary metabolites on cellular metabolism would allow diets to be designed to improve cellular function and overall health.

Generation and analysis of metabolomic data present several challenges. Untargeted metabolomics (or discovery metabolomics) creates a comprehensive analysis of a biological sample. While this gives the most complete picture of cellular metabolism, it comes with several challenges such as identification of known metabolites for biological interpretation and data analysis. While metabolomics is a very powerful tool for exploring cellular metabolism, it is a finicky technology and process. The inclusion of quality control samples provides a method for minimizing variation amongst results. First, normalization of the experimental samples against the pooled quality control samples should eliminate unwanted non-biological variation. Unwanted non-biological variation such as signal drift and batch effect in metabolomics experiments pose a challenge when interpreting biological significance. Signal drift refers to measurement fluctuations due to changes in instrument sensitivity, chromatograph retention time, and sample preparation. Batch effect refers to the technical variation that occurs between "batches" of samples analyzed at different times. Quality control samples serve as a measure of repeatability within an analytical batch. These samples aid in the removal of metabolic features with excessive drift in accurate mass, retention time, or signal. Pooled quality control samples serve as technical replicates as theoretically, their biological composition is

similar to the experimental samples. The purpose of a quality control sample is to equilibrate the analytical platform before analysis of experimental samples to ensure reproducible data, to calculate technical precision within each batch, and to provide data to use for signal correction within and between batches [68]. Comparison of quality control samples before and after normalization demonstrates variability between batches raising concerns that an error in instrumentation may be present prompting samples to be rerun.

The second challenge of performing untargeted metabolomics is the identification of metabolites. In this study, thousands of metabolite ion peaks were generated; however, a limited number of peaks were identified. Initially, significant metabolite ion peaks were separated into inclusion lists for MS/MS fragmentation for metabolite identification. However, following generation of the MS/MS data, an error in the experimental sample master key was identified. This error may have resulted in the inclusion of non-significant metabolite ion peaks or exclusion of significant metabolite ion peaks. Further, the lack of an equine-specific library coupled with an outdated human metabolite library contributed to a small proportion of identifications. Additional identifications are expected as the pooled quality control samples will be analyzed by MS/MS and all metabolite ion peaks within those samples will be identified which should lead to a more complete biological interpretation. In addition, an updated National Institute of Standards and Technology (NIST) database should be available by December 2017.

Further data analysis is required to overcome these challenges and limitations. First, identification and rerunning of samples with significant variability that cannot be corrected with normalization needs to be performed. Second, all metabolite ion peaks in the pooled quality control samples need to undergo MS/MS fragmentation which will allow for additional metabolite identifications. It is anticipated that once these limitations are overcome, based on human studies, that metabolite differences will provide insight into age-associated and diet-associated changes in cellular metabolism.

FOOTNOTES

[a]MARS Horsecare US Inc, Dalton, Ohio, USA.

[b]Sigma-Aldrich, Saint Louis, Missouri, USA.

[c]The Coburn Company, Whitewater, Wisconsin, USA.

[d]ACH Food Companies Inc., Cordova, Tennessee, USA.

[e]Thermo Fisher Scientific, Waltham, Massachusetts, USA.

[f]EMD Millipore Corporation, Billerica, Massachusetts, USA.

[g]Agilent, Santa Clara, California, USA.

[h]Nonlinear Dynamics, Durham, North Carolina, USA.

[i]R Core Team, Vienna, AUSTRIA.

[j]National Institute of Standards and Technology, U.S. Department of Commerce, Gaithersburg, Maryland, USA.

[k]Wishart Research Group, University of Alberta, Edmonton, CANADA.

[l]Fiehn Laboratory, University of California – Davis, Davis, California, USA.

[m]The Scripps Research Institute, La Jolla, California, USA.

APPENDIX

Table 4.1 *Significant ($P \leq 0.05$) amino acid, cofactors and vitamins, lipid, nucleotide, peptide, xenobiotic, and unknown pathway metabolites indicated by higher (↑) concentrations and lower (↓) concentrations before (Day 0) and after (Day 42) dietary adaptation in aged horses compared to adult horses independent of diet and the interaction term (age*diet).*

AMINO ACID	DAY 0	DAY 42
Choline Metabolism		
trimethylamine N-oxide	↑	
Urea Cycle		
D-ornithine	↑	
Lysine Metabolism		
N-epsilon-acetyl-L-lysine	↑	
Methionine Metabolism		
methionine sulfoxide	↑	
Phenylalanine and Tyrosine Metabolism		
L-tyrosine	↓	
Tryptophan Metabolism		
indolelactic acid	↓	
L-kynurenine	↓	
indoxyl sulfate		↑
Tyrosine Metabolism		
retinol	↓	

COFACTORS AND VITAMINS	DAY 0	DAY 42
Hemoglobin, Porphyrin and Chlorophyll Metabolism		
bilirubin	↓	

LIPID	DAY 0	DAY 42
Arachidonic Acid Metabolism		
15-HETE		↑
Fatty Acid Metabolism		
1-o-hexadecyl-2-o-acetyl-sn-glyceryl-3-phosphorylcholine	↓	
1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-N,N-dimethylethanolamine	↑	
traumatic acid		↓
PC-M6		↑
lysophospholipid (16:0)		↑
hydroxybutyrylcarnitine	↑	
3-hydroxysuberic acid	↓	
L-carnitine	↓	
cis-5,8,11-eicosatrienoic acid		↑
Sphingolipid Metabolism		
sphinganine		↑

Table 4.1 (cont'd)

NUCLEOTIDE	DAY 0	DAY 42
Purine Metabolism		
allantoic acid	↑	
Pyrimidine Metabolism		
uridine		↑
Uracil Derivative		
5-hydroxymethyl-4-methyluracil		↑

PEPTIDE	DAY 0	DAY 42
Dipeptide		
prolyl-tyrosine	↑	
tyrosyl-aspartate	↑	
valine-glycine	↑	

XENOBIOTIC	DAY 0	DAY 42
Chemical Compound		
1-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine	↓	
1-heptadecanoyl-sn-glycero-3-phosphocholine		↑
1-pentadecanoyl-sn-glycero-3-phosphocholine		↑
1-hexadecyl-sn-glycero-3-phosphocholine		↑
Drug		
dimethyl sulfoxide		↑
Food Component/Plant		
juzirine	↓	↑
1-hexadecanoyl-sn-glycerol		↑
daphniphylline		↑
glycerol 1-stearate		↑

UNKNOWN	DAY 0	DAY 42
1-stearoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine	↑	
3,4-dihydroxyphenylglycol o-sulfate	↑	
1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-(1'-myo-inositol)		↑
1-stearoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine		↑

Table 4.2 *Significant ($P \leq 0.05$) amino acid, lipid, nucleotide, xenobiotic, and unknown pathway metabolites indicated by higher (↑) concentrations and lower (↓) concentrations before (Day 0) and after (Day 42) dietary adaptation to the fiber diet compared to the control diet.*

AMINO ACID	DAY 0	DAY 42
Choline Metabolism		
trimethylamine N-oxide	↑	
Tyrosine Metabolism		
retinol	↓	

LIPID	DAY 0	DAY 42
Fatty Acid Metabolism		
phosphatidylethanolamine (22:2/0:0)	↓	
3-hydroxysuberic acid		↓
hydroxybutyrylcarnitine		↑
phosphatidylethanolamine (20:1/0:0)	↓	
Steroid		
glycoursodeoxycholic acid		↓

NUCLEOTIDE	DAY 0	DAY 42
Uracil Derivative		
5-hydroxymethyl-4-methyluracil		↓

XENOBIOTIC		
Chemical Compound		
1-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine	↓	
Food Component/Plant		
hydrocotarnine		↑

UNKNOWN		
1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	↓	↓
3-methoxybenzenepropanoic acid		↑
o-nitrobenzoic acid		↑

Table 4.3 *Significant ($P \leq 0.05$) amino acid, lipid, peptide, xenobiotic, and unknown pathway metabolites indicated by higher (↑) concentrations and lower (↓) concentrations before (Day 0) and after (Day 42) dietary adaptation to the starch diet compared to the control diet.*

AMINO ACID	DAY 0	DAY 42
Arginine and Proline Metabolism		
N-acetylornithine		↓
Histidine Metabolism		
L-glutamic acid	↑	
Lysine Metabolism		
N-alpha-acetyl-L-lysine	↑	
N-epsilon-acetyl-L-lysine	↓	
Methionine Metabolism		
5-methylthioribose	↑	
Tryptophan Metabolism		
L-kynurenine		↑

LIPID	DAY 0	DAY 42
Fatty Acid Metabolism		
lysophospholipid (16:0)		↓
phosphatidylethanolamine (20:1/0:0)	↓	↑
phosphatidylethanolamine (20:4/0:0)	↓	

PEPTIDE	DAY 0	DAY 42
Dipeptide		
aspartyl-tryptophan		↑
prolyl-tyrosine	↑	
tyrosyl-aspartate		↑

XENOBIOTIC	DAY 0	DAY 42
Chemical Compounds		
1-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine	↓	
1-pentadecanoyl-sn-glycero-3-phosphocholine		↓
Food Component/Plant		
glycerol 1-stearate		↑

UNKNOWN	DAY 0	DAY 42
1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	↓	

Table 4.4 *Significant ($P \leq 0.05$) amino acid, lipid, peptide, xenobiotic, and unknown pathway metabolites indicated by higher (↑) concentrations and lower (↓) concentrations before (Day 0) and after (Day 42) dietary adaptation to the sugar diet compared to the control diet.*

AMINO ACID	DAY 0	DAY 42
Amino Sugar Metabolism		
L-glutamine		↑
D-Arginine and D-Ornithine Metabolism; Urea Cycle		
D-ornithine	↓	
Histidine Metabolism		
L-histidine		↑
Phenylalanine and Tyrosine Metabolism		
p-cresol sulfate		↓
Tryptophan Metabolism		
indoxyl sulfate		↓

CARBOHYDRATE	DAY 0	DAY 42
Glucose Derivative		
butyl (S)-3-hydroxybutyrate glucoside	↑	

LIPID	DAY 0	DAY 42
Fatty Acid Metabolism		
phosphatidylethanolamine (20:4/0:0)	↓	

NUCLEOTIDE	DAY 0	DAY 42
Pyrimidine Metabolism		
uridine		↑

XENOBIOTIC	DAY 0	DAY 42
Drug		
indoleacrylic acid	↑	
Food Component/Plant		
isoferulic acid		↑

OTHER	DAY 0	DAY 42
Dopamine and Norepinephrine Metabolism		
DOPA sulfate		↓
norepinephrine sulfate		↓
Polyphenol Metabolite		
caffeic acid 4-sulfate		↓
cholest-4-en-26-oic acid, 7-alpha-hydroxy-3-oxo		

Table 4.5 *Significant ($P \leq 0.05$) amino acid, carbohydrate, cofactors and vitamins, lipid, nucleotide, peptide, xenobiotic, other, and unknown pathway metabolites indicated by higher (↑) concentrations and lower (↓) concentrations before (0 minutes) and during (75 minutes) the oral sugar test in aged horses compared to adult horses independent of diet and the interaction term (age*diet).*

AMINO ACID	0 MINUTES	75 MINUTES
Arginine and Proline Metabolism		
N-acetylornithine		↑
citrulline		↑
Urea Cycle		
D-ornithine		↑
Lysine Metabolism		
N-alpha-acetyl-L-lysine	↑	
Phenylalanine and Tyrosine Metabolism		
DL-phenylalanine		↓
L-tyrosine		↓
Pyrimidine Metabolism		
2-aminoisobutyric acid		↑
Tryptophan Metabolism		
indolelactic acid	↓	
L-kynurenine		↓
indoxyl sulfate	↑	
Tyrosine Metabolism		
retinol	↑	

CARBOHYDRATE	0 MINUTES	75 MINUTES
Glucose Derivative		
butyl (S)-3-hydroxybutyrate glucoside	↓	
Starch and Sucrose Metabolism		
2-phenylethanol glucuronide		↓
D-lyxose		↑

COFACTORS AND VITAMINS	0 MINUTES	75 MINUTES
bilirubin	↑	

Table 4.5 (cont'd)

LIPID	0 MINUTES	75 MINUTES
Arachidonic Acid Metabolism		
15-HETE	↑	
Fatty Acid Metabolism		
L-carnitine	↓	
lysophospholipid (16:0)	↑	↑
lysophosphatidylethanolamine (20:1/0:0)		↑
1-nonadecanoyl-2-(9Z-octadecenoyl)-glycero-3-phosphocholine	↑	
1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho-N, N-dimethylethanolamine	↓	↑
cis-5,8,11-eicosatrienoic acid	↑	
traumatic acid	↑	
N-oleoylethanolamine	↑	
1-o-hexadecyl-2-o-acetyl-sn-glyceryl-3-phosphorylcholine	↑	
Fatty Acyls		
10,20-dihydroxyeicosanoic acid	↑	
(E)-2-methyl-2-buten-1-ol o-beta-D-glucopyranoside		↑
Sphingolipid Metabolism		
sphinganine	↑	

NUCLEOTIDE	0 MINUTES	75 MINUTES
Purine Metabolism		
allantoic acid	↑	
Pyrimidine Metabolism		
uridine	↓	

PEPTIDE	0 MINUTES	75 MINUTES
Dipeptide		
prolyl-tyrosine	↑	
tyrosyl-aspartate	↑	↑
valine-glycine	↑	↑
isoleucine-arginine		↓

Table 4.5 (cont'd)

XENOBIOTIC	0 MINUTES	75 MINUTES
Chemical Compound		
1-heptadecanoyl-sn-glycero-3-phosphocholine	↑	
1-hexadecanoyl-sn-glycero-3-phosphoethanolamine	↑	
1-hexadecyl-sn-glycero-3-phosphocholine	↑	
1-pentadecanoyl-sn-glycero-3-phosphocholine	↑	
1-stearoyl-2-hydroxy-sn-glycero-3-phosphocholine	↑	
1-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine	↑	↑
Drug		
indoleacrylic acid		↓
lidocaine	↑	
Food Component/Plant		
daphniphylline	↑	
1-hexadecanoyl-sn-glycerol	↑	
cyclohexylamine		↑

OTHER	0 MINUTES	75 MINUTES
Lactone		
3-hydroxyadipic acid 3,6-lactone		↑

UNKNOWN	0 MINUTES	75 MINUTES
1-hexadecanoyl-2-sn-glycero-3-phosphate	↓	
3-methoxybenzenepropanoic acid	↑	
9(10)-EpOME	↑	
1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine		↑
cholest-4-en-26-oic acid, 7-alpha-hydroxy-3-oxo	↑	

Table 4.6 *Significant ($P \leq 0.05$) amino acid, lipid, xenobiotic, other pathway metabolites indicated by higher (↑) concentrations and lower (↓) concentrations before (0 minutes) and during (75 minutes) the oral sugar test following adaptation to the fiber diet compared to the control diet.*

AMINO ACID	**0 MINUTES**	**75 MINUTES**
Urea Cycle		
D-ornithine		↓
Tryptophan Metabolism		
indoxyl sulfate	↓	

LIPID	**0 MINUTES**	**75 MINUTES**
Fatty Acid Metabolism		
lysophosphatidylethanolamine (20:1/0:0)		↓

XENOBIOTIC	**0 MINUTES**	**75 MINUTES**
Drug		
indoleacrylic acid		↑
salicylic acid	↓	↓

OTHER	**0 MINUTES**	**75 MINUTES**
Benzene Derivative		
homoveratric acid		↓
Dopamine and Norepinephrine Metabolism		
norepinephrine sulfate	↓	↓

Table 4.7 *Significant ($P \leq 0.05$) amino acid, lipid, nucleotide, peptide, xenobiotic, and other pathway metabolites indicated by higher (↑) concentrations and lower (↓) concentrations before (0 minutes) and during (75 minutes) the oral sugar test following adaptation to the starch diet compared to the control diet.*

AMINO ACID	0 MINUTES	75 MINUTES
Arginine and Proline Metabolism		
N-acetylornithine	↓	↓
Leucine, Isoleucine and Valine Metabolism		
isovalerylglucuronide	↓	
Lysine Metabolism		
N-alpha-acetyl-L-lysine	↓	
N (6)-methyllysine		↓
Phenylalanine and Tyrosine Metabolism		
p-cresol sulfate		↓
Tryptophan Metabolism		
DL-indole-3-lactic acid	↓	
Tyrosine Metabolism		
retinol	↓	

LIPID	0 MINUTES	75 MINUTES
Fatty Acid Metabolism		
hydroxybutyrylcarnitine	↓	↓

NUCLEOTIDE	0 MINUTES	75 MINUTES
Pyrimidine Metabolism		
uridine	↑	

PEPTIDE	0 MINUTES	75 MINUTES
Dipeptide		
glucose-phenylalanine	↓	
leucyl-alanine	↓	
L-gamma-glutamyl-L-isoleucine	↓	
isoleucine-arginine		↓
prolyl-tyrosine	↑	

Table 4.7 (cont'd)

XENOBIOTIC	0 MINUTES	75 MINUTES
Benzoate Metabolism		
hippuric acid	↓	↓
Drug		
salicylic acid	↓	
p-acetaminobenzoic acid	↓	↓
Food Component/Plant		
cyclohexylamine	↓	↓
(9Z,12Z,14E)-16-Hydroxy-9,12,14-octadecatrienoic acid		↓

OTHER	0 MINUTES	75 MINUTES
Dopamine and Norepinephrine Metabolism		
norepinephrine sulfate		↓

Table 4.8 *Significant ($P \leq 0.05$) amino acid, carbohydrate, cofactors and vitamins, lipid, xenobiotic, other, and unknown pathway metabolites indicated by higher (↑) concentrations and lower (↓) concentrations before (0 minutes) and during (75 minutes) the oral sugar test following adaptation to the sugar diet compared to the control diet.*

AMINO ACID	0 MINUTES	75 MINUTES
Arginine and Proline Metabolism		
N-acetylornithine		↓
Urea Cycle		
citrulline	↑	
D-ornithine		↓
Phenylalanine and Tyrosine Metabolism		
p-cresol sulfate	↓	↓
Tryptophan Metabolism		
L-kynurenine		↑
indoxyl sulfate	↑	↓

CARBOHYDRATE	0 MINUTES	75 MINUTES
Starch and Sucrose Metabolism		
2-phenylethanol glucuronide		↑
tyramine glucuronide		↑

COFACTORS AND VITAMINS	0 MINUTES	75 MINUTES
Hemoglobin, Porphyrin and Chlorophyll Metabolism		
bilirubin		↑

LIPID	0 MINUTES	75 MINUTES
Fatty Acid Metabolism		
hydroxybutyrylcarnitine		↓
lysophosphatidylethanolamine (20:1/0:0)		↓

XENOBIOTIC	0 MINUTES	75 MINUTES
Chemical Compound		
1-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine		↓
Drug		
indoleacrylic acid	↑	
Food Compound/Plant		
polyethylene	↑	
(1xi,3xi)-1,2,3,4-tetrahydro-1-methyl-beta-carboline-3-carboxylic acid		↓

Table 4.8 (cont'd)

OTHER	0 MINUTES	75 MINUTES
Aminobenzoate Degradation; Microbial Metabolism		
o-nitrobenzoic acid		
Dopamine and Norepinephrine Metabolism		
DOPA sulfate		↓
norepinephrine sulfate	↓	↓

UNKNOWN	0 MINUTES	75 MINUTES
1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine		↓

Figure 4.1 *Metabolite ion peaks before and after standard vector regression normalization in pooled quality control samples and experimental samples. Normalization improved the variability in the samples.* The vertical black line separates the analytical batches.

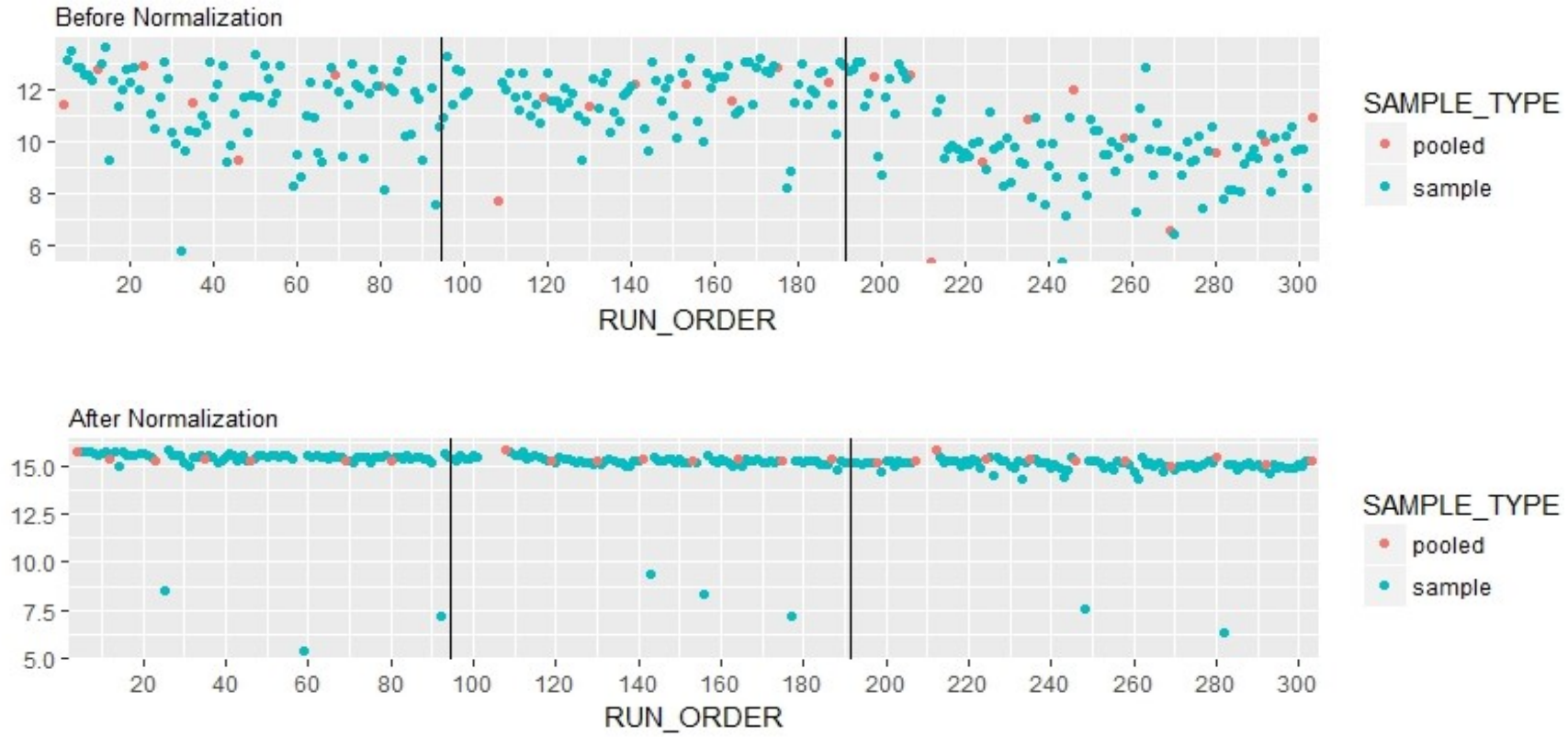
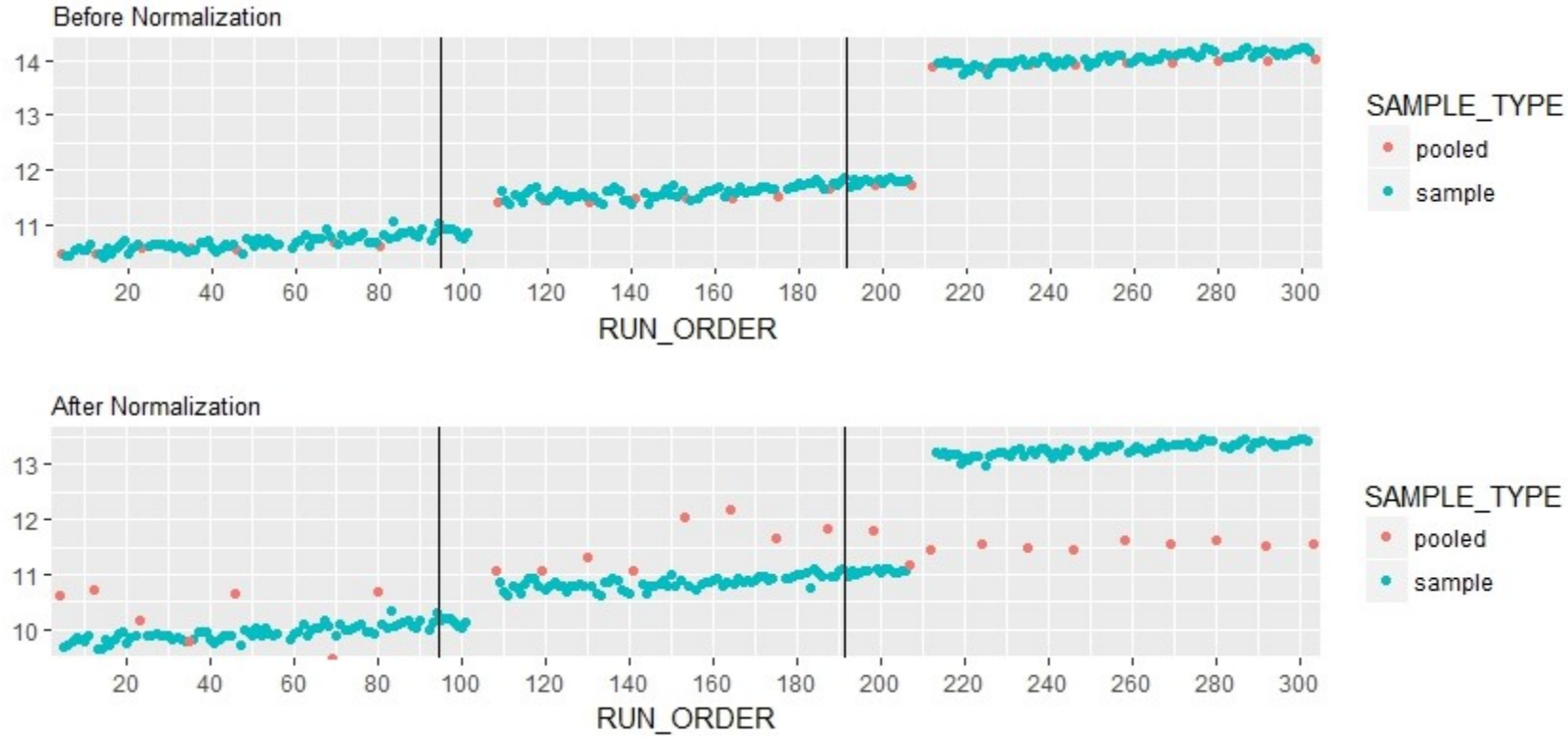


Figure 4.2 *Metabolite ion peaks before and after standard vector regression normalization in pooled quality control samples and experimental samples. Normalization did not correct for batch effects in the samples.* The vertical black line separates the analytical batches.



CHAPTER 5

Insight into Metabolic Perturbations in Welsh Ponies with Insulin Dysregulation, Obesity, and Laminitis

SUMMARY

Background: Metabolomics, the study of small-molecule metabolites, has increased understanding of human metabolic diseases but has not been used to study equine metabolic syndrome.

Objectives: 1) To examine the serum metabolome of Welsh Ponies with and without insulin dysregulation before and during an oral sugar test. 2) To identify differences in metabolites in ponies with insulin dysregulation, obesity, or history of laminitis.

Study Design: In a case-control study, twenty Welsh Ponies (mean ± SD; 13.8 ± 9.0 years) classified as non-insulin dysregulated [CON] (n = 10, insulin < 30 mU/L) or insulin dysregulated [ID] (n = 10, insulin > 60 mU/L) at 75 minutes post administration of corn syrup (Karo® light), obese (n = 6) or non-obese (n = 14), and history of laminitis (n = 9) or no history of laminitis (n = 11).

Methods: Metabolomic analysis was performed on serum obtained at 0 minutes (baseline) and 75 minutes during the oral sugar test. Data were analyzed with multivariable mixed linear models with significance set at $P \leq 0.05$.

Results: Mean insulin concentrations were significantly higher in insulin dysregulated when compared to non-insulin dysregulated ponies at baseline (ID: 15.4 ± 5.6 mU/L and CON: 4.0 ± 2.6 mU/L; $P = 0.001$) and 75 minutes (ID: 112.7 ± 29.1 mU/L and CON: 16.3 ± 8.3 mU/L; $P < 0.001$). Metabolomic analysis revealed a total of 646 metabolites of which 506 were of known identity based on homology with human metabolites. Significant metabolite differences, primarily in the lipid and amino acid pathways, were detected between groups (insulin response, obesity status, laminitis history).

Main Limitations: Samples were collected from client-owned Welsh Ponies in five different locations.

Conclusions: Data provides insight into a possible distinct pattern of metabolites that may have diagnostic utility for early detection of equine metabolic syndrome and provide new knowledge regarding the pathophysiology of metabolic perturbations associated with this condition.

INTRODUCTION

Insulin dysregulation, generalized obesity, regional adiposity and a predisposition for laminitis are central features of equine metabolic syndrome (EMS). Insulin dysregulation, defined as an abnormal resting insulin (hyperinsulinemia) and/or abnormal insulin response to intravenous or oral glucose challenge or feeding, is thought to be the central pathophysiologic feature of EMS. Complex multifactorial disease processes such as human metabolic syndrome and EMS result from disruption of metabolic processes across multiple tissues that sum together to create clinical disease [166]. Due to the complex nature, measurement of glycemic and insulinemic responses to oral or intravenous glucose and/or insulin challenges is likely inadequate to distinguish between hyperinsulinemia caused by exaggerated pancreatic responses, tissue insulin resistance, or reduced insulin clearance [167]. Yet, except for studies addressing the lamina during experimentally induced laminitis [6,8] and dynamic assessment of insulin resistance [168,169], few studies have attempted to identify the metabolic derangements of EMS at a tissue or cellular level. Our current understanding of EMS is based on clinical assays that do not directly assess the altered cellular and molecular pathophysiology within major metabolic tissues (muscle, adipose, liver) and are therefore insufficient to unravel EMS pathophysiology.

Metabolomics, the study of molecules involved in cellular metabolism (i.e. nucleotides, amino acids, fatty acids, carbohydrates, etc.), refers to the global interrogation of the biochemical components in a biological sample (serum, plasma, urine, saliva, cerebrospinal fluid). Because metabolite abundance in the serum can provide information about disruption in metabolic processes across the tissues, evaluation of the serum metabolome is a logical place to start investigating the molecular perturbations relevant to EMS [170]. More than 4,000 metabolites have been identified in human serum using high-throughput mass spectrometry and chromatography [67]. Several human studies have identified plasma metabolites and distinct metabolomic signatures associated with insulin resistance, glucose intolerance, obesity, and type-II diabetes mellitus [21,25,97].

In addition to elucidating alterations in novel metabolic pathways implicated in disease development, serum metabolomics can be used to identify biomarkers that can effectively pinpoint animals

at-risk for EMS and laminitis. Several human studies have demonstrated that metabolite biomarkers identified in cross-sectional data are useful for the detection of subclinical/pre-clinical disease months to years before the onset of clinically identifiable insulin resistance [88,171–174]. Thus, serum metabolites hold promise as potential biomarkers that would allow timely identification of metabolic derangements in animals at-risk for insulin dysregulation.

Although metabolomic analysis is a potentially powerful tool to study the complex molecular pathophysiology of EMS, the measurement of metabolites is costly. Therefore, our objectives were to demonstrate the potential of serum metabolomics to explore the pathophysiology of metabolic dysregulation and to differentiate between individuals with and without evidence of insulin dysregulation, obesity and/or history of laminitis by characterizing differences in the serum metabolome before and during an oral sugar test (OST) in a small cohort of Welsh Ponies. To our knowledge, this is the first attempt to examine the pathophysiology of insulin dysregulation in horses using metabolomics.

MATERIALS AND METHODS

Animals

In a case-control study, twenty Welsh Ponies classified as non-insulin dysregulated [CON] (n = 10, insulin < 30 mU/L) or insulin dysregulated [ID] (n = 10, insulin > 60 mU/L) at 75 minutes post administration of Karo® light corn syrup[a] were used for this study. The cohort was comprised of client-owned ponies located in Virginia, Maryland, Mississippi, Arkansas, and California. Additional information on diet, exercise, management, body condition score [119], laminitis history, and biochemical measures (non-esterified fatty acids (NEFAs), triglycerides, leptin, adiponectin) was obtained for each pony. All methods were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and Michigan State University.

Oral Sugar Test

An OST was administered to all ponies as previously described [62]. Briefly, oral administration of commercially available corn syrup (Karo® light[a]) was given using a 60cc catheter tip syringe (dose: 0.15 mL/kg bodyweight). Blood was collected via an indwelling jugular catheter at 0 minutes (baseline) and 75 minutes. The intravenous catheter was placed one hour prior to commencement of the oral sugar test following subcutaneous administration of lidocaine. Blood was centrifuged, and serum separated and stored at -80°C until analysis.

Determination of Insulin and Glucose Measurements

Insulin concentrations were determined in duplicate by radioimmunoassay (Coat-A-Count®[b]) previously validated for equids [125]. Glucose concentrations were determined in duplicate via a membrane-based glucose oxidase method (YSI 2300 STAT Plus™ Glucose & Lactate Analyzer[c]).

Determination of Other Hormonal and Biochemical Measurements

NEFA concentrations were determined using an in-vitro quantitative enzymatic colorimetric method assay (NEFA-HR[d]). Triglyceride concentrations were determined using the Serum Triglyceride Determination Kit (TR0100[e]). Leptin concentrations were determined using a radioimmunoassay (Multi-Species Leptin RIA[f]). Adiponectin concentrations were determined using the Human High Molecular Weight (HMW) Adiponectin ELISA[f] previously validated for equine serum [175].

Metabolomics

Forty serum samples, 0 minutes (baseline) and 75 minutes from each pony, were analyzed at Metabolon® Inc. using a combination of chromatography and mass spectrometry following sample preparation as described previously [176].

Sample Preparation for Global Metabolomics

Samples were divided into five fractions: analysis by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; positive ionization), analysis by UPLC-MS/MS (negative ionization), analysis by UPLC-MS/MS polar platform (negative ionization), analysis by gas chromatography-mass spectrometry (GC-MS), and one sample was reserved for repeat analysis. A targeted analysis utilized three types of controls in concert with the experimental samples: 1) samples generated from a small portion of each experimental sample of interest served as a technical replicate throughout the dataset; 2) extracted water samples served as process blanks; and 3) a combination of standards spiked into every analyzed sample allowed instrument performance monitoring. Instrument variability was determined by calculating the median relative standard deviation for the standards that were added to each sample prior to injection into the mass spectrometers.

Mass Spectrometry Analysis

The UPLC-MS/MS [177] platform utilized a Waters Acquity UPLC[g] and a Thermo Scientific Q-Exactive[h] high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. Three sample extracts were dried then reconstituted in acidic or basic liquid chromatography-compatible solvents. The first aliquot was analyzed using acidic, positive ion-optimized conditions (n = 254 metabolites), the second aliquot used basic, negative ion-optimized conditions (n = 284 metabolites), and the third aliquot was analyzed via negative ionization following elution from a hydrophilic interaction liquid chromatography (HILIC) column (n = 54 metabolites).

Gas chromatography-mass spectrometry (GC-MS) [178] was performed with a Thermo-Finnigan Trace DSQ[h] mass spectrometer with electron impact ionization (EI). Samples were dried, derivatized, and separated on a fused silica column with helium as the carrier gas (n = 54 metabolites).

Compound Identification, Quantification, and Data Curation

Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight, preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon® Inc. [179]. Metabolon maintains a library of molecules based on authenticated standards that contain the retention time, mass to charge ratio, and chromatographic data. Identification of known chemical entities was based on comparison to metabolomic library entries of more than 3,300 commercially available purified standards. Peaks were quantified using area-under-the-curve. Raw area counts for each metabolite in each sample were normalized to correct for variation resulting from instrument inter-day tuning differences. Subsequent quality control and curation processes were designed to ensure accurate, consistent identification, and to minimize system artifacts, misassignments, and background noise. Pathways were assigned for each metabolite which allowed for examination of overrepresented pathways.

Statistical Analysis

Statistical analysis, performed in the statistical program R[i], following log transformation to ensure normality included multivariable mixed linear models with sex as a covariate and examined metabolite differences between insulin dysregulated and non-insulin dysregulated ponies, obese and non-obese ponies, and ponies with and without a history of laminitis. Correlations between metabolites and biochemical parameters (basal glucose, basal insulin, NEFAs, triglycerides, leptin, adiponectin) were explored using mixed linear models. A simple t-test was performed to determine significance between insulin dysregulated ponies and non-insulin dysregulated ponies for each biochemical parameter. Significance was set at $P \leq 0.05$. Network pathway interaction diagrams were generated using Metscape [180].

Unsupervised principal components analysis (PCA) was performed to visualize the distribution of metabolic profiles within and between groups. Supervised least absolute shrinkage and selection operator (LASSO) penalized generalized linear models were fitted for optimal feature selection for classification of

each group – insulin response, obesity status, and laminitis history. LASSO regressions were fitted using the 'glmnet' R package. Model parameters were tuned using leave-one-out cross-validation and the optimal subset of features was selected from the model with minimal mean cross-validation error. No further model diagnostics could be performed due to an insufficient number of observations.

RESULTS

Animals

Non-insulin dysregulated ponies (mean ± SD; 13.8 ± 9.0 years) were a combination of mares (n = 6), geldings (n = 3), and stallions (n = 1) while the insulin dysregulated ponies (11.3 ± 6.1 years) were exclusively mares (n = 10). All ponies were in moderate to obese body condition (median [range]; CON: 5.5 [5.0 – 8.0] and ID: 6.8 [5.0 – 8.5] out of 9). Ponies did not have clinical laminitis at the time of testing; however, a history of laminitis was reported in both non-insulin dysregulated (n = 1) and insulin dysregulated ponies (n = 8).

Insulin and Glucose Concentrations

Mean insulin concentrations were significantly higher in insulin dysregulated ponies compared to non-insulin dysregulated ponies at 0 minutes (mean ± SD; ID: 15.4 ± 5.6 mU/L and CON: 4.0 ± 2.6 mU/L; $P = 0.001$) and at 75 minutes (ID: 112.7 ± 29.1 mU/L and CON: 16.3 ± 8.3 mU/L; $P < 0.001$) whereas mean glucose concentrations did not differ between groups at baseline (ID: 73.9 ± 9.0 mg/dL and CON: 73.9 ± 7.2 mg/dL) or at 75 minutes (ID: 108.1 ± 12.6 mg/dL and CON: 106.3 ± 23.4 mg/dL).

Other Hormonal and Biochemical Concentrations

Mean non-esterified fatty acid concentrations (ID: 0.4 ± 0.2 mEq/L and CON: 0.3 ± 0.2 mEq/L), triglyceride concentrations (ID: 92.9 ± 101.8 mg/dL and CON: 42.0 ± 56.4 mg/dL), and leptin concentrations (ID: 6.6 ± 3.9 ng/mL and CON: 5.8 ± 5.1 ng/mL) did not differ between insulin dysregulated ponies and non-insulin dysregulated ponies. Adiponectin concentrations (ID: 2692.3 ± 1553.5 ng/mL and

CON: 7838.5 ± 3281.5 ng/mL) were significantly lower in insulin dysregulated ponies compared to non-insulin dysregulated ponies.

Metabolomics

Metabolomic analysis revealed a total of 646 metabolites of which 506 were of known identity based on homology with human metabolites (**Table 5.1**). The 506 known metabolites were classified into eight metabolic pathways (lipid, amino acid, carbohydrate, cofactors and vitamins, energy, nucleotide, peptide, xenobiotics) and 71 sub-pathways based on the Kyoto Encyclopedia of Genes and Genomes **(**KEGG) pathway classification system (**Table 5.2**).

Metabolite Changes During an Oral Sugar Test

To determine if the oral sugar test elicited metabolite alterations in addition to the expected glycemic and insulinemic responses, metabolites were compared at 0 minutes (baseline) and 75 minutes in all twenty ponies regardless of phenotype (insulin response, obesity status, laminitis history). Seventeen (17) metabolites had increased concentrations while 107 metabolites had decreased concentrations post administration of Karo® light corn syrup when compared to baseline concentrations. Metabolites (n = 124) significantly different between these time points are listed by metabolic pathways and sub-pathways (**Figure 5.1**): lipid (n = 31), amino acid (n = 46), carbohydrate (n = 5), cofactors and vitamins (n = 6), energy (n = 3), nucleotide (n = 3), peptide (n = 9), and xenobiotics (n = 21). After administration of Karo® syrup, glucose, fructose, and mannose increased, whereas lactate decreased relative to baseline. The tricarboxylic acid (TCA) cycle intermediates (alpha-ketoglutarate, malate, succinate) were also significantly decreased at 75 minutes (**Figure 5.2**). Thirty-one (31) lipid metabolites were significantly different between time points. Most lipid metabolites (26 / 31), including acylcarnitines, monohydroxy long-chain fatty acids and polyunsaturated fatty acids, were decreased at 75 minutes indicating an overall decrease in fatty acid metabolism after administration of Karo® syrup. Similarly, amino acid metabolism metabolites (44 / 46), including branched-chain amino acids, were reduced (**Figure 5.3**). The most dramatic

decrease in metabolites from baseline to 75 minutes was a decrease in lidocaine from the subcutaneous administration for catheter placement.

Metabolite Differences Between Insulin Dysregulated and Non-Insulin Dysregulated Ponies

When comparing the insulin dysregulated ponies versus the non-insulin dysregulated ponies, 55 metabolites at 0 minutes (baseline) and 51 metabolites at 75 minutes were statistically different. Metabolites (n = 82) significantly different between insulin dysregulated and non-insulin dysregulated ponies are listed based on their metabolic pathways and sub-pathways: lipid (**Figure 5.4**; n = 45), amino acid (**Figure 5.5**; n = 16), and carbohydrate, cofactors and vitamins, energy, nucleotide, peptide, and xenobiotics (**Figure 5.6**; n = 21). At baseline, 12 metabolites had increased concentrations while 43 metabolites had decreased concentrations in insulin dysregulated ponies compared to non-insulin dysregulated ponies. Further, at baseline, most metabolites with decreased concentrations were in the lipid (n = 22) and amino acid (n = 9) pathways. At 75 minutes, 7 metabolites had increased concentrations and 44 metabolites had decreased concentrations in insulin dysregulated ponies compared to non-insulin dysregulated ponies. Similar to baseline, insulin dysregulated ponies had decreased concentrations of lipid metabolites (n = 22) and amino acid metabolites (n = 7) after administration of Karo® syrup. In addition, at 75 minutes, insulin dysregulated ponies had lower concentrations of TCA cycle intermediates (citrate, fumarate, malate) compared to non-insulin dysregulated ponies. Insulin dysregulated ponies had a significantly higher concentration of serotonin (5HT) relative to non-insulin dysregulated ponies.

Metabolite Differences Between Obese and Non-Obese Ponies

To determine if the same or similar metabolites were different in obese (body condition score ≥ 7.0) ponies (n = 6) versus non-obese ponies (n = 14), metabolite measurements were compared in these two groups. Overall, 91 metabolites at 0 minutes (baseline) and 102 metabolites at 75 minutes were statistically different between these groups. Metabolites (n = 145) significantly different between groups are listed based on their metabolic pathways and sub-pathways: lipid (**Figure 5.7**; n = 79), amino acid (**Figure 5.8**; n = 41),

and carbohydrate, cofactors and vitamins, nucleotide, peptide, xenobiotics (**Figure 5.9**; n = 25). At baseline, 65 metabolites had increased concentrations while 26 metabolites had decreased concentrations in obese ponies compared to non-obese ponies. Obese ponies compared to non-obese ponies had significantly higher baseline concentrations of long-chain fatty acids (n = 8) and acylcarnitines (n = 8). Obese ponies also had significantly higher baseline concentrations of branched-chain amino acids (isoleucine, leucine, valine). At 75 minutes, 57 metabolites had increased concentrations and 45 metabolites had decreased concentrations in obese ponies compared to non-obese ponies. Similar to baseline, obese ponies had elevated concentrations of several acylcarnitines and branched-chain amino acids compared to non-obese ponies following administration of Karo® syrup. Obese ponies also had significantly lower concentrations of polyunsaturated fatty acids at 75 minutes. The most dramatic metabolite increases in obese ponies compared to non-obese ponies was 1,5 – anhydroglucitol.

Metabolite Differences Between Ponies With and Without a History of Laminitis

To determine if the same or similar metabolites were different due to laminitis history, ponies with a history of laminitis (n = 9) were compared to ponies without a history of laminitis (n = 11). One-hundred-thirty-six (136) metabolites at 0 minutes (baseline) and 124 metabolites at 75 minutes were statistically different between previously laminitic and non-laminitic ponies. Metabolites (n = 182) significantly different between these groups are listed based on their metabolic pathways and sub-pathways: lipid (**Figure 5.10**; n = 91), amino acid (**Figure 5.11**; n = 50), and carbohydrate, cofactors and vitamins, energy, nucleotide, peptide, xenobiotics (**Figure 5.12**; n = 41). At baseline, 62 metabolites had increased concentrations while 74 metabolites had decreased concentrations in ponies with a history of laminitis compared to ponies without a history of laminitis. At baseline, long-chain fatty acids (n = 10), polyunsaturated fatty acids (n = 7) and monoacylglycerols (n = 8) were consistently higher in previously laminitic compared to non-laminitic ponies. At 75 minutes, 60 metabolites had increased concentrations and 64 metabolites had decreased concentrations in ponies with a history of laminitis compared to ponies without a history of laminitis. These differences were characterized by greater concentrations of

polyunsaturated fatty acids (n = 6) and monoacylglycerols (n = 6) in previously laminitic compared to non-laminitic ponies.

Metabolite Similarities Between Insulin Response, Obesity Status, and Laminitis History

The overlaps between the significant metabolites identified when ponies were parsed by insulin response, obesity status, or laminitis history are depicted in **Figure 5.13**. A total of six (6) metabolites were shared in all three groups. Twenty-five (25) metabolites overlap when ponies were parsed by obesity status or laminitis history, five (5) metabolites overlap when ponies were parsed by laminitis history or insulin response, and three (3) metabolites overlap when ponies were parsed by obesity status or insulin response.

Metabolite Correlations to Clinical Parameters

Additional analysis revealed subsets of measured metabolites correlated ($r \geq 0.5$) to the clinical parameters commonly measured in equids with suspected metabolic dysfunction (basal glucose, basal insulin, NEFAs, triglycerides, leptin, adiponectin; **Table 5.4** and **Table 5.5**). Seven (7) metabolites were correlated to basal glucose, eighty-five (85) metabolites were correlated to basal insulin, seven (7) metabolites were correlated to NEFAs, thirteen (13) metabolites were correlated to triglycerides, fifty-four (54) metabolites were correlated to leptin, and twelve (12) metabolites were correlated to adiponectin. Thirty (30) compounds were correlated to more than one parameter. Regardless of the parameter most of the metabolites arise from the lipid and amino acid pathways.

Metabolites as Potential Biomarkers

To show the utility of serum metabolites to distinguish between ponies grouped by insulin response, obesity status or laminitis history, unsupervised principal components analysis (PCA) was performed. The first two principal components depicted capture approximately 34.2% (principal component 1 – 22.8%, principal component 2 – 11.4%) of the variation in the data and separate the ponies into two groups. Plots of the first two dimensions from unsupervised PCA allow for visualization of the relationships between the

metabolic profiles of each group when ponies are labeled by insulin response (**Figure 5.14a**), obesity status (**Figure 5.14b**) or laminitis history (**Figure 5.14c**). The optimal number of metabolites (i.e. biomarkers) necessary to distinguish individuals based on insulin response, obesity status, and laminitis history was determined using LASSO regression. The number of metabolites needed to differentiate ponies based on insulin response (n = 23), obesity status (n = 14), and laminitis history (n = 21) is listed in **Table 5.3**. Minimal overlap was observed between these metabolite lists with only three metabolites (2-margaroyl-GPC, oleoyl-linoleoyl-glycerophosphocholine, phenylcarnitine) shared between the insulin response and laminitis history biomarker lists.

DISCUSSION

While insulin dysregulation is thought to be the central pathophysiologic mechanism of EMS, the lack of information regarding cellular and molecular pathophysiology make the underlying molecular mechanisms unclear. For complex diseases that span multiple tissues, metabolomics from biologic fluid samples including serum, plasma, and urine, provide an opportunity to obtain additional quantitative biologic information that may help decipher disease mechanisms and identify potentially useful disease biomarkers. Here we demonstrate the utility of serum metabolomics to contribute to our understanding of EMS pathophysiology by 1) establishing the power of serum metabolomics to give insight into the metabolic responses to an oral sugar test beyond measurement of glucose and insulin concentrations, 2) identifying a list of metabolites that differentiates between the three EMS phenotypes (insulin response, obesity status, laminitis history), 3) identifying metabolites that correlate to other typical measures of metabolic dysfunction (basal glucose, basal insulin, NEFAs, triglycerides, leptin, adiponectin), and 4) pinpointing metabolites that can potentially be used as biomarkers for disease.

The oral sugar test is a relatively simple test used in horses and ponies to provide an indication of glycemic and insulinemic responses to an oral sugar bolus. Similar to an oral glucose tolerance test (OGTT) performed in humans, an oral sugar test should provide a physiologic stimulus that results in metabolite flux through specific metabolic reactions/pathways. Comparable to findings in humans, the metabolite

concentrations in ponies (regardless of phenotype) indicate a switching from a relatively catabolic state (baseline/fasting) to an anabolic state after administration of Karo® syrup. Many of these changes can be attributed to four key areas of insulin action — an increase in glycolysis, and decreases in lipolysis, ketogenesis and proteolysis [181,182]. First, following administration of an oral sugar bolus, the cytosolic pathways of glucose disposal are overloaded as demonstrated by significant increases in glucose, fructose, and mannose. Second, increases in pyruvate concentrations above baseline values indicate increases in glycolysis [182]. In humans, a switch to glycolysis is also indicated by elevations in lactate which occurs approximately 30 minutes after peak insulin values [182]; however, lactate did not significantly increase at 75 minutes in our study. Our previous work has demonstrated that insulin peaks between 60 – 90 minutes post Karo® syrup administration [10,12]; therefore the 75 minute sample may have been too early to detect increases in lactate associated with insulin action. The switch to glycolysis from beta-oxidation is also supported by changes in acylcarnitines. Carnitine conjugation of long-chain fatty acids is a required step for import into the mitochondria prior to beta-oxidation and acylcarnitine accumulation. Acylcarnitine release into the plasma reflects substrate flux through beta-oxidation; decreases in acylcarnitines at 75 minutes relative to baseline suggest a decrease in beta-oxidation [183]. Decreases in saturated and monounsaturated long-chain fatty acids and polyunsaturated fatty acids indicate an inhibition of lipolysis [183]. Inhibition of ketogenesis and decrease TCA cycle flux is evidenced by decreases in ketones (3-hydroxybutyrate) and TCA cycle intermediates (alpha-ketoglutarate, malate, succinate). Lastly, a decrease in amino acid concentrations, including branched-chain amino acid concentrations, after administration of Karo® syrup indicates an inhibition of proteolysis and possible usage for protein synthesis.

Comparison of ponies with and without insulin dysregulation primarily identified differences in lysolipids, TCA cycle intermediates and urea cycle metabolites. Several glycerophosphocholines, such as oleoyl-linoleoyl-glycerophosphocholine, were decreased in insulin dysregulated ponies at both time points. In humans, individuals with low concentrations of oleoyl-linoleoyl-glycerophosphocholine develop glucose intolerance and type-II diabetes mellitus [184]. In addition, decreases in the TCA cycle intermediates (citrate, malate, fumarate) in insulin dysregulated ponies mirror decreased TCA cycle intermediates in type-

II diabetes mellitus patients [181,182,185]. Similar to humans with insulin resistance and type-II diabetes mellitus, decreases in urea cycle metabolites were present in insulin dysregulated ponies [184,185]. Finally, decreases in polyunsaturated fatty acids (linoleate) and bile acids (cholate), which were both decreased in insulin dysregulated ponies, are important biomarkers of insulin resistance in humans [95,184].

In humans, branched-chain amino acids, non-esterified fatty acids, acylcarnitines, and phospholipids have been identified as potential biomarkers for obesity [89,186,187]. For some of the metabolites, group differences were evident at baseline, while for others the difference was only evident after an oral sugar test which is a similar finding in human studies [181,182]. Analogous to obese humans, obese ponies compared to non-obese ponies have elevated serum concentrations of several long-chain fatty acids at baseline [188]. However, unlike obese humans, long-chain fatty acid concentrations were not different between obese and non-obese ponies at 75 minutes [83]. Obese ponies also had increased concentrations of several long-chain acylcarnitines (C16, C18, C18:1) at both time points when compared to non-obese ponies. These findings parallel findings in obese humans and may indicate lipid oversupply resulting in saturation of the mitochondrial capacity for beta-oxidation and incomplete long-chain fatty acid oxidation [188]. Obese ponies had higher carnitine concentrations at baseline and 75 minutes which has been associated with increased body mass index and waist circumference as well as insulin resistance and elevated triglycerides in humans [189]. Similar to findings in obese humans [190], obese ponies had lower lysolipid concentrations relative to non-obese ponies post administration of an oral sugar bolus. In addition, obese ponies had higher concentrations of branched-chain amino acids (isoleucine, leucine, valine) compared to non-obese ponies, indicating a delayed suppression of BCAA oxidation after an oral sugar test [83,189,190]. The most dramatic metabolite increases in obese ponies compared to non-obese ponies was 1,5 – anhydroglucitol, a metabolite that competes with glucose for filtration and elimination by the kidneys. This metabolite is a recognized marker of postprandial glucose control in humans [191].

The largest number of significant differences in metabolites were identified when ponies with a history of laminitis were compared to ponies without a history of laminitis; however, with the exception of increases in monoacylglycerols and polyunsaturated fatty acids at baseline and 75 minutes, many of the

differences in previously laminitic compared to non-laminitic ponies overlapped with important metabolic differences in the insulin dysregulation and obesity analyses, or overlapped between all three phenotypes. The metabolite overlap between phenotypes is not surprising given that these phenotypes often occur concurrently in equine metabolic dysfunction. Elevated circulating free fatty acids and hyperinsulinemia have been reported in obese horses with insulin resistance [192] and triglyceride accumulation in muscle rather than adipose tissue [193] was seen in healthy horses challenged with super-physiologic levels of insulin. Metabolites involved in fatty acid metabolism and amino acid metabolism were elevated in insulin dysregulated ponies, obese ponies, and ponies with a history of laminitis. In addition, the tricarboxylic acid cycle was less efficient in insulin dysregulated ponies and ponies with a history of laminitis as citrate, malate and fumarate, metabolites associated with the mitochondrial use of pyruvate, were lower in these phenotypes post administration of oral sugar suggesting that the mitochondria were less able to remove acetyl-CoA equivalents through energy production. Levels of homoarginine were lower in obese and previously laminitic ponies when compared to non-obese or non-laminitic ponies. In humans, low homoarginine levels are a risk factor for cardiovascular, cerebrovascular, and renal diseases potentially due to effects on nitric oxide and cellular energy metabolism [194]. The overlap in metabolite changes between these three phenotypes is also evident in the principal components analysis. In this analysis, more than 30% of the variation was captured by the first two principal components and plotting the first versus the second principal component separated the ponies into two clusters. However, the two clusters did not align completely with any of the three clinical phenotype groups (insulin response, obesity status, laminitis history), suggesting that these clinical phenotypes alone are inadequate to separate metabolic differences between the ponies.

Much focus has been directed towards identification of animals at-risk for EMS and the identification of biomarkers that can provide prognostic information about laminitis risk. An ideal diagnostic test for EMS would be based on measurements at a single time point, would not be impacted by environmental variables, would be minimally confounded by individual factors (gender, age, breed, genetics), and would accurately classify horses and/or predict laminitis risk, allowing appropriate early

intervention and disease prevention. In humans, serum metabolites have been identified that predict metabolic diseases up to a decade before clinical onset. In this study, LASSO analysis yielded a subset of compounds that differentiate between disease and healthy individuals that could play a role as diagnostic tests. Identification of differing metabolites between the non-insulin dysregulated and insulin dysregulated phenotypes in a baseline sample may eliminate the need for a dynamic challenge test in the future. In addition, the ability to define a metabolomic signature may reveal specific biomarkers that predict and/or diagnose insulin dysregulation leading to a better understanding of disease processes that may help identify new therapeutic targets.

This study has provided evidence that metabolomic profiling is a relevant approach for further defining metabolic alterations due to insulin dysregulation and obesity in horses. Examination of the serum metabolome of this Welsh Pony cohort demonstrated significant differences in metabolites primarily derived from the lipid and amino acid pathways when comparing ponies grouped by each EMS phenotype (insulin response, obesity status, laminitis history). Further, examination of the metabolite list against currently used metabolic dysregulation measurements (basal glucose, basal insulin, NEFAs, triglycerides, leptin, adiponectin) revealed a strong correlation to leptin and triglycerides, suggesting that metabolites may be useful for linking obesity and insulin dysregulation to other components of the EMS phenotype. However, despite the parallel between the findings from this study and findings in humans with insulin resistance, type-II diabetes mellitus and obesity, results from this cohort should be interpreted with caution. First, this is a small cohort restricted to a single breed. Second, ponies were initially included based on insulinemic responses to an oral sugar test thus there is significant overlap between the insulin dysregulation, obesity and laminitis groups limiting our analyses. When ponies were parsed by the three phenotypes (insulin response, obesity status, laminitis history) metabolomics showed that there were similarities and distinct differences which coincides with our understanding that equine metabolic syndrome is complex. The results presented here should be confirmed in a large cohort of animals that will allow for metabolite differences due to pathologic factors such as insulin dysregulation and obesity and physiologic factors such as age, gender, and breed to be differentiated. Despite these limitations, our results

clearly demonstrate the potential of serum metabolomics to provide insight into the molecular pathophysiology and to define a metabolomic signature for EMS.

FOOTNOTES

[a]ACH Food Companies Inc., Cordova, Tennessee, USA.

[b]Siemens Diagnostics, Los Angeles, California, USA.

[c]YSI Incorporated Life Sciences, Yellow Springs, Ohio, USA.

[d]Wako Chemicals USA, Richmond, Virginia, USA.

[e]Sigma-Aldrich® Company, St. Louis, Missouri, USA.

[f]EMD Millipore Corporation, Billerica, Massachusetts, USA.

[g]Waters Corporation, Milford, Massachusetts, USA.

[h]Thermo Fisher Scientific, Waltham, Massachusetts, USA.

[i]R Core Team, Vienna, AUSTRIA.

APPENDIX

Table 5.1 *List of measured metabolites.*

Metabolic Pathway	Sub Pathway	Biochemical Name	Platform	Comp ID	KEGG	HMDB	PUBCHEM
Amino Acid	Glycine, Serine and Threonine Metabolism	glycine	LC/MS pos	58	C00037	HMDB00123	750
Amino Acid	Glycine, Serine and Threonine Metabolism	N-acetylglycine	GC/MS	27710		HMDB00532	10972
Amino Acid	Glycine, Serine and Threonine Metabolism	sarcosine (N-methylglycine)	GC/MS	1516	C00213	HMDB00271	1088
Amino Acid	Glycine, Serine and Threonine Metabolism	betaine	LC/MS pos	3141	C00719	HMDB00043	247
Amino Acid	Glycine, Serine and Threonine Metabolism	serine	LC/MS pos	1648	C00065	HMDB00187	5951
Amino Acid	Glycine, Serine and Threonine Metabolism	N-acetylserine	LC/MS polar	37076		HMDB02931	65249
Amino Acid	Glycine, Serine and Threonine Metabolism	threonine	LC/MS pos	1284	C00188	HMDB00167	6288
Amino Acid	Glycine, Serine and Threonine Metabolism	N-acetylthreonine	LC/MS neg	33939	C01118		152204
Amino Acid	Alanine and Aspartate Metabolism	alanine	LC/MS polar	1126	C00041	HMDB00161	5950
Amino Acid	Alanine and Aspartate Metabolism	N-acetylalanine	LC/MS polar	1585	C02847	HMDB00766	88064
Amino Acid	Alanine and Aspartate Metabolism	aspartate	LC/MS neg	443	C00049	HMDB00191	5960
Amino Acid	Alanine and Aspartate Metabolism	asparagine	LC/MS polar	512	C00152	HMDB00168	6267
Amino Acid	Alanine and Aspartate Metabolism	N-acetylasparagine	LC/MS pos	33942		HMDB06028	99715
Amino Acid	Alanine and Aspartate Metabolism	N-acetylaspartate (NAA)	LC/MS polar	22185	C01042	HMDB00812	65065
Amino Acid	Glutamate Metabolism	glutamate	LC/MS pos	57	C00025	HMDB00148	611
Amino Acid	Glutamate Metabolism	glutamine	LC/MS pos	53	C00064	HMDB00641	5961
Amino Acid	Glutamate Metabolism	N-acetylglutamate	LC/MS pos	15720	C00624	HMDB01138	70914
Amino Acid	Glutamate Metabolism	N-acetylglutamine	LC/MS pos	33943	C02716	HMDB06029	182230

Table 5.1 (cont'd)

Amino Acid	Glutamate Metabolism	N-acetyl-aspartyl-glutamate (NAAG)	LC/MS pos	35665	C12270	HMDB01067	5255
Amino Acid	Glutamate Metabolism	pyroglutamine	LC/MS pos	46225			134508
Amino Acid	Histidine Metabolism	histidine	LC/MS neg	59	C00135	HMDB00177	6274
Amino Acid	Histidine Metabolism	N-acetylhistidine	LC/MS neg	33946	C02997	HMDB32055	75619
Amino Acid	Histidine Metabolism	1-methylhistidine	LC/MS neg	30460	C01152	HMDB00001	92105
Amino Acid	Histidine Metabolism	3-methylhistidine	LC/MS neg	15677	C01152	HMDB00479	64969
Amino Acid	Histidine Metabolism	trans-urocanate	LC/MS pos	607	C00785	HMDB00301	736715
Amino Acid	Histidine Metabolism	cis-urocanate	LC/MS neg	40410			1549103
Amino Acid	Histidine Metabolism	imidazole propionate	LC/MS pos	40730		HMDB02271	70630
Amino Acid	Histidine Metabolism	imidazole lactate	LC/MS pos	15716	C05568	HMDB02320	440129
Amino Acid	Histidine Metabolism	1-methylimidazoleacetate	LC/MS pos	32350	C05828	HMDB02820	75810
Amino Acid	Histidine Metabolism	4-imidazoleacetate	LC/MS pos	32349	C02835	HMDB02024	96215
Amino Acid	Lysine Metabolism	lysine	LC/MS polar	1301	C00047	HMDB00182	5962
Amino Acid	Lysine Metabolism	N6-acetyllysine	LC/MS pos	36752	C02727	HMDB00206	92832
Amino Acid	Lysine Metabolism	N-6-trimethyllysine	LC/MS pos	1498	C03793	HMDB01325	440120
Amino Acid	Lysine Metabolism	2-aminoadipate	LC/MS pos	6146	C00956	HMDB00510	469
Amino Acid	Lysine Metabolism	glutarate (pentanedioate)	LC/MS polar	396	C00489	HMDB00661	743
Amino Acid	Lysine Metabolism	glutarylcarnitine (C5)	LC/MS pos	44664		HMDB13130	71464488
Amino Acid	Lysine Metabolism	3-methylglutarylcarnitine (1)	LC/MS pos	46547		HMDB00552	128145

Table 5.1 (cont'd)

Amino Acid	Lysine Metabolism	pipecolate	LC/MS pos	1444	C00408	HMDB00070	849
Amino Acid	Phenylalanine and Tyrosine Metabolism	phenylalanine	LC/MS pos	64	C00079	HMDB00159	6140
Amino Acid	Phenylalanine and Tyrosine Metabolism	N-acetylphenylalanine	LC/MS neg	33950	C03519	HMDB00512	74839
Amino Acid	Phenylalanine and Tyrosine Metabolism	phenylpyruvate	LC/MS neg	566	C00166	HMDB00205	997
Amino Acid	Phenylalanine and Tyrosine Metabolism	phenyllactate (PLA)	LC/MS neg	22130	C05607	HMDB00779	3848
Amino Acid	Phenylalanine and Tyrosine Metabolism	4-hydroxyphenylacetate	GC/MS	541	C00642	HMDB00020	127
Amino Acid	Phenylalanine and Tyrosine Metabolism	phenylacetylglycine	LC/MS pos	33945	C05598	HMDB00821	68144
Amino Acid	Phenylalanine and Tyrosine Metabolism	tyrosine	LC/MS pos	1299	C00082	HMDB00158	6057
Amino Acid	Phenylalanine and Tyrosine Metabolism	N-acetyltyrosine	LC/MS neg	32390		HMDB00866	68310
Amino Acid	Phenylalanine and Tyrosine Metabolism	4-hydroxyphenylpyruvate	LC/MS neg	1669	C01179	HMDB00707	979
Amino Acid	Phenylalanine and Tyrosine Metabolism	3-(4-hydroxyphenyl) lactate	LC/MS neg	32197	C03672	HMDB00755	9378
Amino Acid	Phenylalanine and Tyrosine Metabolism	phenol sulfate	LC/MS neg	32553	C02180	HMDB60015	74426
Amino Acid	Phenylalanine and Tyrosine Metabolism	p-cresol sulfate	LC/MS neg	36103	C01468	HMDB11635	4615423
Amino Acid	Phenylalanine and Tyrosine Metabolism	o-cresol sulfate	LC/MS neg	36845			11615528
Amino Acid	Phenylalanine and Tyrosine Metabolism	3-methoxytyrosine	LC/MS pos	12017		HMDB01434	1670
Amino Acid	Phenylalanine and Tyrosine Metabolism	gentisate	LC/MS neg	18280	C00628	HMDB00152	3469
Amino Acid	Phenylalanine and Tyrosine Metabolism	phenylpropionylglycine	LC/MS neg	35434		HMDB00860	152323
Amino Acid	Phenylalanine and Tyrosine Metabolism	3-[3-(sulfooxy)phenyl] propanoic acid	LC/MS neg	45415			187488
Amino Acid	Phenylalanine and Tyrosine Metabolism	3-hydroxy-3-phenylpropionate	GC/MS	43497			92959

Table 5.1 (cont'd)

Amino Acid	Phenylalanine and Tyrosine Metabolism	3-(3-hydroxyphenyl) propionate	LC/MS neg	35635	C11457	HMDB00375	91
Amino Acid	Phenylalanine and Tyrosine Metabolism	3-(4-hydroxyphenyl) propionate	LC/MS neg	39587	C01744	HMDB02199	10394
Amino Acid	Phenylalanine and Tyrosine Metabolism	3-phenylpropionate (hydrocinnamate)	LC/MS neg	15749	C05629	HMDB00764	107
Amino Acid	Phenylalanine and Tyrosine Metabolism	thyroxine	LC/MS pos	46079	C01829	HMDB01918	5819
Amino Acid	Tryptophan Metabolism	tryptophan	LC/MS pos	54	C00078	HMDB00929	6305
Amino Acid	Tryptophan Metabolism	indolelactate	GC/MS	18349	C02043	HMDB00671	92904
Amino Acid	Tryptophan Metabolism	indoleacetate	LC/MS pos	27513	C00954	HMDB00197	802
Amino Acid	Tryptophan Metabolism	indolepropionate	LC/MS pos	32405		HMDB02302	3744
Amino Acid	Tryptophan Metabolism	3-indoxyl sulfate	LC/MS neg	27672		HMDB00682	10258
Amino Acid	Tryptophan Metabolism	kynurenine	LC/MS pos	15140	C00328	HMDB00684	161166
Amino Acid	Tryptophan Metabolism	picolinate	LC/MS pos	1512	C10164	HMDB02243	1018
Amino Acid	Tryptophan Metabolism	5-hydroxyindoleacetate	LC/MS pos	437	C05635	HMDB00763	1826
Amino Acid	Tryptophan Metabolism	serotonin (5HT)	LC/MS pos	2342	C00780	HMDB00259	5202
Amino Acid	Tryptophan Metabolism	tryptophan betaine	LC/MS neg	37097	C09213	HMDB61115	442106
Amino Acid	Tryptophan Metabolism	C-glycosyltryptophan	LC/MS pos	32675			
Amino Acid	Tryptophan Metabolism	indole-3-carboxylic acid	LC/MS pos	38116	C19837	HMDB03320	69867
Amino Acid	Leucine, Isoleucine and Valine Metabolism	leucine	LC/MS pos	60	C00123	HMDB00687	6106
Amino Acid	Leucine, Isoleucine and Valine Metabolism	N-acetylleucine	LC/MS pos	1587	C02710	HMDB11756	70912
Amino Acid	Leucine, Isoleucine and Valine Metabolism	4-methyl-2-oxopentanoate	LC/MS neg	22116	C00233	HMDB00695	70

Table 5.1 (cont'd)

Amino Acid	Leucine, Isoleucine and Valine Metabolism	isovalerate	LC/MS neg	44656	C08262	HMDB00718	10430
Amino Acid	Leucine, Isoleucine and Valine Metabolism	isovalerylglycine	LC/MS neg	35107		HMDB00678	546304
Amino Acid	Leucine, Isoleucine and Valine Metabolism	isovalerylcarnitine	LC/MS pos	34407		HMDB00688	6426851
Amino Acid	Leucine, Isoleucine and Valine Metabolism	beta-hydroxyisovalerate	LC/MS polar	12129		HMDB00754	69362
Amino Acid	Leucine, Isoleucine and Valine Metabolism	beta-hydroxyisovaleroylcarnitine	LC/MS pos	35433			
Amino Acid	Leucine, Isoleucine and Valine Metabolism	alpha-hydroxyisovaleroyl carnitine	LC/MS pos	46263			
Amino Acid	Leucine, Isoleucine and Valine Metabolism	3-methylglutaconate	LC/MS pos	38667		HMDB00522	1551553
Amino Acid	Leucine, Isoleucine and Valine Metabolism	alpha-hydroxyisovalerate	LC/MS neg	33937		HMDB00407	99823
Amino Acid	Leucine, Isoleucine and Valine Metabolism	methylsuccinate	LC/MS polar	15745		HMDB01844	10349
Amino Acid	Leucine, Isoleucine and Valine Metabolism	isoleucine	LC/MS pos	1125	C00407	HMDB00172	6306
Amino Acid	Leucine, Isoleucine and Valine Metabolism	allo-isoleucine	GC/MS	46552			6950182; 99288
Amino Acid	Leucine, Isoleucine and Valine Metabolism	3-methyl-2-oxovalerate	LC/MS neg	15676	C00671	HMDB03736	47
Amino Acid	Leucine, Isoleucine and Valine Metabolism	2-methylbutyrylcarnitine (c5)	LC/MS pos	45095		HMDB00378	6426901
Amino Acid	Leucine, Isoleucine and Valine Metabolism	2-methylbutyrylglycine	LC/MS pos	31928		HMDB00339	193872
Amino Acid	Leucine, Isoleucine and Valine Metabolism	tiglyl carnitine	LC/MS pos	35428		HMDB02366	22833596
Amino Acid	Leucine, Isoleucine and Valine Metabolism	tigloylglycine	LC/MS pos	1598		HMDB00959	6441567
Amino Acid	Leucine, Isoleucine and Valine Metabolism	2-hydroxy-3-methylvalerate	LC/MS neg	36746		HMDB00317	164623
Amino Acid	Leucine, Isoleucine and Valine Metabolism	3-hydroxy-2-ethylpropionate	GC/MS	32397		HMDB00396	188979
Amino Acid	Leucine, Isoleucine and Valine Metabolism	ethylmalonate	LC/MS polar	15765		HMDB00622	11756

Table 5.1 (cont'd)

Amino Acid	Leucine, Isoleucine and Valine Metabolism	valine	LC/MS pos	1649	C00183	HMDB00883	6287
Amino Acid	Leucine, Isoleucine and Valine Metabolism	N-acetylvaline	LC/MS neg	1591		HMDB11757	66789
Amino Acid	Leucine, Isoleucine and Valine Metabolism	3-methyl-2-oxobutyrate	LC/MS polar	44526	C00141	HMDB00019	49
Amino Acid	Leucine, Isoleucine and Valine Metabolism	isobutyrylcarnitine	LC/MS pos	33441		HMDB00736	168379
Amino Acid	Leucine, Isoleucine and Valine Metabolism	isobutyrylglycine	LC/MS neg	35437		HMDB00730	10855600
Amino Acid	Leucine, Isoleucine and Valine Metabolism	3-hydroxyisobutyrate	LC/MS polar	1549	C06001	HMDB00336	87
Amino Acid	Leucine, Isoleucine and Valine Metabolism	alpha-hydroxyisocaproate	LC/MS neg	22132	C03264	HMDB00746	83697
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	methionine	LC/MS pos	1302	C00073	HMDB00696	6137
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	N-acetylmethionine	LC/MS neg	1589	C02712	HMDB11745	448580
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	N-formylmethionine	LC/MS neg	2829	C03145	HMDB01015	439750
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	methionine sulfoxide	LC/MS pos	18374	C02989	HMDB02005	158980
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	2-aminobutyrate	LC/MS pos	42374	C02261	HMDB00650	439691
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	2-hydroxybutyrate (AHB)	GC/MS	21044	C05984	HMDB00008	440864
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	cysteine	GC/MS	31453	C00097	HMDB00574	5862
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	cystine	GC/MS	31454	C00491	HMDB00192	67678
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	S-methylcysteine	LC/MS pos	39592		HMDB02108	24417
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	cysteine s-sulfate	LC/MS neg	22176	C05824	HMDB00731	115015
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	hypotaurine	LC/MS polar	590	C00519	HMDB00965	107812
Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	taurine	LC/MS polar	2125	C00245	HMDB00251	1123

Table 5.1 (cont'd)

Amino Acid	Methionine, Cysteine, SAM and Taurine Metabolism	N-acetyltaurine	LC/MS neg	48187			159864
Amino Acid	Urea cycle; Arginine and Proline Metabolism	arginine	LC/MS pos	1638	C00062	HMDB00517	232
Amino Acid	Urea cycle; Arginine and Proline Metabolism	urea	LC/MS pos	1670	C00086	HMDB00294	1176
Amino Acid	Urea cycle; Arginine and Proline Metabolism	ornithine	GC/MS	1493	C00077	HMDB03374	6262
Amino Acid	Urea cycle; Arginine and Proline Metabolism	proline	LC/MS pos	1898	C00148	HMDB00162	145742
Amino Acid	Urea cycle; Arginine and Proline Metabolism	citrulline	LC/MS pos	2132	C00327	HMDB00904	9750
Amino Acid	Urea cycle; Arginine and Proline Metabolism	homoarginine	LC/MS polar	22137	C01924	HMDB00670	9085
Amino Acid	Urea cycle; Arginine and Proline Metabolism	homocitrulline	LC/MS pos	22138	C02427	HMDB00679	65072
Amino Acid	Urea cycle; Arginine and Proline Metabolism	dimethylarginine (SDMA + ADMA)	LC/MS pos	36808	C03626	HMDB01539	123831
Amino Acid	Urea cycle; Arginine and Proline Metabolism	N-acetylarginine	LC/MS pos	33953	C02562	HMDB04620	67427
Amino Acid	Urea cycle; Arginine and Proline Metabolism	N-delta-acetylornithine	LC/MS pos	43249			9920500
Amino Acid	Urea cycle; Arginine and Proline Metabolism	N-methyl proline	LC/MS pos	37431			557
Amino Acid	Urea cycle; Arginine and Proline Metabolism	trans-4-hydroxyproline	LC/MS pos	32306	C01157	HMDB00725	5810
Amino Acid	Urea cycle; Arginine and Proline Metabolism	pro-hydroxy-pro	LC/MS pos	35127		HMDB06695	11673055
Amino Acid	Creatine Metabolism	creatine	LC/MS pos	27718	C00300	HMDB00064	586
Amino Acid	Creatine Metabolism	creatinine	LC/MS pos	513	C00791	HMDB00562	588
Amino Acid	Creatine Metabolism	guanidinoacetate	LC/MS pos	43802	C00581	HMDB00128	763
Amino Acid	Polyamine Metabolism	N-acetylputrescine	LC/MS pos	37496	C02714	HMDB02064	122356
Amino Acid	Polyamine Metabolism	4-acetamidobutanoate	LC/MS pos	1558	C02946	HMDB03681	18189

Table 5.1 (cont'd)

Amino Acid	Guanidino and Acetamido Metabolism	4-guanidinobutanoate	LC/MS pos	15681	C01035	HMDB03464	500
Amino Acid	Glutathione Metabolism	cysteine-glutathione disulfide	LC/MS pos	46734		HMDB00656	4247235
Amino Acid	Glutathione Metabolism	5-oxoproline	LC/MS neg	1494	C01879	HMDB00267	7405
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylalanine	LC/MS pos	37063			440103
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylglutamine	LC/MS pos	2730	C05283	HMDB11738	150914
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylisoleucine	LC/MS pos	34456		HMDB11170	14253342
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylleucine	LC/MS pos	18369		HMDB11171	151023
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamyllysine	LC/MS pos	33934		HMDB03869	65254; 14284565
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylmethionine	LC/MS pos	44872			7009567
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylphenylalanine	LC/MS pos	33422		HMDB00594	111299
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylthreonine	LC/MS pos	33364			
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamyltyrosine	LC/MS pos	2734		HMDB11741	94340
Peptide	Gamma-glutamyl Amino Acid	gamma-glutamylvaline	LC/MS pos	43829		HMDB11172	7015683
Peptide	Dipeptide Derivative	carnosine	LC/MS neg	1768	C00386	HMDB00033	439224
Peptide	Dipeptide Derivative	N-acetylcarnosine	LC/MS pos	43488		HMDB12881	9903482
Peptide	Dipeptide Derivative	anserine	LC/MS neg	15747	C01262	HMDB00194	112072
Peptide	Dipeptide	alpha-glutamylglutamate	LC/MS pos	22166	C01425	HMDB28818	439500
Peptide	Dipeptide	aspartylleucine	LC/MS pos	40068			332962
Peptide	Dipeptide	aspartylvaline	LC/MS pos	41373			4991131

Table 5.1 (cont'd)

Peptide	Dipeptide	cyclo(gly-pro)	LC/MS pos	37077			126154
Peptide	Dipeptide	cyclo(leu-pro)	LC/MS pos	37104			7074739
Peptide	Dipeptide	glycylleucine	LC/MS pos	34398	C02155	HMDB00759	92843
Peptide	Dipeptide	glycylphenylalanine	LC/MS pos	33954		HMDB28848	92953
Peptide	Dipeptide	glycylvaline	LC/MS pos	18357		HMDB28854	97417
Peptide	Dipeptide	histidylvaline	LC/MS pos	42069			7021871
Peptide	Dipeptide	isoleucylaspartate	LC/MS pos	42982			
Peptide	Dipeptide	isoleucylglycine	LC/MS pos	40008			342532
Peptide	Dipeptide	leucylleucine	LC/MS pos	36756	C11332	HMDB28933	76807
Peptide	Dipeptide	phenylalanylleucine	LC/MS pos	40192			4078229
Peptide	Dipeptide	phenylalanyltryptophan	LC/MS neg	41377			
Peptide	Dipeptide	prolylglycine	LC/MS pos	40703			7408076; 6426709
Peptide	Dipeptide	pyroglutamylvaline	LC/MS neg	32394			152416
Peptide	Dipeptide	tryptophylglutamate	LC/MS pos	41401			3634442
Peptide	Dipeptide	cis-Cyclo[L-ala-L-Pro]	LC/MS pos	47098			6428987
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1,5-anhydroglucitol (1,5-AG)	LC/MS neg	20675	C07326	HMDB02712	64960
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	glucose	GC/MS	20488	C00031	HMDB00122	79025
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	pyruvate	LC/MS neg	42582	C00022	HMDB00243	1060
Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	lactate	GC/MS	527	C00186	HMDB00190	612

Table 5.1 (cont'd)

Carbohydrate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	glycerate	LC/MS polar	1572	C00258	HMDB00139	752
Carbohydrate	Pentose Metabolism	ribulose	GC/MS	35855	C00309	HMDB00621	151261
Carbohydrate	Pentose Metabolism	ribose	GC/MS	12083	C00121	HMDB00283	5779
Carbohydrate	Pentose Metabolism	ribitol	LC/MS polar	15772	C00474	HMDB00508	6912
Carbohydrate	Pentose Metabolism	ribonate	LC/MS polar	27731			5460677
Carbohydrate	Pentose Metabolism	xylulose	GC/MS	18344	C00310	HMDB00654	5289590
Carbohydrate	Pentose Metabolism	xylonate	GC/MS	35638	C05411	HMDB60256	6602431
Carbohydrate	Pentose Metabolism	xylose	GC/MS	15835	C00181	HMDB00098	135191
Carbohydrate	Pentose Metabolism	xylitol	GC/MS	4966	C00379	HMDB02917	6912
Carbohydrate	Pentose Metabolism	arabinose	GC/MS	575	C00216	HMDB00646	66308
Carbohydrate	Pentose Metabolism	threitol	GC/MS	35854	C16884	HMDB04136	169019
Carbohydrate	Pentose Metabolism	arabitol	GC/MS	38075	C01904	HMDB01851	94154
Carbohydrate	Disaccharides and Oligosaccharides	sucrose	LC/MS neg	1519	C00089	HMDB00258	5988
Carbohydrate	Fructose, Mannose and Galactose Metabolism	fructose	GC/MS	577	C00095	HMDB00660	5984
Carbohydrate	Fructose, Mannose and Galactose Metabolism	sorbitol	GC/MS	15053	C00794	HMDB00247	5780
Carbohydrate	Fructose, Mannose and Galactose Metabolism	mannose	GC/MS	584	C00159	HMDB00169	18950
Carbohydrate	Fructose, Mannose and Galactose Metabolism	mannitol	GC/MS	15335	C00392	HMDB00765	6251
Carbohydrate	Aminosugar Metabolism	glucuronate	LC/MS polar	15443	C00191	HMDB00127	444791
Carbohydrate	Aminosugar Metabolism	N-acetylneuraminate	LC/MS polar	32377	C00270	HMDB00230	439197

Table 5.1 (cont'd)

Carbohydrate	Aminosugar Metabolism	erythronate	LC/MS polar	42420		HMDB00613	2781043
Energy	TCA Cycle	citrate	LC/MS neg	1564	C00158	HMDB00094	311
Energy	TCA Cycle	alpha-ketoglutarate	LC/MS polar	528	C00026	HMDB00208	51
Energy	TCA Cycle	succinylcarnitine	LC/MS pos	37058			
Energy	TCA Cycle	succinate	LC/MS polar	1437	C00042	HMDB00254	1110
Energy	TCA Cycle	fumarate	GC/MS	1643	C00122	HMDB00134	444972
Energy	TCA Cycle	malate	GC/MS	1303	C00149	HMDB00156	525
Energy	TCA Cycle	tricarballylate	LC/MS neg	15729	C19806	HMDB31193	14925
Energy	Oxidative Phosphorylation	phosphate	GC/MS	11438	C00009	HMDB01429	1061
Lipid	Short Chain Fatty Acid	valerate	LC/MS neg	33443	C00803	HMDB00892	7991
Lipid	Medium Chain Fatty Acid	caproate (6:0)	LC/MS neg	32489	C01585	HMDB00535	8892
Lipid	Medium Chain Fatty Acid	heptanoate (7:0)	LC/MS neg	1644	C17714	HMDB00666	8094
Lipid	Medium Chain Fatty Acid	caprylate (8:0)	GC/MS	32492	C06423	HMDB00482	379
Lipid	Medium Chain Fatty Acid	pelargonate (9:0)	LC/MS neg	12035	C01601	HMDB00847	8158
Lipid	Medium Chain Fatty Acid	10-undecenoate (11:1n1)	LC/MS neg	32497			
Lipid	Medium Chain Fatty Acid	5-dodecenoate (12:1n7)	LC/MS neg	33968		HMDB00529	5312378
Lipid	Long Chain Fatty Acid	myristate (14:0)	LC/MS neg	1365	C06424	HMDB00806	11005
Lipid	Long Chain Fatty Acid	myristoleate (14:1n5)	LC/MS neg	32418	C08322	HMDB02000	5281119
Lipid	Long Chain Fatty Acid	palmitate (16:0)	LC/MS neg	1336	C00249	HMDB00220	985

Table 5.1 (cont'd)

Lipid	Long Chain Fatty Acid	palmitoleate (16:1n7)	LC/MS neg	33447	C08362	HMDB03229	445638
Lipid	Long Chain Fatty Acid	margarate (17:0)	LC/MS neg	1121		HMDB02259	10465
Lipid	Long Chain Fatty Acid	10-heptadecenoate (17:1n7)	LC/MS neg	33971			5312435
Lipid	Long Chain Fatty Acid	stearate (18:0)	LC/MS neg	1358	C01530	HMDB00827	5281
Lipid	Long Chain Fatty Acid	oleate (18:1n9)	GC/MS	1359	C00712	HMDB00207	445639
Lipid	Long Chain Fatty Acid	cis-vaccenate (18:1n7)	GC/MS	33970	C08367	HMDB03231	5282761
Lipid	Long Chain Fatty Acid	10-nonadecenoate (19:1n9)	LC/MS neg	33972		HMDB13622	5312513
Lipid	Long Chain Fatty Acid	arachidate (20:0)	LC/MS neg	1118	C06425	HMDB02212	10467
Lipid	Long Chain Fatty Acid	eicosenoate (20:1n9 or 11)	LC/MS neg	33587			5282768
Lipid	Long Chain Fatty Acid	erucate (22:1n9)	LC/MS neg	1552	C08316	HMDB02068	5281116
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	stearidonate (18:4n3)	LC/MS neg	33969	C16300	HMDB06547	5312508
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	eicosapentaenoate (EPA; 20:5n3)	LC/MS neg	18467	C06428	HMDB01999	446284
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	docosapentaenoate (n3 DPA; 22:5n3)	LC/MS neg	32504	C16513	HMDB01976	6441454
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	docosahexaenoate (DHA; 22:6n3)	LC/MS neg	44675	C06429	HMDB02183	445580
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	docosatrienoate (22:3n3)	LC/MS neg	32417	C16534	HMDB02823	5312556
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	linoleate (18:2n6)	LC/MS neg	1105	C01595	HMDB00673	5280450
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	linolenate [alpha or gamma; (18:3n3 or 6)]	LC/MS neg	34035	C06427		5280934
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	dihomo-linolenate (20:3n3 or n6)	LC/MS neg	35718	C03242	HMDB02925	5280581
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	arachidonate (20:4n6)	LC/MS neg	1110	C00219	HMDB01043	444899

Table 5.1 (cont'd)

Lipid	Polyunsaturated Fatty Acid (n3 and n6)	docosapentaenoate (n6 DPA; 22:5n6)	LC/MS pos	37478	C16513	HMDB13123	6441454
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	docosadienoate (22:2n6)	LC/MS neg	32415	C16533		5282807
Lipid	Polyunsaturated Fatty Acid (n3 and n6)	dihomo-linoleate (20:2n6)	LC/MS neg	17805	C16525	HMDB05060	6439848
Lipid	Fatty Acid, Branched	15-methylpalmitate (isobar with 2-methylpalmitate)	LC/MS neg	38768			17903417
Lipid	Fatty Acid, Branched	17-methylstearate	LC/MS neg	38296			3083779
Lipid	Fatty Acid, Dicarboxylate	2-hydroxyglutarate	GC/MS	37253	C02630	HMDB00606	43
Lipid	Fatty Acid, Dicarboxylate	azelate (nonanedioate)	LC/MS neg	18362	C08261	HMDB00784	2266
Lipid	Fatty Acid, Dicarboxylate	sebacate (decanedioate)	LC/MS neg	32398	C08277	HMDB00792	5192
Lipid	Fatty Acid, Dicarboxylate	dodecanedioate	LC/MS neg	32388	C02678	HMDB00623	12736
Lipid	Fatty Acid, Dicarboxylate	octadecanedioate	LC/MS neg	36754		HMDB00782	70095
Lipid	Fatty Acid, Methyl Ester	linoleate, methyl ester	GC/MS	36801			5284421
Lipid	Fatty Acid, Amino	2-aminoheptanoate	LC/MS pos	43761			227939
Lipid	Fatty Acid, Amino	2-aminooctanoate	LC/MS pos	43343		HMDB00991	69522
Lipid	Fatty Acid Synthesis	malonylcarnitine	LC/MS pos	37059		HMDB02095	22833583
Lipid	Fatty Acid Synthesis	2-methylmalonyl carnitine	LC/MS pos	35482		HMDB13133	53481628
Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	butyrylcarnitine	LC/MS pos	32412	C02862	HDMB02013	439829
Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	butyrylglycine	LC/MS neg	31850		HMDB00808	88412
Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	propionylcarnitine	LC/MS pos	32452	C03017	HMDB00824	107738
Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	propionylglycine	LC/MS neg	31932		HMDB00783	98681

Table 5.1 (cont'd)

Lipid	Fatty Acid Metabolism (also BCAA Metabolism)	methylmalonate (MMA)	LC/MS polar	1496	C02170	HMDB00202	487
Lipid	Fatty Acid Metabolism (Acyl Glycine)	hexanoylglycine	LC/MS neg	35436		HMDB00701	99463
Lipid	Fatty Acid Metabolism (Acyl Glycine)	N-octanoylglycine	LC/MS neg	43502		HMDB00832	84290
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	acetylcarnitine	LC/MS pos	32198	C02571	HMDB00201	1
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	hydroxybutyrylcarnitine	LC/MS pos	43264		HMDB13127	53481617
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	hexanoylcarnitine	LC/MS pos	32328		HMDB00705	6426853
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	octanoylcarnitine	LC/MS pos	33936	C02838	HMDB00791	123701
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	decanoylcarnitine	LC/MS pos	33941		HMDB00651	10245190
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	cis-4-decenoyl carnitine	LC/MS pos	38178			
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	laurylcarnitine	LC/MS pos	34534		HMDB02250	10427569
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	myristoylcarnitine	LC/MS pos	33952		HMDB05066	53477791
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	palmitoylcarnitine	LC/MS pos	44681	C02990	HMDB00222	461
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	stearoylcarnitine	LC/MS pos	34409		HMDB00848	6426855
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	oleoylcarnitine	LC/MS pos	35160		HMDB05065	6441392; 53477789
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	linoleoylcarnitine	LC/MS pos	46223		HMDB06469	6450015
Lipid	Fatty Acid Metabolism (Acyl Carnitine)	myristoleoylcarnitine	LC/MS pos	48182			
Lipid	Carnitine Metabolism	deoxycarnitine	LC/MS pos	36747	C01181	HMDB01161	134
Lipid	Carnitine Metabolism	carnitine	LC/MS pos	15500	C00318	HMDB00062	10917
Lipid	Ketone Bodies	3-hydroxybutyrate (BHBA)	GC/MS	542	C01089	HMDB00357	441

Table 5.1 (cont'd)

Lipid	Fatty Acid, Monohydroxy	4-hydroxybutyrate (GHB)	GC/MS	34585	C00989	HMDB00710	10413
Lipid	Fatty Acid, Monohydroxy	alpha-hydroxycaproate	LC/MS neg	37073		HMDB01624	99824
Lipid	Fatty Acid, Monohydroxy	2-hydroxyoctanoate	LC/MS neg	22036		HMDB02264	94180
Lipid	Fatty Acid, Monohydroxy	2-hydroxydecanoate	LC/MS neg	42489			21488
Lipid	Fatty Acid, Monohydroxy	2-hydroxypalmitate	LC/MS neg	35675		HMDB31057	92836
Lipid	Fatty Acid, Monohydroxy	2-hydroxystearate	LC/MS neg	17945	C03045		69417
Lipid	Fatty Acid, Monohydroxy	3-hydroxydecanoate	LC/MS neg	22053		HMDB02203	26612
Lipid	Fatty Acid, Monohydroxy	5-hydroxyhexanoate	LC/MS neg	31938		HMDB00525	170748
Lipid	Fatty Acid, Monohydroxy	16-hydroxypalmitate	LC/MS neg	39609	C18218	HMDB06294	10466
Lipid	Fatty Acid, Monohydroxy	13-HODE + 9-HODE	LC/MS neg	37752			43013
Lipid	Eicosanoid	12-HETE	LC/MS neg	37536		HMDB06111	5312983
Lipid	Endocannabinoid	oleic ethanolamide	LC/MS neg	38102		HMDB02088	5283454
Lipid	Endocannabinoid	N-oleoyltaurine	LC/MS neg	39732			6437033
Lipid	Endocannabinoid	N-stearoyltaurine	LC/MS neg	39730			168274
Lipid	Endocannabinoid	N-palmitoyltaurine	LC/MS neg	39835			
Lipid	Inositol Metabolism	myo-inositol	GC/MS	19934	C00137	HMDB00211	892
Lipid	Inositol Metabolism	chiro-inositol	GC/MS	37112	C19891	HMDB34220	
Lipid	Inositol Metabolism	inositol 1-phosphate (I1P)	GC/MS	1481	C04006	HMDB00213	440194
Lipid	Phospholipid Metabolism	choline	LC/MS pos	15506	C00114	HMDB00097	305

Table 5.1 (cont'd)

Lipid	Phospholipid Metabolism	glycerophosphorylcholine (GPC)	LC/MS pos	15990	C00670	HMDB00086	71920
Lipid	Phospholipid Metabolism	glycerophosphoethanolamine	LC/MS polar	37455	C01233	HMDB00114	123874
Lipid	Lysolipid	1-myristoylglycerophosphocholine (14:0)	LC/MS pos	45453	C04230	HMDB10379	460604
Lipid	Lysolipid	2-myristoylglycerophosphocholine*	LC/MS pos	35626			
Lipid	Lysolipid	1-pentadecanoylglycerophosphocholine (15:0)	LC/MS pos	37418			
Lipid	Lysolipid	1-palmitoylglycerophosphocholine (16:0)	LC/MS pos	33955			86554
Lipid	Lysolipid	2-palmitoylglycerophosphocholine	LC/MS pos	35253			15061532
Lipid	Lysolipid	1-palmitoleoylglycerophosphocholine (16:1)	LC/MS pos	33230			24779461
Lipid	Lysolipid	2-palmitoleoylglycerophosphocholine	LC/MS pos	35819			
Lipid	Lysolipid	1-margaroylglycerophosphocholine (17:0)	LC/MS pos	44682	C04230	HMDB12108	24779463
Lipid	Lysolipid	2-margaroylglycerophosphocholine	LC/MS pos	44683			
Lipid	Lysolipid	1-stearoylglycerophosphocholine (18:0)	LC/MS pos	33961			497299
Lipid	Lysolipid	2-stearoylglycerophosphocholine	LC/MS pos	35255			10208382
Lipid	Lysolipid	1-oleoylglycerophosphocholine (18:1)	LC/MS pos	48258			16081932
Lipid	Lysolipid	2-oleoylglycerophosphocholine	LC/MS pos	48259			
Lipid	Lysolipid	1-linoleoylglycerophosphocholine (18:2n6)	LC/MS pos	34419	C04100		11988421
Lipid	Lysolipid	2-linoleoylglycerophosphocholine	LC/MS pos	35257			
Lipid	Lysolipid	1-linolenoylglycerophosphocholine (18:3n3)	LC/MS pos	45951			
Lipid	Lysolipid	2-linolenoylglycerophosphocholine(18:3n3)	LC/MS pos	45869			

Table 5.1 (cont'd)

Lipid	Lysolipid	1-nonadecanoylglycerophosphocholine (19:0)	LC/MS pos	47087			
Lipid	Lysolipid	1-dihomo-linoleoylglycerophosphocholine (20:2n6)	LC/MS pos	33871			
Lipid	Lysolipid	1-arachidoylglycerophosphocholine (20:0)	LC/MS pos	45456	C04230	HMDB10390	24779473
Lipid	Lysolipid	2-arachidoylglycerophosphocholine	LC/MS pos	35623			
Lipid	Lysolipid	1-eicosenoylglycerophosphocholine (20:1n9)	LC/MS pos	44560			
Lipid	Lysolipid	2-eicosenoylglycerophosphocholine(20:1n9)	LC/MS pos	48119			
Lipid	Lysolipid	1-eicosatrienoylglycerophosphocholine (20:3)	LC/MS pos	33821			
Lipid	Lysolipid	2-eicosatrienoylglycerophosphocholine	LC/MS pos	35884			
Lipid	Lysolipid	1-arachidonoylglycerophosphocholine (20:4n6)	LC/MS pos	33228	C05208		
Lipid	Lysolipid	2-arachidonoylglycerophosphocholine	LC/MS pos	35256			
Lipid	Lysolipid	1-eicosapentaenoylglycerophosphocholine (20:5n3)	LC/MS pos	44563			
Lipid	Lysolipid	1-docosapentaenoylglycerophosphocholine (22:5n3)	LC/MS pos	37231			
Lipid	Lysolipid	2-docosapentaenoylglycerophosphocholine (22:5n3)	LC/MS pos	37366			
Lipid	Lysolipid	1-docosapentaenoylglycerophosphocholine (22:5n6)	LC/MS pos	45675			
Lipid	Lysolipid	1-docosahexaenoylglycerophosphocholine (22:6n3)	LC/MS pos	33822			
Lipid	Lysolipid	2-docosahexaenoylglycerophosphocholine	LC/MS pos	35883			
Lipid	Lysolipid	1-palmitoylplasmenylethanolamine	LC/MS neg	39270			
Lipid	Lysolipid	1-stearoylplasmenylethanolamine	LC/MS neg	39271			
Lipid	Lysolipid	1-oleoylplasmenylethanolamine	LC/MS neg	44621			

Table 5.1 (cont'd)

Lipid	Lysolipid	1-palmitoylglycerophosphoethanolamine	LC/MS neg	35631		HMDB11503	9547069
Lipid	Lysolipid	2-palmitoylglycerophosphoethanolamine	LC/MS neg	45452			
Lipid	Lysolipid	1-stearoylglycerophosphoethanolamine	LC/MS neg	42398		HMDB11130	9547068
Lipid	Lysolipid	2-stearoylglycerophosphoethanolamine	LC/MS neg	41220			
Lipid	Lysolipid	1-oleoylglycerophosphoethanolamine	LC/MS neg	35628		HMDB11506	9547071
Lipid	Lysolipid	2-oleoylglycerophosphoethanolamine	LC/MS neg	45455			
Lipid	Lysolipid	1-palmitoleoylglycerophosphoethanolamine	LC/MS neg	34565			
Lipid	Lysolipid	1-linoleoylglycerophosphoethanolamine	LC/MS neg	32635		HMDB11507	52925130
Lipid	Lysolipid	2-linoleoylglycerophosphoethanolamine	LC/MS neg	36593			
Lipid	Lysolipid	1-arachidonoylglycerophosphoethanolamine	LC/MS neg	35186		HMDB11517	42607465
Lipid	Lysolipid	2-arachidonoylglycerophosphoethanolamine	LC/MS neg	32815			
Lipid	Lysolipid	1-eicosatrienoylglycerophosphoethanolamine	LC/MS neg	44630			
Lipid	Lysolipid	1-palmitoylglycerophosphoinositol	LC/MS neg	35305			
Lipid	Lysolipid	1-stearoylglycerophosphoinositol	LC/MS neg	19324			
Lipid	Lysolipid	2-stearoylglycerophosphoinositol	LC/MS neg	39223			
Lipid	Lysolipid	1-oleoylglycerophosphoinositol	LC/MS neg	36602			
Lipid	Lysolipid	1-linoleoylglycerophosphoinositol	LC/MS neg	36594			
Lipid	Lysolipid	1-arachidonoylglycerophosphoinositol	LC/MS neg	34214			
Lipid	Lysolipid	1-stearoylglycerophosphoserine	LC/MS neg	45966			9547101

Table 5.1 (cont'd)

Lipid	Lysolipid	1-oleoylglycerophosphoserine	LC/MS neg	19260			9547099
Lipid	Lysolipid	1-linoleoylglycerophosphoserine	LC/MS neg	43676			
Lipid	Lysolipid	1-palmitoylglycerophosphate	LC/MS neg	34428	C04036	HMDB00327	6419701
Lipid	Lysolipid	1-arachidonoylglyercophosphate	LC/MS neg	46325			
Lipid	Lysolipid	1-palmitoylglycerophosphoglycerol	LC/MS neg	45970			3300276
Lipid	Lysolipid	1-oleoylglycerophosphoglycerol	LC/MS neg	45968			
Lipid	Lysolipid	2-nonadecanoylglycerophosphocholine (19:0)	LC/MS pos	47088			
Lipid	Lysolipid	oleoyl-linoleoyl-glycerophosphoinositol (1)	LC/MS polar	48303			
Lipid	Lysolipid	palmitoyl-arachidonoyl-glycerophosphocholine (1)	LC/MS polar	48383			
Lipid	Lysolipid	palmitoyl-arachidonoyl-glycerophosphocholine (2)	LC/MS polar	48392			
Lipid	Lysolipid	palmitoyl-linoleoyl-glycerophosphocholine (1)	LC/MS polar	48385			
Lipid	Lysolipid	palmitoyl-linoleoyl-glycerophosphocholine (2)	LC/MS polar	48269			
Lipid	Lysolipid	palmitoyl-linoleoyl-glycerophosphoinositol (1)	LC/MS polar	48304			
Lipid	Lysolipid	palmitoyl-oleoyl-glycerophosphocholine (1)	LC/MS polar	48267			
Lipid	Lysolipid	stearoyl-arachidonoyl-glycerophosphocholine (1)	LC/MS polar	48386			
Lipid	Lysolipid	stearoyl-arachidonoyl-glycerophosphocholine (2)	LC/MS polar	48393			
Lipid	Lysolipid	stearoyl-arachidonoyl-glycerophosphoinositol (1)	LC/MS polar	48282			
Lipid	Lysolipid	oleoyl-linoleoyl-glycerophosphocholine (1)	LC/MS polar	48270			
Lipid	Lysolipid	oleoyl-linoleoyl-glycerophosphocholine (2)	LC/MS polar	48390			

Table 5.1 (cont'd)

Lipid	Lysolipid	palmitoyl-palmitoyl-glycerophosphocholine (1)	LC/MS polar	48276			
Lipid	Lysolipid	palmitoyl-palmitoyl-glycerophosphocholine (2)	LC/MS polar	48389			
Lipid	Lysolipid	stearoyl-linoleoyl-glycerophosphocholine (1)	LC/MS polar	48387			
Lipid	Lysolipid	stearoyl-linoleoyl-glycerophosphocholine (2)	LC/MS polar	48388			
Lipid	Lysolipid	stearoyl-arachidonoyl-glycerophosphoethanolamine (1)	LC/MS polar	48497			
Lipid	Lysolipid	stearoyl-linoleoyl-glycerophosphoethanolamine (1)	LC/MS polar	48499			
Lipid	Glycerolipid Metabolism	glycerol	LC/MS neg	15122	C00116	HMDB00131	753
Lipid	Glycerolipid Metabolism	glycerol 3-phosphate (G3P)	GC/MS	15365	C00093	HMDB00126	754
Lipid	Monoacylglycerol	1-myristoylglycerol (1-monomyristin)	LC/MS neg	35625	C01885	HMDB11561	79050
Lipid	Monoacylglycerol	1-palmitoylglycerol (1-monopalmitin)	LC/MS pos	21127		HMDB31074	14900
Lipid	Monoacylglycerol	2-palmitoylglycerol (2-monopalmitin)	LC/MS neg	33419		HMDB11533	123409
Lipid	Monoacylglycerol	1-margaroylglycerol (1-monoheptadecanoin)	LC/MS pos	34391			107036
Lipid	Monoacylglycerol	1-stearoylglycerol (1-monostearin)	GC/MS	21188	D01947	HMDB31075	24699
Lipid	Monoacylglycerol	2-stearoylglycerol (2-monostearin)	LC/MS pos	34059			79075
Lipid	Monoacylglycerol	1-oleoylglycerol (1-monoolein)	LC/MS pos	21184		HMDB11567	5283468
Lipid	Monoacylglycerol	2-oleoylglycerol (2-monoolein)	LC/MS neg	21232			5319879
Lipid	Monoacylglycerol	1-linoleoylglycerol (1-monolinolein)	LC/MS neg	27447			5283469
Lipid	Monoacylglycerol	2-linoleoylglycerol (2-monolinolein)	LC/MS neg	32506		HMDB11538	5365676
Lipid	Monoacylglycerol	1-linolenoylglycerol	LC/MS pos	34393		HMDB11569	53480978

Table 5.1 (cont'd)

Lipid	Monoacylglycerol	1-dihomo-linolenylglycerol (alpha, gamma)	LC/MS pos	48341			
Lipid	Sphingolipid Metabolism	sphinganine	LC/MS pos	17769	C00836	HMDB00269	3126
Lipid	Sphingolipid Metabolism	palmitoyl sphingomyelin	LC/MS polar	37506			9939941
Lipid	Sphingolipid Metabolism	stearoyl sphingomyelin	LC/MS polar	19503	C00550	HMDB01348	6453725
Lipid	Sphingolipid Metabolism	sphingosine 1-phosphate	LC/MS pos	34445	C06124	HMDB00277	5283560
Lipid	Sphingolipid Metabolism	sphingosine	LC/MS pos	17747	C00319	HMDB00252	5353955
Lipid	Sphingolipid Metabolism	myristoyl sphingomyelin	LC/MS polar	42463			11433862
Lipid	Sphingolipid Metabolism	nervonoyl sphingomyelin	LC/MS polar	47153			
Lipid	Mevalonate Metabolism	3-hydroxy-3-methylglutarate	LC/MS polar	531	C03761	HMDB00355	1662
Lipid	Sterol	cholesterol	GC/MS	63	C00187	HMDB00067	11025495
Lipid	Sterol	7-alpha-hydroxy-3-oxo-4-cholestenoate (7-Hoca)	LC/MS neg	36776	C17337	HMDB12458	3081085
Lipid	Sterol	cholestanol	GC/MS	21131	C12978	HMDB00908	6665
Lipid	Sterol	beta-sitosterol	GC/MS	27414	C01753	HMDB00852	222284
Lipid	Sterol	campesterol	GC/MS	33997	C01789	HMDB02869	173183
Lipid	Steroid	5alpha-pregnan-3alpha,20beta-diol disulfate 1	LC/MS neg	37201			
Lipid	Steroid	cortisol	LC/MS pos	1712	C00735	HMDB00063	5754
Lipid	Steroid	corticosterone	LC/MS pos	5983	C02140	HMDB01547	5753
Lipid	Steroid	cortisone	LC/MS pos	1769	C00762	HMDB02802	222786
Lipid	Steroid	estrone 3-sulfate	LC/MS neg	18474	C02538	HMDB01425	3001028

Table 5.1 (cont'd)

Lipid	Primary Bile Acid Metabolism	cholate	LC/MS neg	22842	C00695	HMDB00619	221493
Lipid	Primary Bile Acid Metabolism	glycocholate	LC/MS neg	18476	C01921	HMDB00138	10140
Lipid	Primary Bile Acid Metabolism	taurocholate	LC/MS neg	18497	C05122	HMDB00036	6675
Lipid	Primary Bile Acid Metabolism	chenodeoxycholate	LC/MS neg	1563	C02528	HMDB00518	10133
Lipid	Primary Bile Acid Metabolism	glycochenodeoxycholate	LC/MS neg	32346	C05466	HMDB00637	12544
Lipid	Primary Bile Acid Metabolism	taurochenodeoxycholate	LC/MS neg	18494	C05465	HMDB00951	387316
Lipid	Primary Bile Acid Metabolism	tauro-alpha-muricholate	LC/MS neg	42605			
Lipid	Primary Bile Acid Metabolism	tauro-beta-muricholate	LC/MS neg	33983		HMDB00932	168408
Lipid	Secondary Bile Acid Metabolism	deoxycholate	LC/MS neg	1114	C04483	HMDB00626	222528
Lipid	Secondary Bile Acid Metabolism	lithocholate	LC/MS neg	1483	C03990	HMDB00761	9903
Lipid	Secondary Bile Acid Metabolism	taurolithocholate	LC/MS neg	31889	C02592	HMDB00722	10595
Lipid	Secondary Bile Acid Metabolism	taurolithocholate 3-sulfate	LC/MS neg	36850	C03642	HMDB02580	440071
Lipid	Secondary Bile Acid Metabolism	ursodeoxycholate	LC/MS neg	1605	C07880	HMDB00946	31401
Lipid	Secondary Bile Acid Metabolism	tauroursodeoxycholate	LC/MS neg	39378		HMDB00874	9848818
Lipid	Secondary Bile Acid Metabolism	taurohyodeoxycholic acid	LC/MS neg	43588			119046
Lipid	Secondary Bile Acid Metabolism	taurocholenate sulfate	LC/MS neg	32807			
Nucleotide	Purine Metabolism (Hypo)Xanthine/Inosine	inosine	LC/MS neg	1123	C00294	HMDB00195	6021
Nucleotide	Purine Metabolism (Hypo)Xanthine/Inosine	hypoxanthine	LC/MS pos	3127	C00262	HMDB00157	790
Nucleotide	Purine Metabolism (Hypo)Xanthine/Inosine	xanthine	LC/MS neg	3147	C00385	HMDB00292	1188

Table 5.1 (cont'd)

Nucleotide	Purine Metabolism (Hypo)Xanthine/Inosine	xanthosine	LC/MS pos	15136	C01762	HMDB00299	64959
Nucleotide	Purine Metabolism (Hypo)Xanthine/Inosine	urate	LC/MS neg	1604	C00366	HMDB00289	1175
Nucleotide	Purine Metabolism (Hypo)Xanthine/Inosine	allantoin	LC/MS pos	1107	C02350	HMDB00462	204
Nucleotide	Purine Metabolism, Adenine containing	adenosine 5'-monophosphate (AMP)	LC/MS pos	32342	C00020	HMDB00045	6083
Nucleotide	Purine Metabolism, Adenine containing	adenosine	LC/MS pos	555	C00212	HMDB00050	60961
Nucleotide	Purine Metabolism, Adenine containing	adenine	LC/MS pos	554	C00147	HMDB00034	190
Nucleotide	Purine Metabolism, Adenine containing	N1-methyladenosine	LC/MS pos	15650	C02494	HMDB03331	27476
Nucleotide	Purine Metabolism, Guanine containing	7-methylguanine	LC/MS pos	35114	C02242	HMDB00897	11361
Nucleotide	Pyrimidine Metabolism, Orotate containing	orotate	LC/MS polar	1505	C00295	HMDB00226	967
Nucleotide	Pyrimidine Metabolism, Uracil containing	uridine	LC/MS neg	606	C00299	HMDB00296	6029
Nucleotide	Pyrimidine Metabolism, Uracil containing	uracil	LC/MS pos	605	C00106	HMDB00300	1174
Nucleotide	Pyrimidine Metabolism, Uracil containing	pseudouridine	LC/MS neg	33442	C02067	HMDB00767	15047
Nucleotide	Pyrimidine Metabolism, Uracil containing	5-methyluridine (ribothymidine)	LC/MS pos	35136		HMDB00884	445408
Nucleotide	Pyrimidine Metabolism, Uracil containing	3-ureidopropionate	LC/MS pos	3155	C02642	HMDB00026	111
Nucleotide	Pyrimidine Metabolism, Uracil containing	beta-alanine	GC/MS	55	C00099	HMDB00056	239
Nucleotide	Pyrimidine Metabolism, Uracil containing	N-acetyl-beta-alanine	LC/MS pos	37432	C01073		76406
Nucleotide	Pyrimidine Metabolism, Cytidine containing	cytidine	LC/MS pos	514	C00475	HMDB00089	6175
Nucleotide	Pyrimidine Metabolism, Thymine containing	thymine	LC/MS neg	604	C00178	HMDB00262	1135
Nucleotide	Pyrimidine Metabolism, Thymine containing	5,6-dihydrothymine	LC/MS pos	1418	C00906	HMDB00079	93556

Table 5.1 (cont'd)

Nucleotide	Pyrimidine Metabolism, Thymine containing	3-aminoisobutyrate	GC/MS	1566	C05145	HMDB03911	64956
Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	quinolinate	LC/MS neg	1899	C03722	HMDB00232	1066
Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	nicotinamide	LC/MS pos	594	C00153	HMDB01406	936
Cofactors and Vitamins	Nicotinate and Nicotinamide Metabolism	trigonelline (N'-methylnicotinate)	LC/MS pos	32401	C01004	HMDB00875	5570
Cofactors and Vitamins	Riboflavin Metabolism	riboflavin (Vitamin B2)	LC/MS neg	1827	C00255	HMDB00244	493570
Cofactors and Vitamins	Pantothenate and CoA Metabolism	pantothenate	LC/MS pos	1508	C00864	HMDB00210	6613
Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	threonate	LC/MS polar	27738	C01620	HMDB00943	151152
Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	oxalate (ethanedioate)	LC/MS neg	20694	C00209	HMDB02329	971
Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	gulonic acid	LC/MS polar	46957			9794176
Cofactors and Vitamins	Tocopherol Metabolism	alpha-tocopherol	GC/MS	1561	C02477	HMDB01893	14985
Cofactors and Vitamins	Tocopherol Metabolism	gamma-tocopherol	GC/MS	33420	C02483	HMDB01492	14986
Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	heme	LC/MS pos	41754			26945
Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	bilirubin (Z,Z)	LC/MS neg	43807	C00486	HMDB00054	5280352
Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	bilirubin (E,E)	LC/MS neg	32586			5315454
Cofactors and Vitamins	Hemoglobin and Porphyrin Metabolism	biliverdin	LC/MS neg	2137	C00500	HMDB01008	5353439
Cofactors and Vitamins	Vitamin B6 Metabolism	pyridoxate	LC/MS neg	31555	C00847	HMDB00017	6723
Xenobiotics	Benzoate Metabolism	hippurate	LC/MS neg	15753	C01586	HMDB00714	464
Xenobiotics	Benzoate Metabolism	2-hydroxyhippurate (salicylurate)	LC/MS neg	18281	C07588	HMDB00840	10253
Xenobiotics	Benzoate Metabolism	3-hydroxyhippurate	LC/MS neg	39600		HMDB06116	450268

Table 5.1 (cont'd)

Xenobiotics	Benzoate Metabolism	4-hydroxyhippurate	LC/MS neg	35527		HMDB13678	151012
Xenobiotics	Benzoate Metabolism	benzoate	LC/MS neg	15778	C00180	HMDB01870	243
Xenobiotics	Benzoate Metabolism	3-hydroxybenzoate	LC/MS neg	15673	C00587	HMDB02466	7420
Xenobiotics	Benzoate Metabolism	catechol sulfate	LC/MS neg	35320	C00090	HMDB59724	3083879
Xenobiotics	Benzoate Metabolism	O-methylcatechol sulfate	LC/MS neg	46111			22473
Xenobiotics	Benzoate Metabolism	3-methyl catechol sulfate (1)	LC/MS neg	46165			
Xenobiotics	Benzoate Metabolism	3-methyl catechol sulfate (2)	LC/MS neg	46164			
Xenobiotics	Benzoate Metabolism	4-methylcatechol sulfate	LC/MS neg	46146			
Xenobiotics	Benzoate Metabolism	methyl-4-hydroxybenzoate	GC/MS	34386	D01400	HMDB32572	7456
Xenobiotics	Benzoate Metabolism	2-ethylphenylsulfate	LC/MS neg	36847			
Xenobiotics	Benzoate Metabolism	4-ethylphenylsulfate	LC/MS neg	36099	C13637		
Xenobiotics	Benzoate Metabolism	4-vinylphenol sulfate	LC/MS neg	36098	C05627	HMDB04072	6426766
Xenobiotics	Food Component/Plant	1,6-anhydroglucose	GC/MS	21049		HMDB00640	2724705
Xenobiotics	Food Component/Plant	1H-quinolin-2-one	LC/MS pos	40464	C06415		6038
Xenobiotics	Food Component/Plant	2,3-dihydroxyisovalerate	LC/MS polar	38276	C04039	HMDB12141	677
Xenobiotics	Food Component/Plant	gluconate	LC/MS polar	587	C00257	HMDB00625	10690
Xenobiotics	Food Component/Plant	cinnamoylglycine	LC/MS neg	38637		HMDB11621	709625
Xenobiotics	Food Component/Plant	dihydroferulic acid	LC/MS neg	40481			14340
Xenobiotics	Food Component/Plant	equol glucuronide	LC/MS neg	41948			

Table 5.1 (cont'd)

Xenobiotics	Food Component/Plant	equol sulfate	LC/MS neg	40478			
Xenobiotics	Food Component/Plant	ergothioneine	LC/MS neg	37459	C05570	HMDB03045	3032311
Xenobiotics	Food Component/Plant	erythritol	GC/MS	20699	C00503	HMDB02994	222285
Xenobiotics	Food Component/Plant	ferulic acid 4-sulfate	LC/MS neg	47114		HMDB29200	6305574
Xenobiotics	Food Component/Plant	homostachydrine	LC/MS pos	33009	C08283	HMDB33433	441447
Xenobiotics	Food Component/Plant	indolin-2-one	LC/MS pos	43374	C12312		321710
Xenobiotics	Food Component/Plant	N-(2-furoyl) glycine	LC/MS neg	31536		HMDB00439	21863
Xenobiotics	Food Component/Plant	quinate	LC/MS polar	18335	C00296	HMDB03072	6508
Xenobiotics	Food Component/Plant	stachydrine	LC/MS pos	34384	C10172	HMDB04827	115244
Xenobiotics	Food Component/Plant	tartarate	LC/MS neg	15336	C00898	HMDB00956	444305
Xenobiotics	Food Component/Plant	thymol sulfate	LC/MS neg	36095	C09908	HMDB01878	
Xenobiotics	Food Component/Plant	4-allylphenol sulfate	LC/MS neg	37181			
Xenobiotics	Food Component/Plant	methyl glucopyranoside (alpha + beta)	LC/MS neg	46144			
Xenobiotics	Bacterial/Fungal	tartronate (hydroxymalonate)	LC/MS neg	20693	C02287	HMDB35227	45
Xenobiotics	Drug	2-hydroxyacetaminophen sulfate	LC/MS neg	33173			
Xenobiotics	Drug	4-acetaminophen sulfate	LC/MS neg	37475	C06804	HMDB59911	83939
Xenobiotics	Drug	4-acetamidophenol	LC/MS pos	12032	C06804	HMDB01859	1983
Xenobiotics	Drug	4-acetylphenol sulfate	LC/MS neg	44620			4684006
Xenobiotics	Drug	6-oxopiperidine-2-carboxylic acid	LC/MS pos	43231			3014237

Table 5.1 (cont'd)

Xenobiotics	Drug	hydroquinone sulfate	LC/MS neg	35322	C00530	HMDB02434	161220
Xenobiotics	Drug	lidocaine	LC/MS pos	35661	D00358	HMDB14426	3676
Xenobiotics	Drug	salicylate	LC/MS neg	1515	C00805	HMDB01895	338
Xenobiotics	Chemical	2-pyrrolidinone	LC/MS pos	31675		HMDB02039	12025
Xenobiotics	Chemical	sulfate	LC/MS neg	46960	C00059	HMDB01448	1118
Xenobiotics	Chemical	O-sulfo-L-tyrosine	LC/MS neg	45413			514186
Xenobiotics	Chemical	2-aminophenol sulfate	LC/MS neg	43266		HMDB61116	181670
Xenobiotics	Chemical	2-ethylhexanoate	LC/MS neg	1554		HMDB31230	8697
Xenobiotics	Chemical	2-hydroxyisobutyrate	GC/MS	22030		HMDB00729	11671
Xenobiotics	Chemical	dimethyl sulfone	LC/MS pos	43424	C11142	HMDB04983	6213
Xenobiotics	Chemical	phenylcarnitine	LC/MS pos	43265			
Xenobiotics	Chemical	trizma acetate	GC/MS	20710	C07182		6503
Xenobiotics	Chemical	N-methylpipecolate	LC/MS pos	47101			11862129; 11286529

Table 5.2 *Number of metabolites in each metabolic pathway and sub-pathway.*

METABOLIC PATHWAY	NUMBER OF METABOLITES
Amino Acid	**135**
Alanine and Aspartate Metabolism	6
Creatine Metabolism	3
Glutamate Metabolism	6
Glutathione Metabolism	2
Glycine, Serine and Threonine Metabolism	8
Guanidino and Acetamido Metabolism	1
Histidine Metabolism	10
Leucine, Isoleucine and Valine Metabolism	29
Lysine Metabolism	8
Methionine, Cysteine, SAM and Taurine Metabolism	13
Phenylalanine and Tyrosine Metabolism	22
Polyamine Metabolism	2
Tryptophan Metabolism	12
Urea cycle; Arginine and Proline Metabolism	13
Carbohydrate	**24**
Amino-sugar Metabolism	3
Disaccharides and Oligosaccharides	1
Fructose, Mannose and Galactose Metabolism	4
Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	5
Pentose Metabolism	11
Cofactors and Vitamins	**15**
Ascorbate and Aldarate Metabolism	3
Hemoglobin and Porphyrin Metabolism	4
Nicotinate and Nicotinamide Metabolism	3
Pantothenate and CoA Metabolism	1
Riboflavin Metabolism	1
Tocopherol Metabolism	2
Vitamin B6 Metabolism	1
Energy	**8**
Oxidative Phosphorylation	1
TCA Cycle	7
Lipid	**216**
Carnitine Metabolism	2
Eicosanoid	1
Endocannabinoid	4
Fatty Acid Metabolism (also BCAA Metabolism)	5
Fatty Acid Metabolism (Acyl Carnitine)	13
Fatty Acid Metabolism (Acyl Glycine)	2

Table 5.2 (cont'd)

Fatty Acid Synthesis	2
Fatty Acid, Amino	2
Fatty Acid, Branched	2
Fatty Acid, Dicarboxylate	5
Fatty Acid, Methyl Ester	1
Fatty Acid, Monohydroxy	10
Glycerolipid Metabolism	2
Inositol Metabolism	3
Ketone Bodies	1
Long Chain Fatty Acid	13
Lysolipid	80
Medium Chain Fatty Acid	6
Mevalonate Metabolism	1
Monoacylglycerol	12
Phospholipid Metabolism	3
Polyunsaturated Fatty Acid (n3 and n6)	12
Primary Bile Acid Metabolism	8
Secondary Bile Acid Metabolism	8
Short Chain Fatty Acid	1
Sphingolipid Metabolism	7
Steroid/Sterol	10
Nucleotide	**23**
Purine Metabolism, (Hypo)Xanthine/Inosine containing	6
Purine Metabolism, Adenine containing	4
Purine Metabolism, Guanine containing	1
Pyrimidine Metabolism, Cytidine containing	1
Pyrimidine Metabolism, Orotate containing	1
Pyrimidine Metabolism, Thymine containing	3
Pyrimidine Metabolism, Uracil containing	7
Peptide	**31**
Dipeptide/Dipeptide Derivative	21
Gamma-glutamyl Amino Acid	10
Xenobiotics	**54**
Bacterial/Fungal	1
Benzoate Metabolism	15
Chemical	10
Drug	8
Food Component/Plant	20
Unknown	**140**
TOTAL	**646**

Table 5.3 *Optimal number of metabolites that distinguish between control and disease status for each variable (insulin response, obesity status, laminitis history) as determined by LASSO analysis.*

INSULIN RESPONSE	OBESITY	LAMINITIS
13-HODE + 9-HODE	1-eicosadienoyl-GPC	1-dihomo-linolenylglycerol
1-eicosatrienoyl-GPC	1-linoleoyl-GPS	1-oleoylglycerol
1-linoleoyl-GPC	arabitol	2-docosahexaenoyl-GPC
2-margaroyl-GPC	betaine	2-ethylphenylsulfate
3-(3-hydroxyphenyl) propionate	carnosine	2-margaroyl-GPC
3-hydroxyisobutyrate	chenodeoxycholate	2-palmitoylglycerol
4-imidazoleacetate	dodecanedioate	2-pyrrolidinone
5-hydroxyindoleacetate	equol glucuronide	4-acetylphenyl sulfate
asparagine	ferulic acid 4-sulfate	4-hydroxybutyrate
cinnamoylglycine	methylmalonate (MMA)	benzoate
glucuronate	N-6-trimethyllysine	carnitine
imidazole propionate	octanoylcarnitine	homocitrulline
indole-3-carboxylic acid	palmitoyl-arachidonoyl-glycerophosphocholine	N-delta-acetylornithine
isovalerylcarnitine	ursodeoxycholate	oleoyl-linoleoyl-glycerophosphocholine
N-palmitoyltaurine		orotate
octadecanedioate		phenylcarnitine
oleoyl-linoleoyl-glycerophosphocholine		propionylglycine
oxalate (ethanedioate)		quinolinate
palmitoyl-linoleoyl-glycerophosphocholine		serotonin
phenylcarnitine		tartarate
sphingomyelin		tauro-alpha-muricholate
stearoyl-arachidonoyl-glycerophosphoinositol		

Table 5.4 *Uphill (positive) correlations between metabolites and clinical parameter measurements (basal glucose, basal insulin, non-esterified fatty acids (NEFAs), triglycerides, leptin, adiponectin).*

BASAL GLUCOSE	BASAL INSULIN	NEFAs	TRIGLYCERIDES	LEPTIN	ADIPONECTIN
	r = 0.7	***r = 0.5***	***r = 0.7***	***r = 0.7***	**r = 0.5**
	hydroquinone sulfate	3-[3-(sulfooxy)phenyl] propanoic acid	cinnamoylglycine	mannitol	1-linoleoylglycerophosphocholine
	arginine	asparagine	phenylcarnitine	tartronate (hydroxymalonate)	2-stearoylglycerophosphocholine
	r = 0.6	stearoyl-arachidonoyl-glycerophosphoinositol	***r = 0.6***	N-acetylvaline	adenine
	N-acetylleucine		2-ethylhexanoate	N-(2-furoyl) glycine	arabinose
	cyclo(leu-pro)		taurolithocholate	tricarballylate	cytidine
	N-(2-furoyl) glycine		taurolithocholate 3-sulfate	hydroquinone sulfate	isovalerylcarnitine
	citrulline		***r = 0.5***	4-allylphenol sulfate	oxalate (ethanedioate)
	4-imidazoleacetate		N-oleoyltaurine	***r = 0.6***	
	guanidinoacetate		taurohyodeoxycholic acid	xylose	
	indoleacetate		13-HODE + 9-HODE	1-dihomo-linoleoylglycerophosphocholine	
	r = 0.5		2-arachidonoylglycerophosphocholine	cyclo(gly-pro)	
	methionine sulfoxide		N-acetylglycine	1-palmitoylglycerophosphoinositol	
	asparagine		2-ethylphenylsulfate	1H-quinolin-2-one	
	dihydroferulic acid			1-linoleoylglycerophosphoserine	
	3-(3-hydroxyphenyl) propionate			1-linoleoylglycerophosphoinositol	
	salicylate			N-acetylleucine	
	gentisate			N-methylpipecolate	
	1-linoleoylglycerophosphoinositol			N-acetylasparagine	
	pyroglutamine			indolin-2-one	
	2-stearoylglycerophosphoinositol			propionylglycine	
	dodecanedioate			isoleucylaspartate	
	1-palmitoylglycerophosphoinositol			isoleucylglycine	
	hippurate			***r = 0.5***	

Table 5.4 (cont'd)

	N-acetylalanine			benzoate	
	3-hydroxybenzoate			salicylate	
	4-hydroxyhippurate			picolinate	
	phenol sulfate			gentisate	
	N-acetylglutamate			3-phenylpropionate (hydrocinnamate)	
				3-hydroxy-3-phenylpropionate	
				2-hydroxydecanoate	
				equol glucuronide	
				4-hydroxybutyrate (GHB)	
				4-acetamidophenol	
				3-hydroxybenzoate	
				hippurate	
				2-pyrrolidinone	
				3-(3-hydroxyphenyl) propionate	
				prolylglycine	
				N-acetylglutamine	
				2-aminophenol sulfate	

Table 5.5 *Downhill (negative) correlations between metabolites and clinical parameter measurements (basal glucose, basal insulin, non-esterified fatty acids (NEFAs), triglycerides, leptin, adiponectin).*

BASAL GLUCOSE	BASAL INSULIN	NEFAs	TRIGLYCERIDES	LEPTIN	ADIPONECTIN
r = -0.6	***r = -0.7***	***r = -0.5***	***r = -0.5***	***r = -0.5***	***r = -0.6***
valine	carnitine	2-hydroxypalmitate	betaine	10-heptadecenoate	allantoin
r = -0.5	homocitrulline	corticosterone	N1-methyladenosine	10-nonadecenoate	asparagine
3-methyl-2-oxobutyrate	beta-alanine	cortisol		10-undecenoate	***r = -0.5***
Cortisone	***r = -0.6***	trans-urocanate		2-aminobutyrate	cholate
N1-methyladenosine	erucate			alanine	hypotaurine
trans-urocanate	1-eicosapentaenoylglycerophosphocholine			carnitine	octadecanedioate
betaine	10-nonadecenoate			cholestanol	
riboflavin (Vitamin B2)	eicosenoate			dihomo-linolenate	
	beta-hydroxyisovaleroylcarnitine			docosahexaenoate (DHA)	
	oleic ethanolamide			docosapentaenoate	
	eicosapentaenoate (EPA)			linolenate	
	5-dodecenoate			lysine	
	cholestanol			margarate	
	isovalerylcarnitine			oleate	
	r = -0.5			palmitoleate	
	1-margaroylglycerophosphocholine			stearidonate	
	phosphate				
	myristoleoylcarnitine				
	10-heptadecenoate				
	16-hydroxypalmitate				
	sphinganine				
	palmitate				
	stearidonate				
	indole-3-carboxylic acid				
	margarate				
	15-methylpalmitate				

Table 5.5 (cont'd)

	4-methyl-2-oxopentanoate				
	1-palmitoylglycerophosphocholine				
	urate				
	cis-vaccenate				
	isoleucine				
	7-alpha-hydroxy-3-oxo-4-cholestenoate				
	myristoylcarnitine				
	1-linolenoylglycerophosphocholine				
	2-margaroylglycerophosphocholine				
	1-palmitoleoylglycerophosphocholine				
	glutarate (pentanedioate)				
	palmitoleate				
	dihomo-linolenate				
	myristate				
	2-hydroxyisobutyrate				
	2-palmitoleoylglycerophosphocholine				
	palmitoylcarnitine				
	1-oleoylglycerophosphocholine				
	1-eicosatrienoylglycerophosphocholine				
	stearate				
	5alpha-pregnan-3alpha,20beta-diol disulfate 1				
	2-arachidoylglycerophosphocholine				
	2-methylbutyrylcarnitine				
	oleate				
	3-hydroxydecanoate				
	2-aminobutyrate				
	alpha-hydroxyisovaleroyl carnitine				
	3-methyl-2-oxovalerate				

Table 5.5 (cont'd)

	docosatrienoate				
	stearoylcarnitine				
	oleoylcarnitine				
	N-delta-acetylornithine				
	linolenate				
	ornithine				
	N-stearoyltaurine				

Figure 5.1a *Tricarboxylic acid (TCA) cycle network pathway at 0 minutes (baseline).*

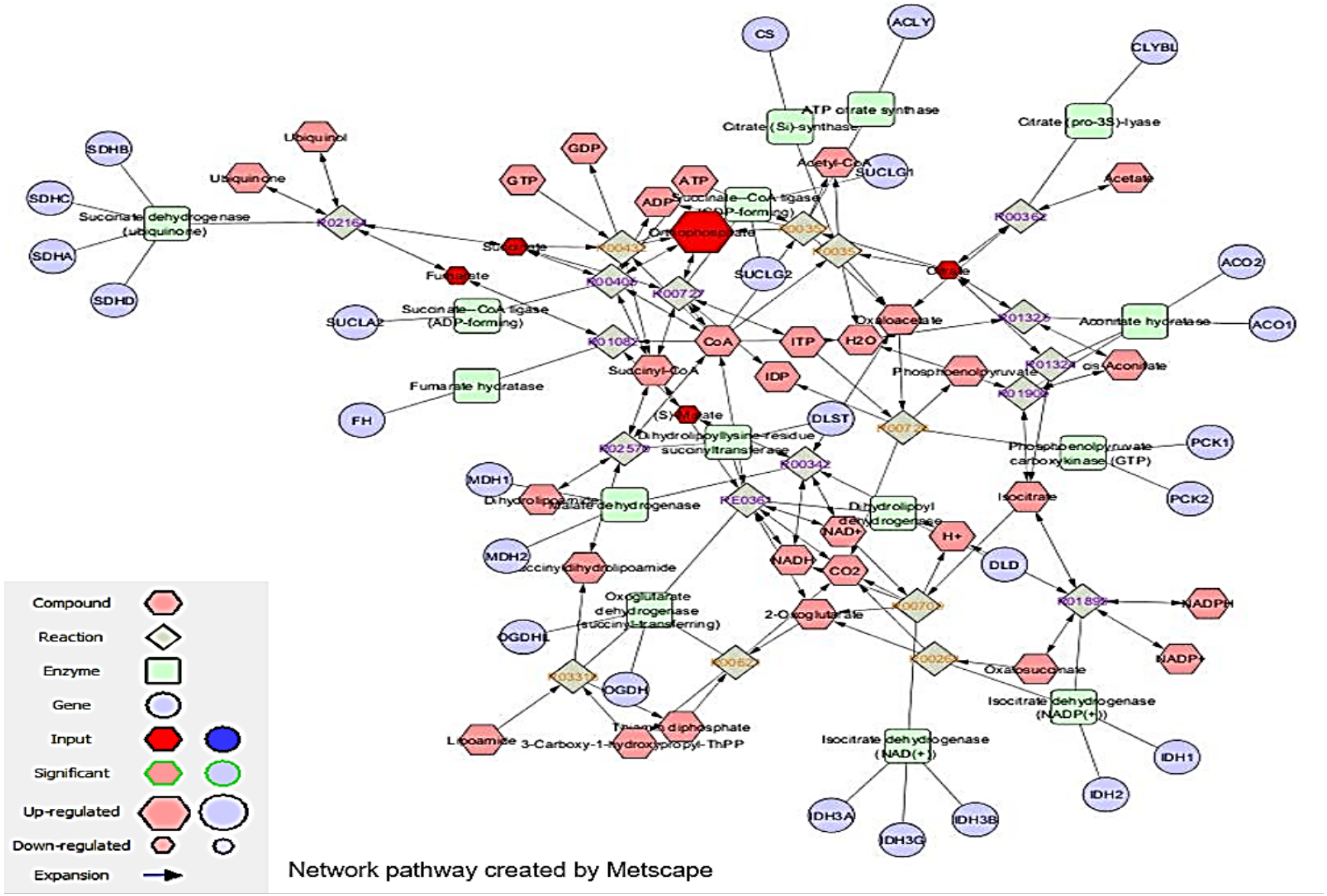
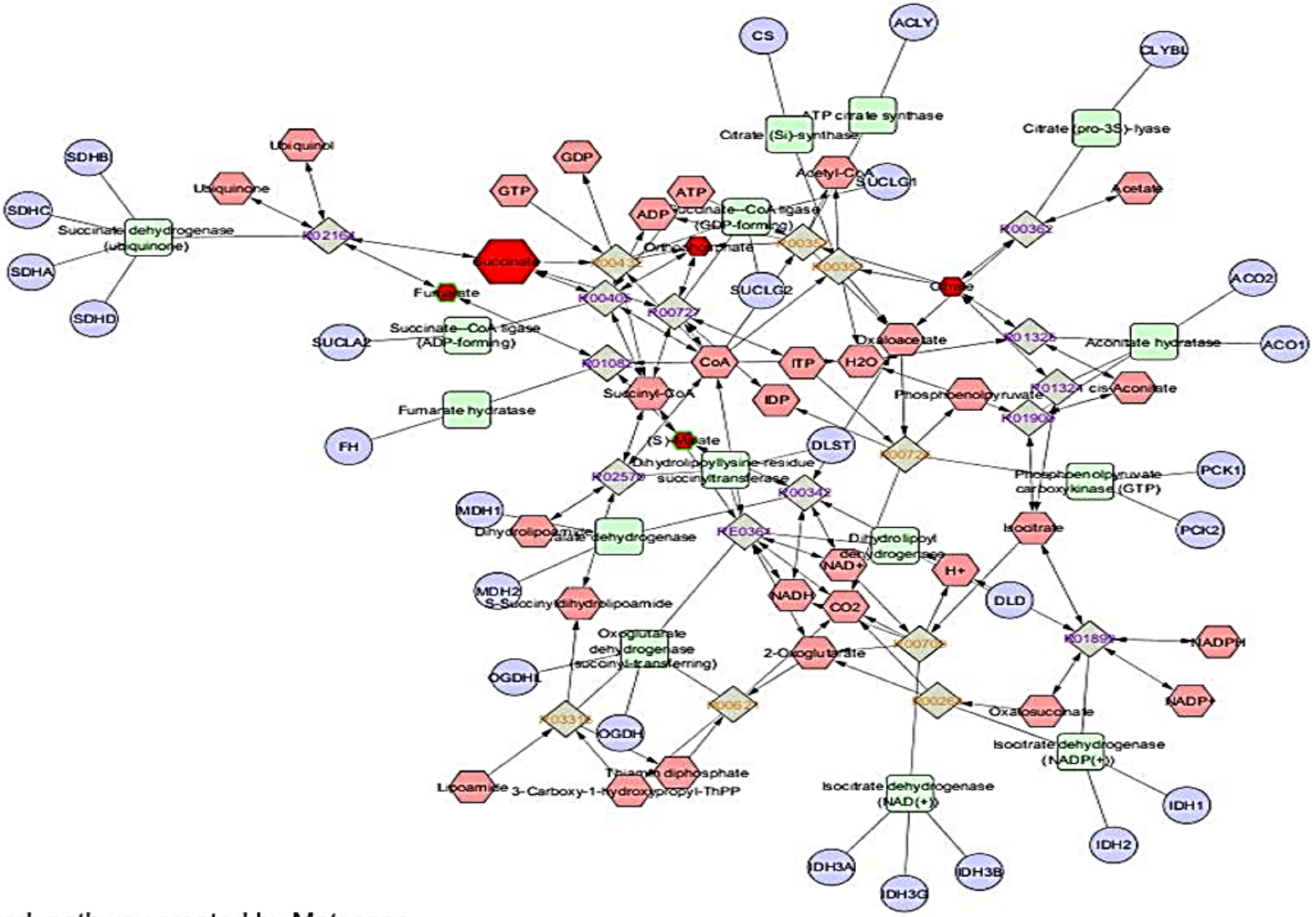


Figure 5.1b *Tricarboxylic acid (TCA) cycle network pathway at 75 minutes.*



Network pathway created by Metscape

Figure 5.2a *Branched-chain amino acid (BCAA) pathway at 0 minutes (baseline).*

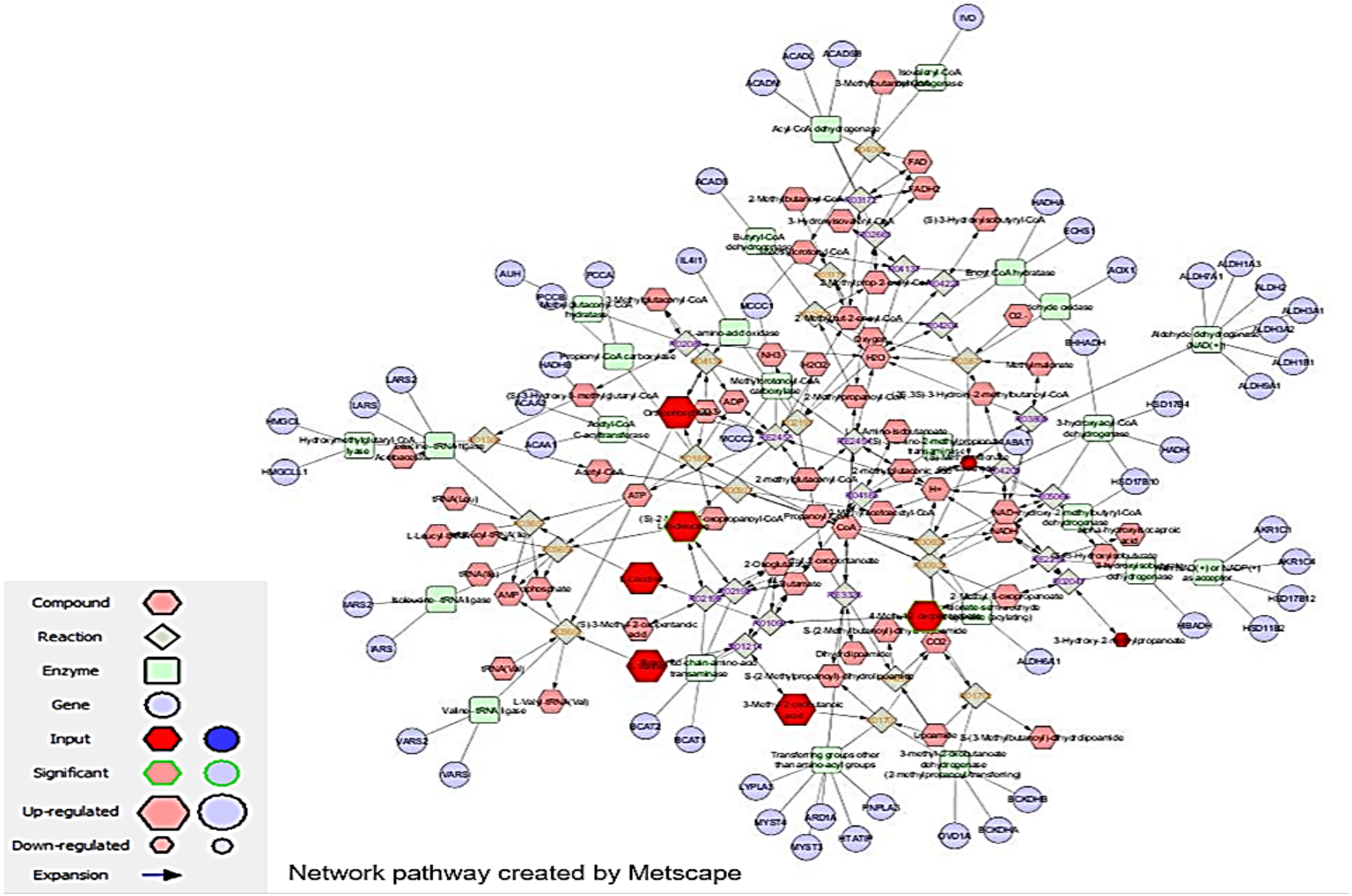


Figure 5.2b *Branched-chain amino acid (BCAA) pathway at 75 minutes.*

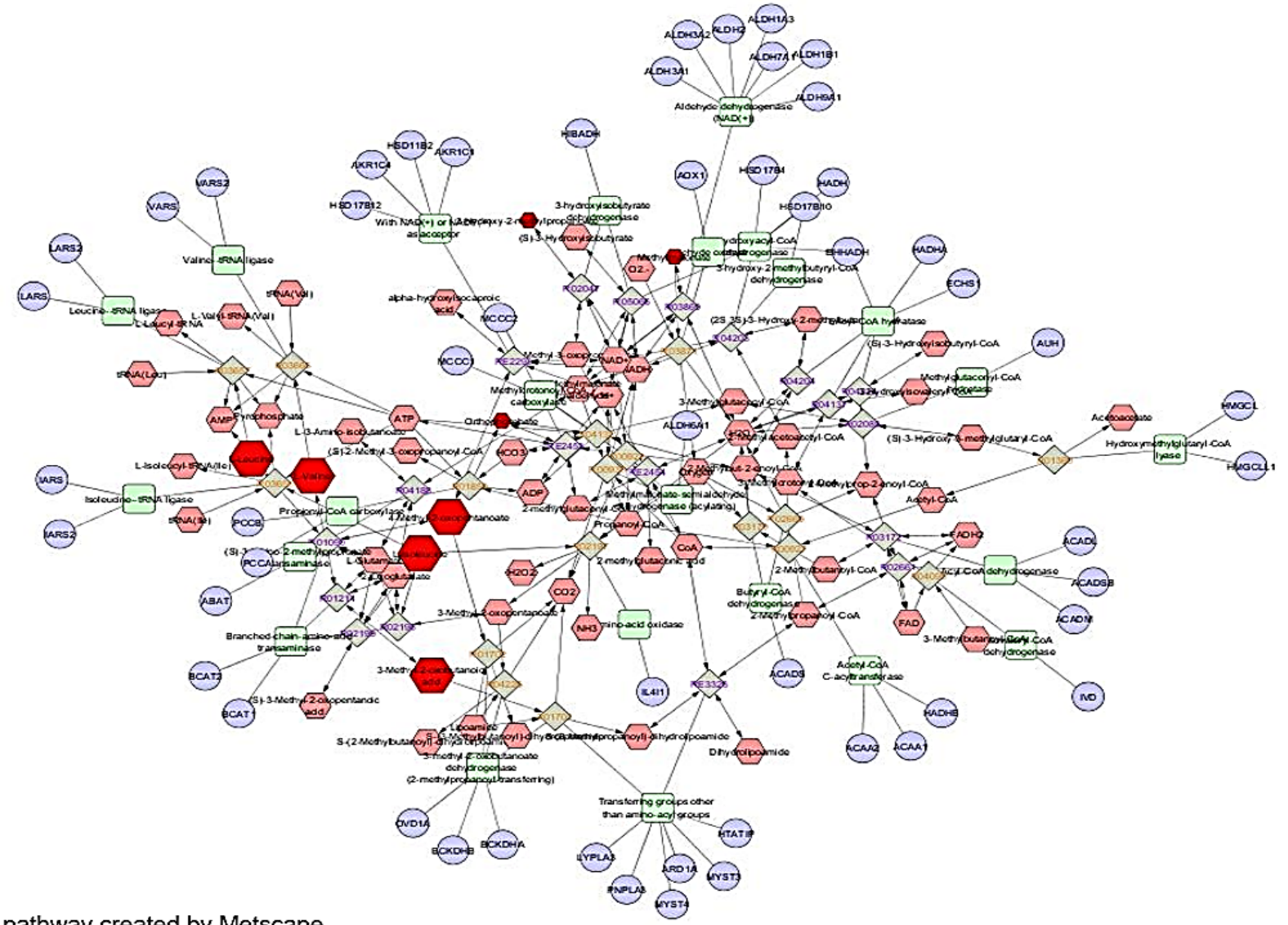


Figure 5.3 *Significant (P < 0.05) metabolite differences at 0 minutes and 75 minutes during an oral sugar test are displayed for the lipid, amino acid, carbohydrate, cofactor and vitamin, energy, nucleotide, peptide, and xenobiotic pathways.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means). For each metabolite, the least squares means estimate for baseline is set to 0 (vertical line). Positive LS means indicate increases in metabolite abundance following administration of Karo® light corn syrup relative to baseline, whereas negative LS means indicate decreases in metabolite abundance following administration of Karo® light corn syrup relative to baseline. All data are represented on a log scale.

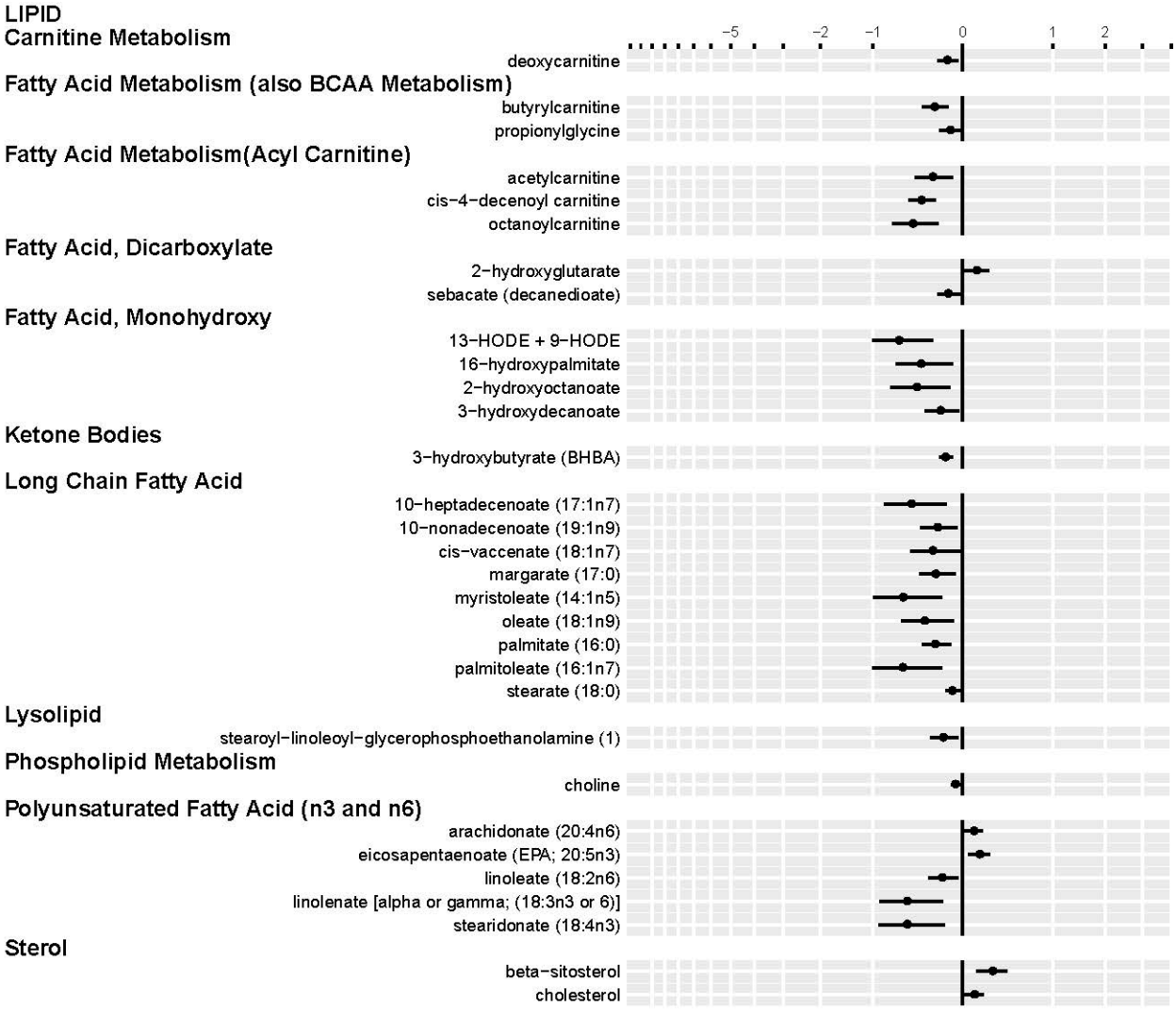


Figure 5.3 (cont'd)

Figure 5.3 (cont'd)

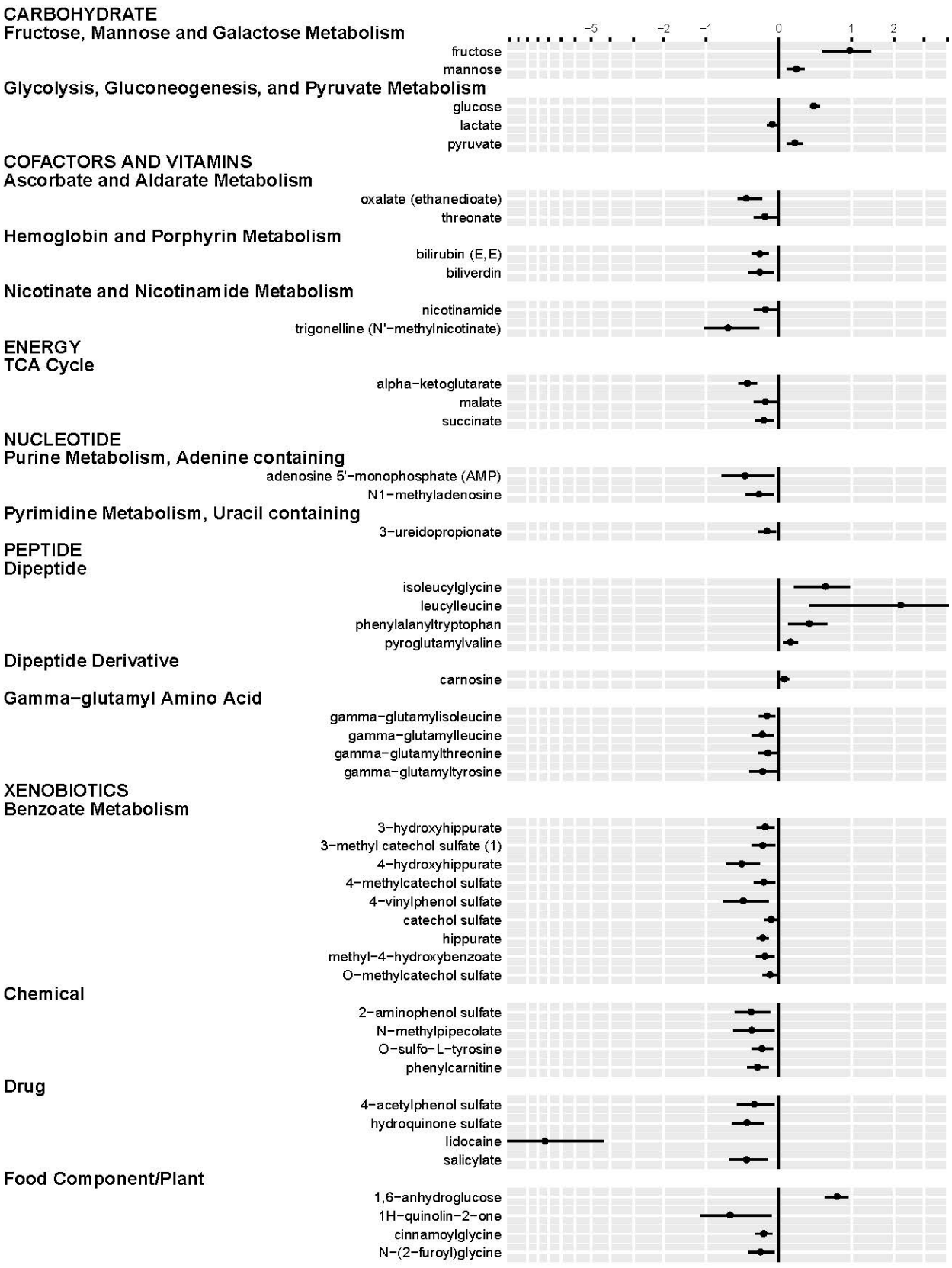


Figure 5.4 *Significant (P < 0.05) metabolite differences in the lipid pathway for ponies with insulin dysregulation compared to non-insulin dysregulated ponies at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for insulin dysregulated ponies. For each metabolite, the least squares means estimate for non-insulin dysregulated ponies is set to 0 (vertical line). Positive LS means in insulin dysregulated ponies indicate increases in metabolite abundance relative to controls, whereas negative LS means in insulin dysregulated ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

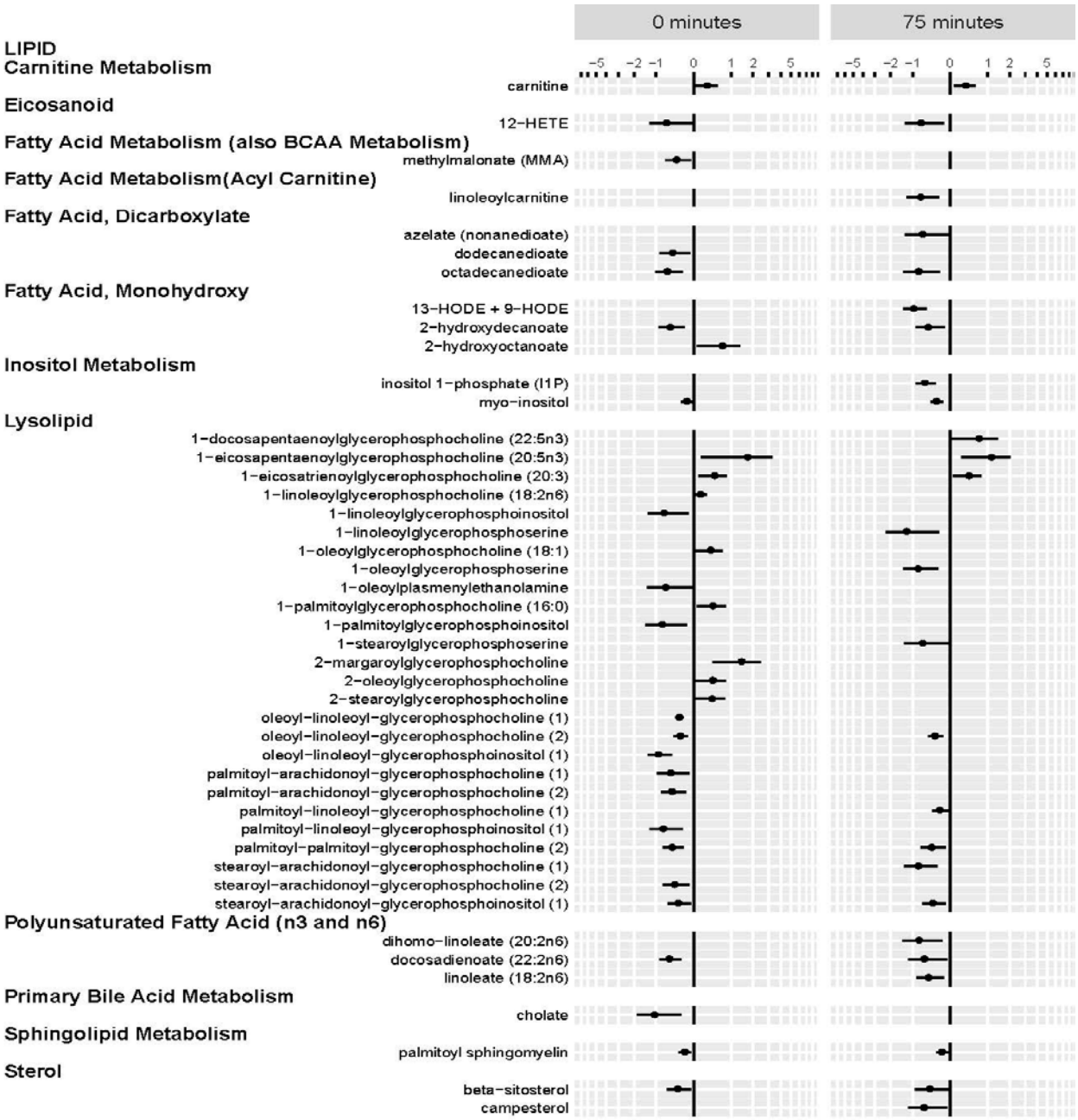


Figure 5.5 *Significant (P < 0.05) metabolite differences in the amino acid pathway for ponies with insulin dysregulation compared to non-insulin dysregulated ponies at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for insulin dysregulated ponies. For each metabolite, the least squares means estimate for non-insulin dysregulated ponies is set to 0 (vertical line). Positive LS means in insulin dysregulated ponies indicate increases in metabolite abundance relative to controls, whereas negative LS means in insulin dysregulated ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

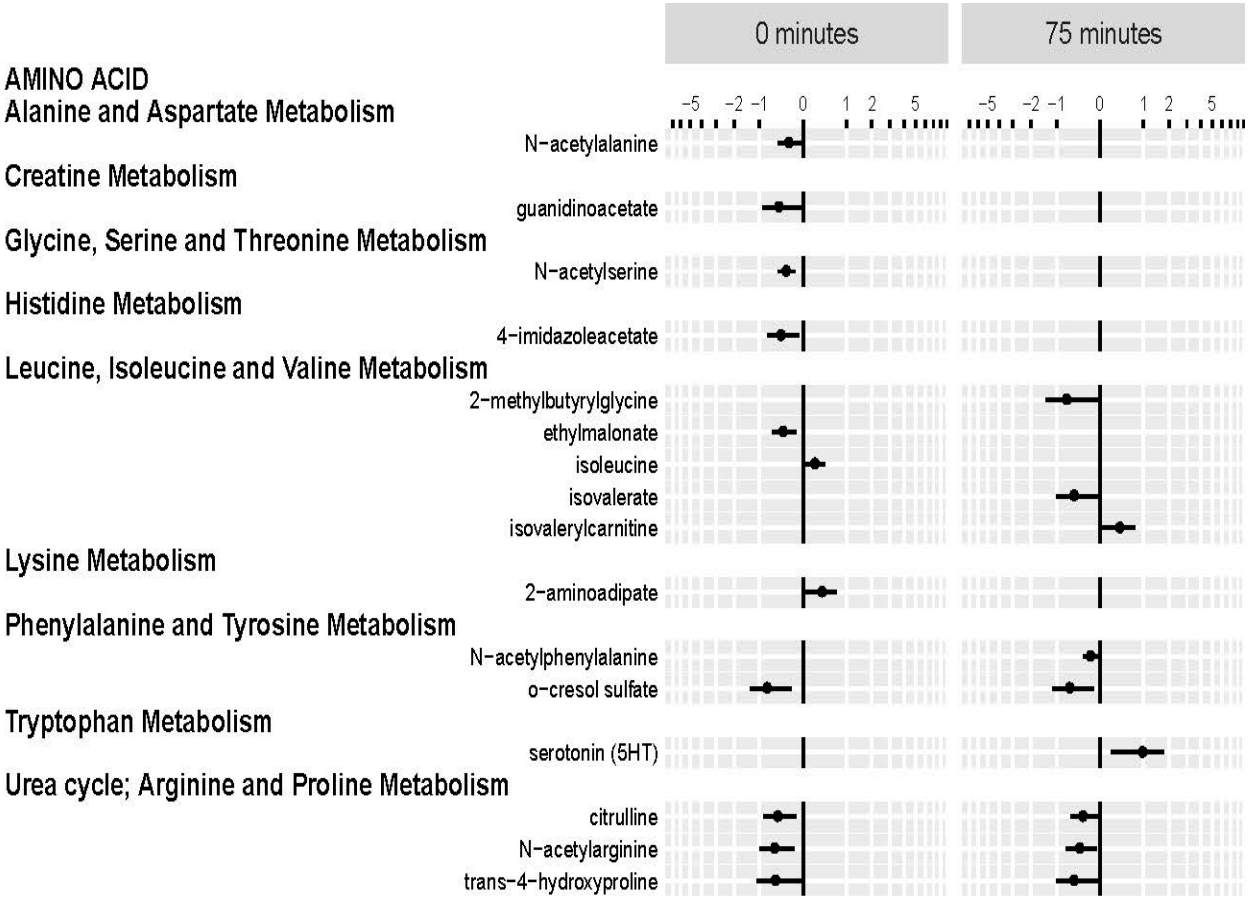


Figure 5.6 *Significant (P < 0.05) metabolite differences in the carbohydrate, cofactor and vitamin, energy, nucleotide, and xenobiotic pathways for ponies with insulin dysregulation compared to non-insulin dysregulated ponies at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for insulin dysregulated ponies. For each metabolite, the least squares means estimate for non-insulin dysregulated ponies is set to 0 (vertical line). Positive LS means in insulin dysregulated ponies indicate increases in metabolite abundance relative to controls, whereas negative LS means in insulin dysregulated ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

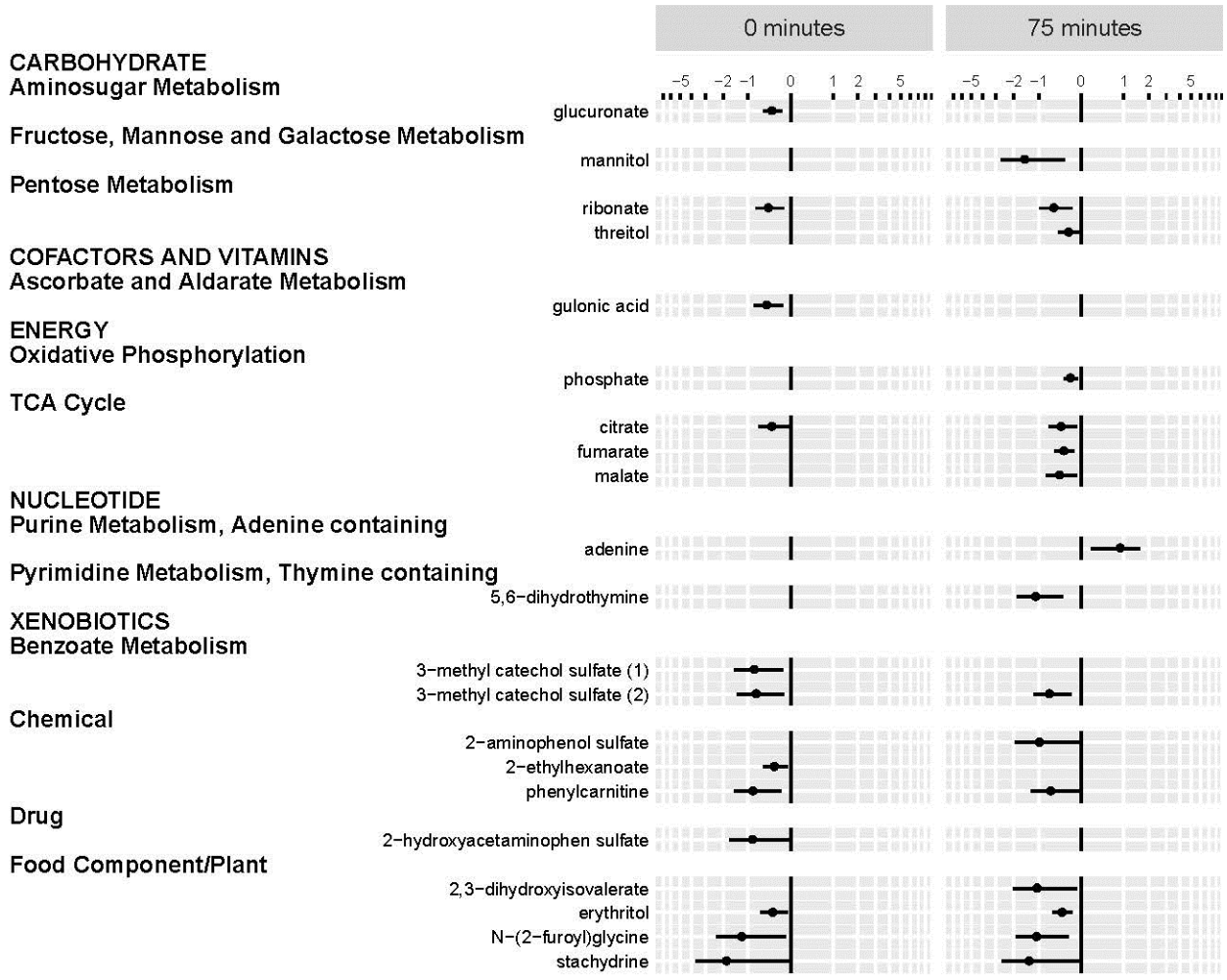


Figure 5.7 *Significant (P < 0.05) metabolite differences in the lipid pathway for obese compared to non-obese ponies at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for obese ponies. For each metabolite, the least squares means estimate for non-obese ponies is set to 0 (vertical line). Positive LS means in obese ponies indicate increases in metabolite abundance relative to controls, whereas negative LS means in obese ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

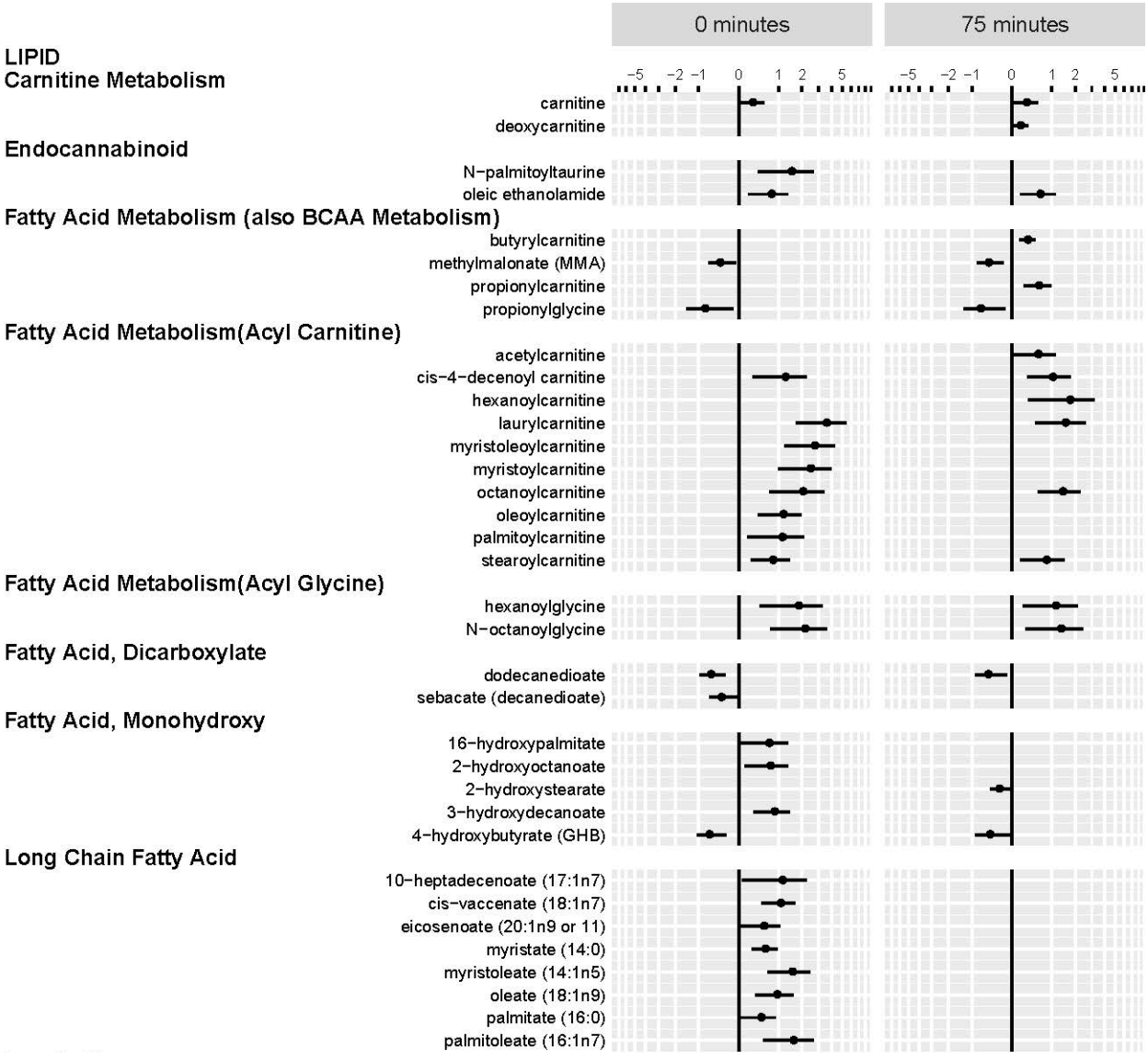


Figure 5.7 (cont'd)

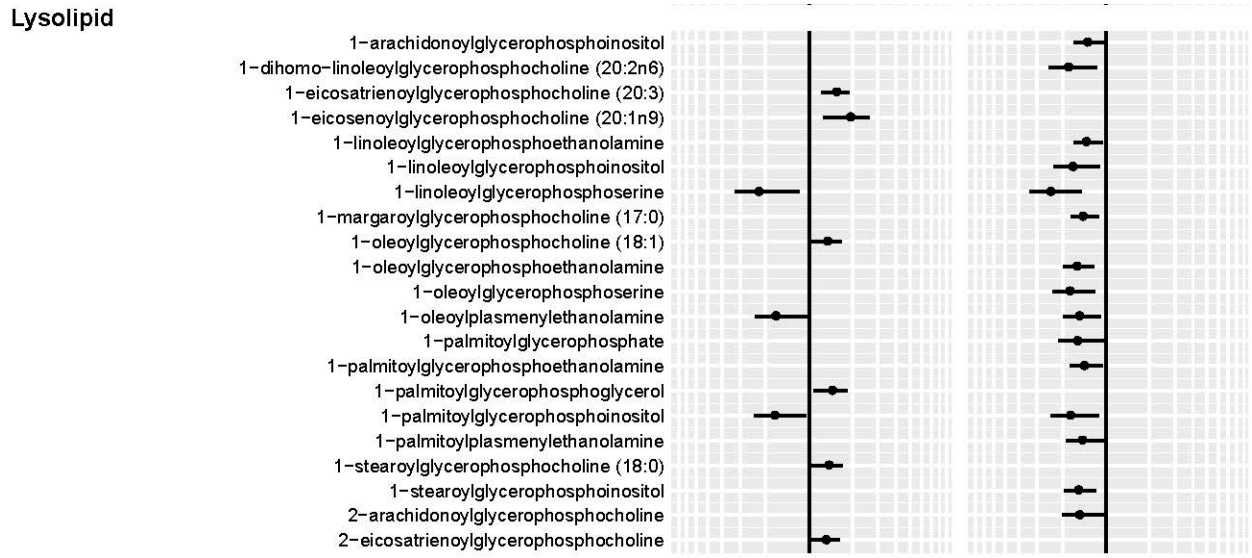


Figure 5.7 (cont'd)

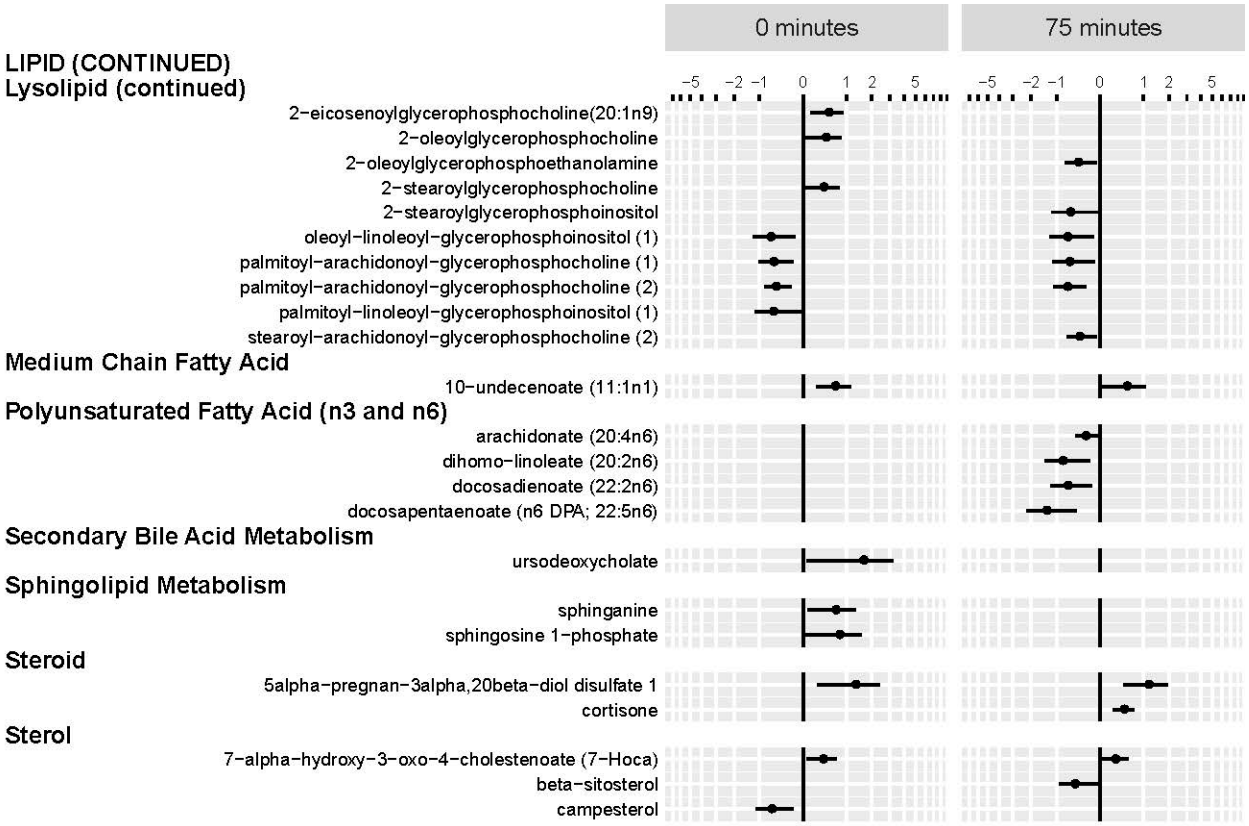


Figure 5.8 *Significant (P < 0.05) metabolites differences in the amino acid pathway for obese compared to non-obese ponies at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for obese ponies. For each metabolite, the least squares means estimate for non-obese ponies is set to 0 (vertical line). Positive LS means in obese ponies indicate increases in metabolite abundance relative to controls, whereas negative LS means in obese ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

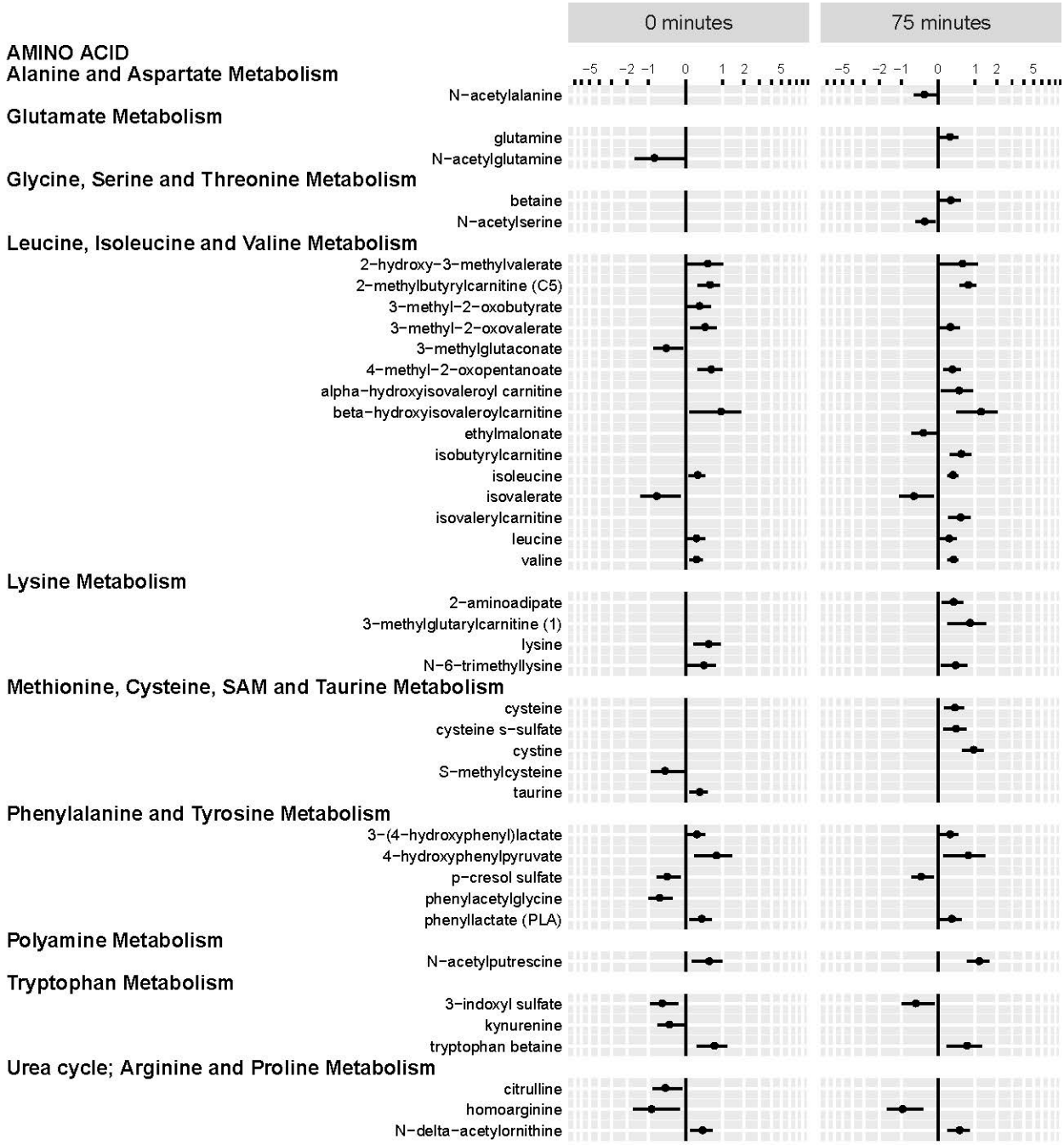


Figure 5.9 *Significant (P < 0.05) metabolite differences in carbohydrate, cofactor and vitamin, nucleotide, peptide, and xenobiotic pathways for obese compared to non-obese ponies at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for obese ponies. For each metabolite, the least squares means estimate for non-obese ponies is set to 0 (vertical line). Positive LS means in obese indicate increases in metabolite abundance relative to controls, whereas negative LS means in obese ponies indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

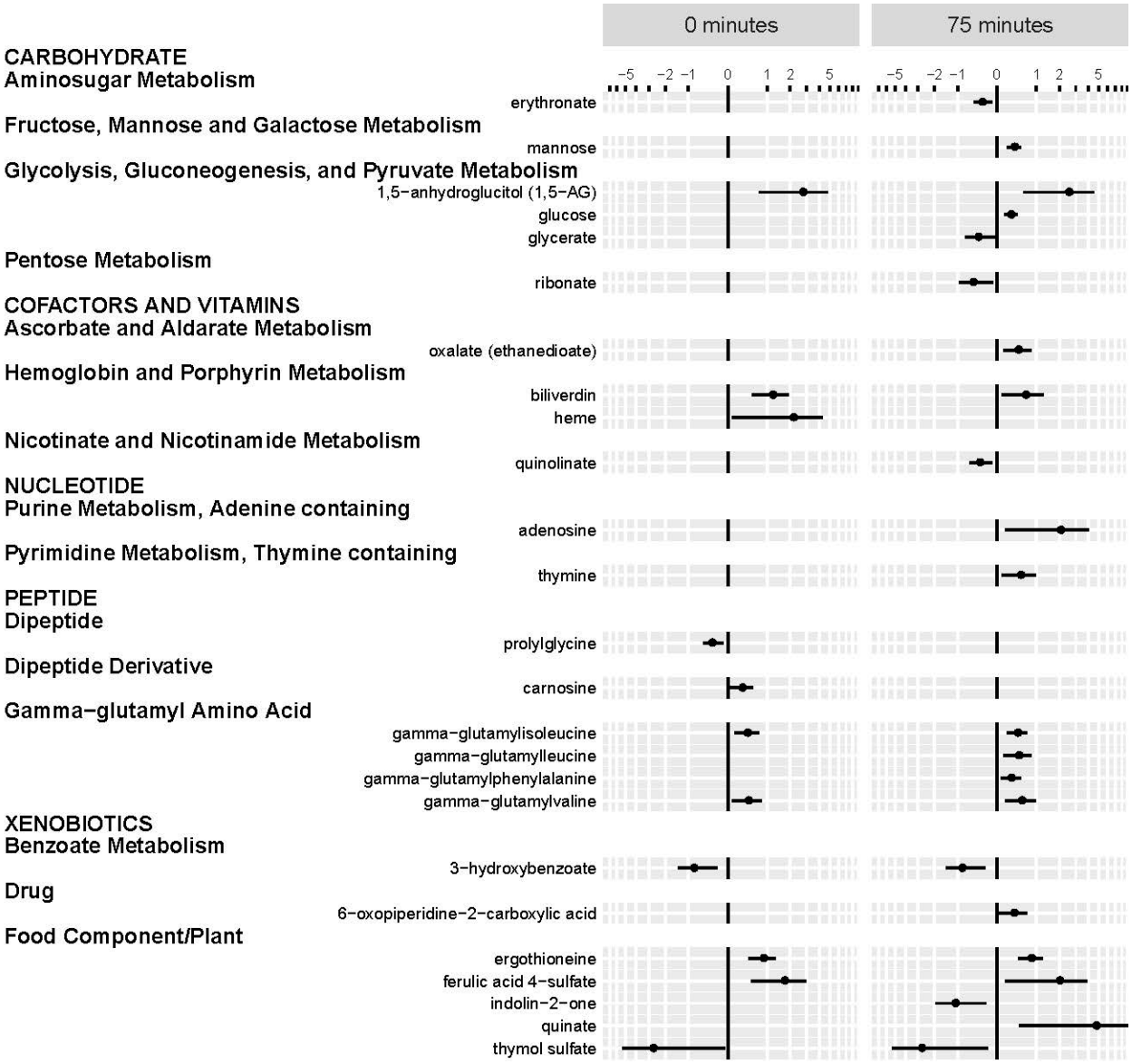


Figure 5.10 *Significant (P < 0.05) metabolites differences in the lipid pathway in ponies with a history of laminitis compared to ponies without a history of laminitis at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for ponies with a history of laminitis. For each metabolite, the least squares means estimate for ponies without a history of laminitis is set to 0 (vertical line). Positive LS means in ponies with a history of laminitis indicate increases in metabolite abundance relative to controls, whereas negative LS means in ponies with a history of laminitis indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

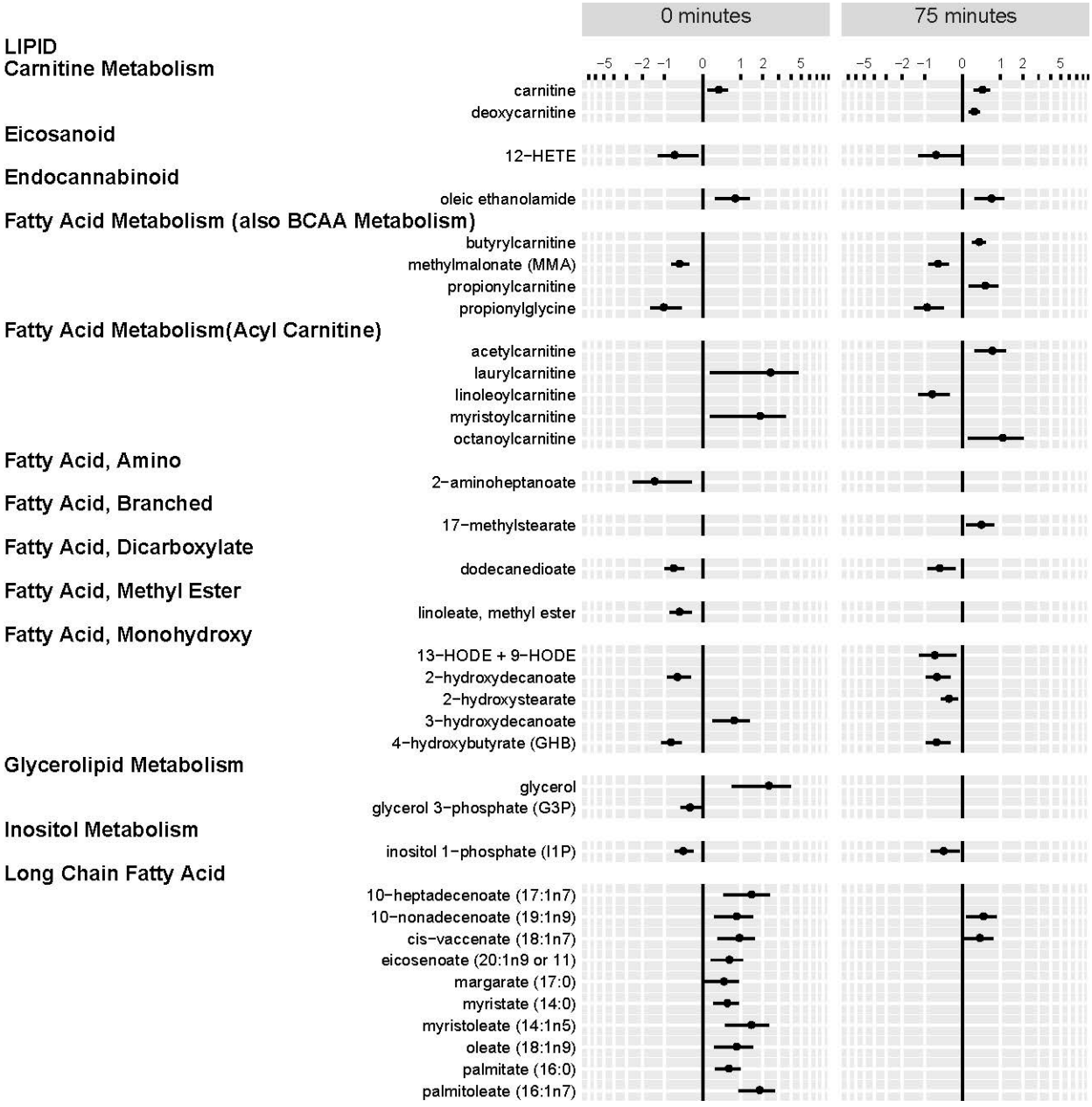


Figure 5.10 (cont'd)

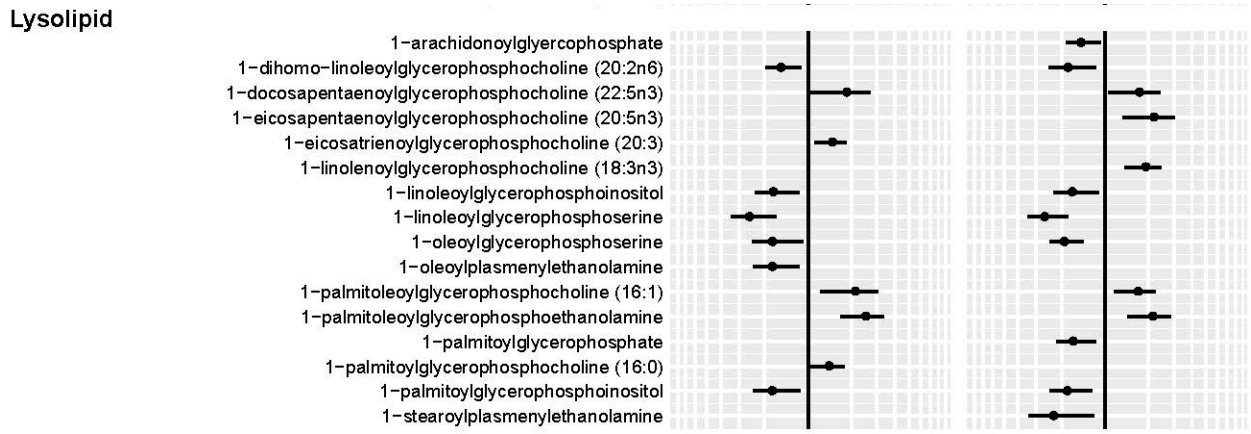


Figure 5.10 (cont'd)

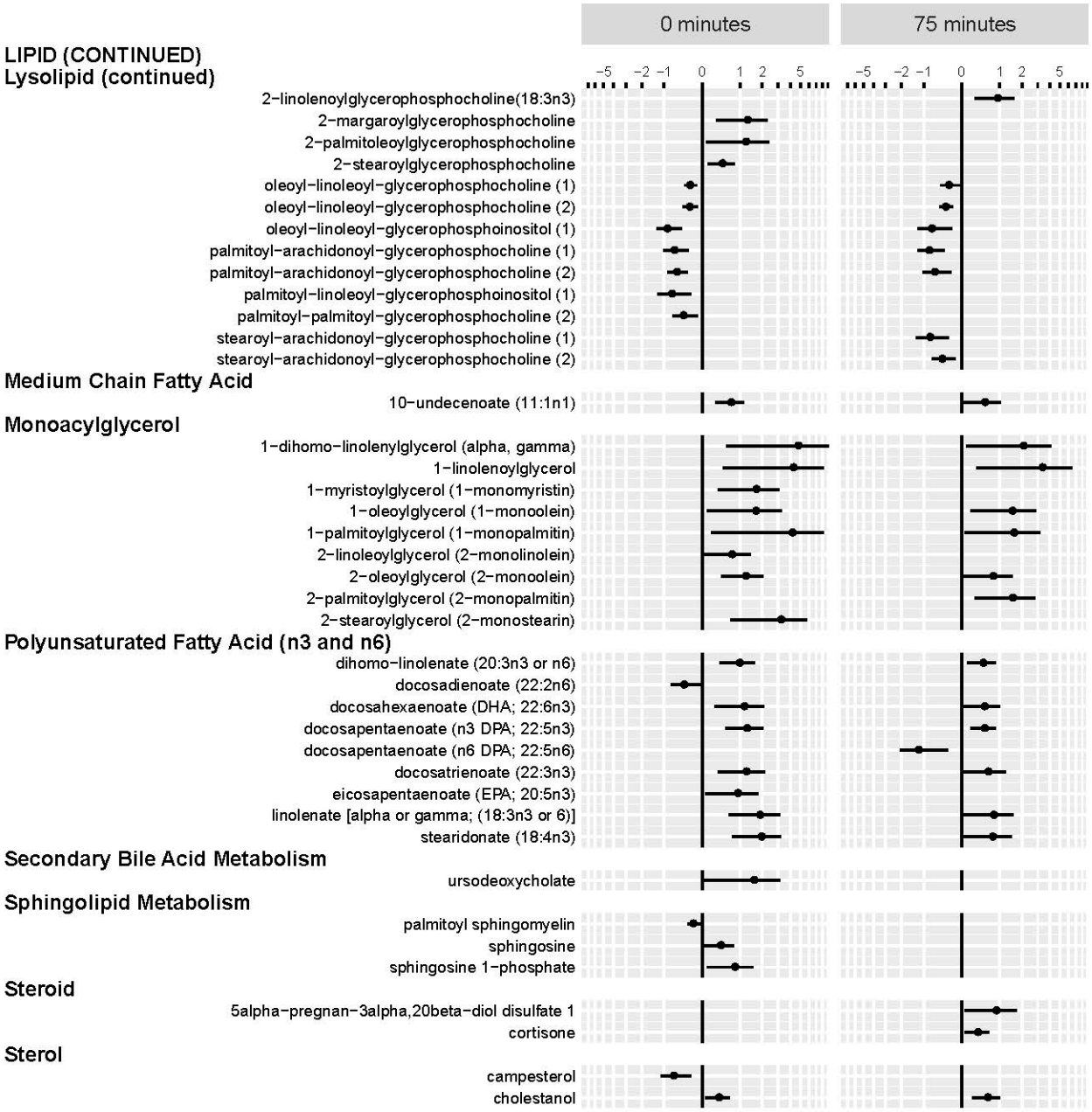


Figure 5.11 *Significant (P < 0.05) metabolite differences in the amino acid pathway in ponies with a history of laminitis compared to ponies without a history of laminitis at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for ponies with a history of laminitis. For each metabolite, the least squares means estimate for ponies without a history of laminitis is set to 0 (vertical line). Positive LS means in ponies with a history of laminitis indicate increases in metabolite abundance relative to controls, whereas negative LS means in ponies with a history of laminitis indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

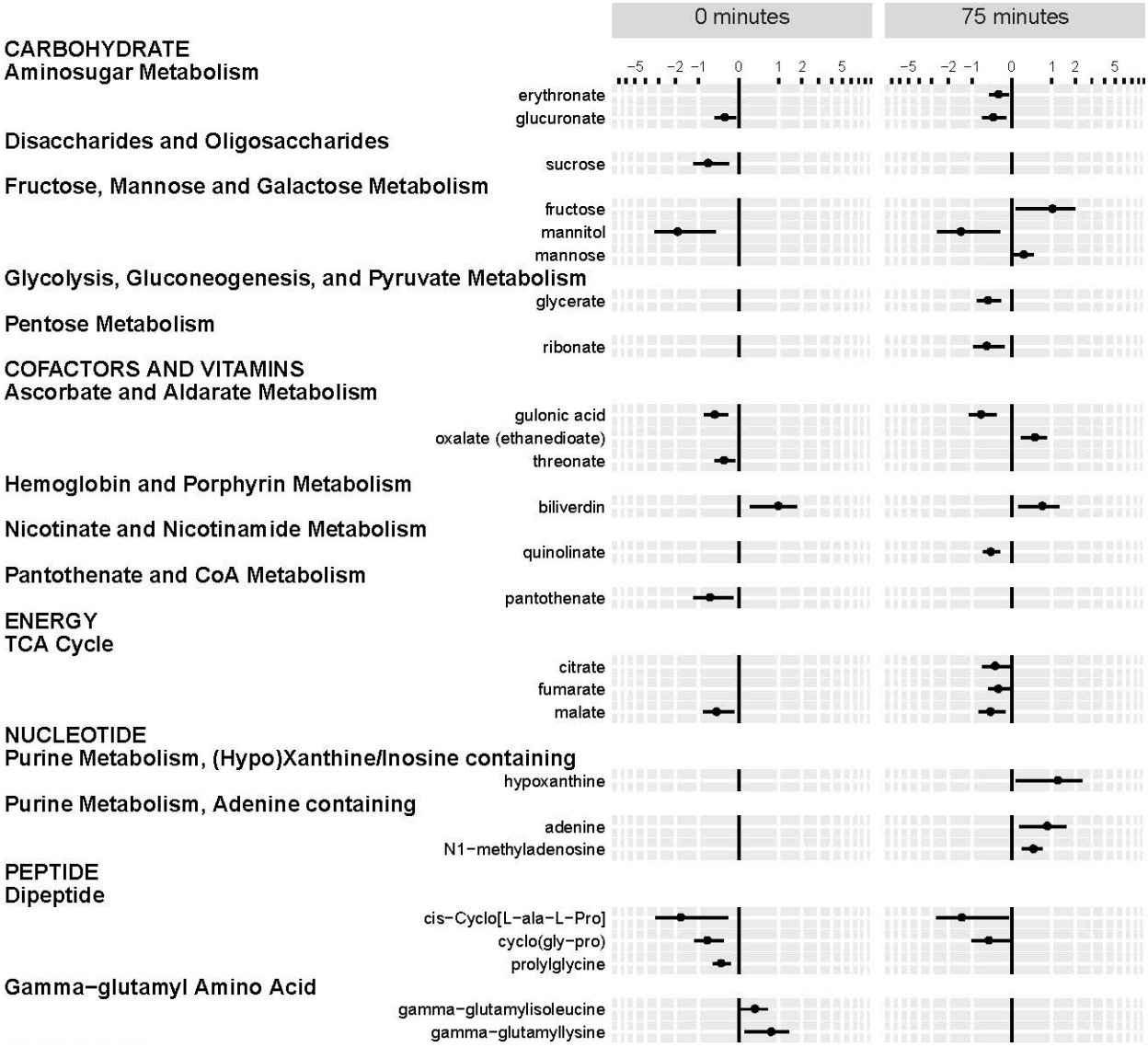


Figure 5.11 (cont'd)

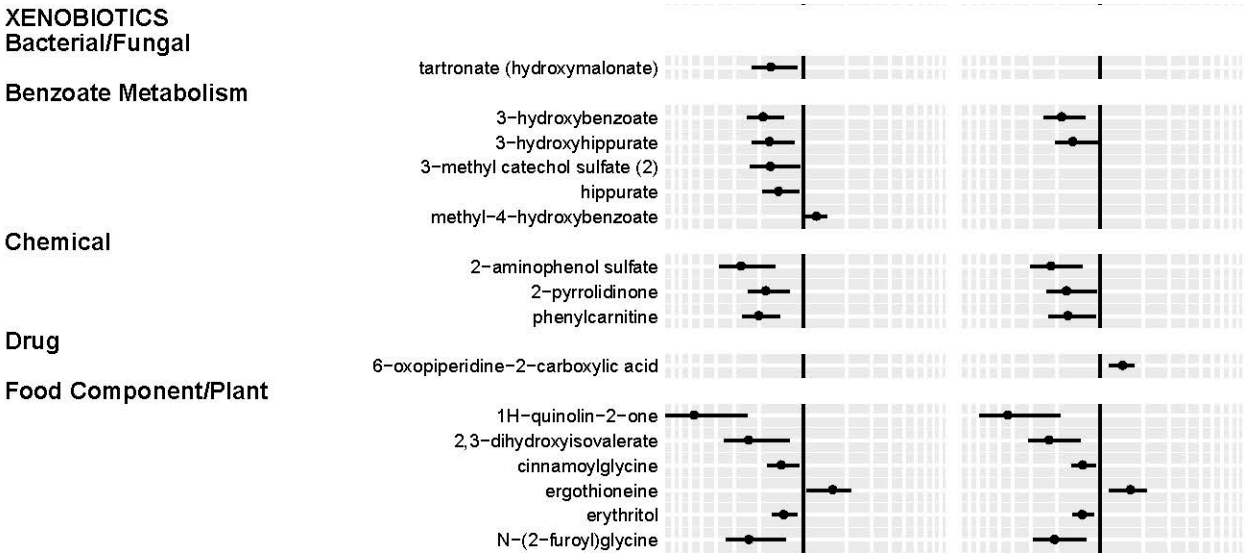


Figure 5.12 *Significant (P < 0.05) metabolite differences in carbohydrate, cofactor and vitamin, energy, nucleotide, peptide, and xenobiotic pathways in ponies with a history of laminitis compared to ponies without a history of laminitis at 0 minutes and 75 minutes.* Filled circles (●) represent the least squares means estimates and horizontal lines (—) represent the confidence interval around the least squares means (LS means) for ponies with a history of laminitis. For each metabolite, the least squares means estimate for ponies without a history of laminitis is set to 0 (vertical line). Positive LS means in ponies with a history of laminitis indicate increases in metabolite abundance relative to controls, whereas negative LS means in ponies with a history of laminitis indicate decreases in metabolite abundance relative to controls. All data are represented on a log scale.

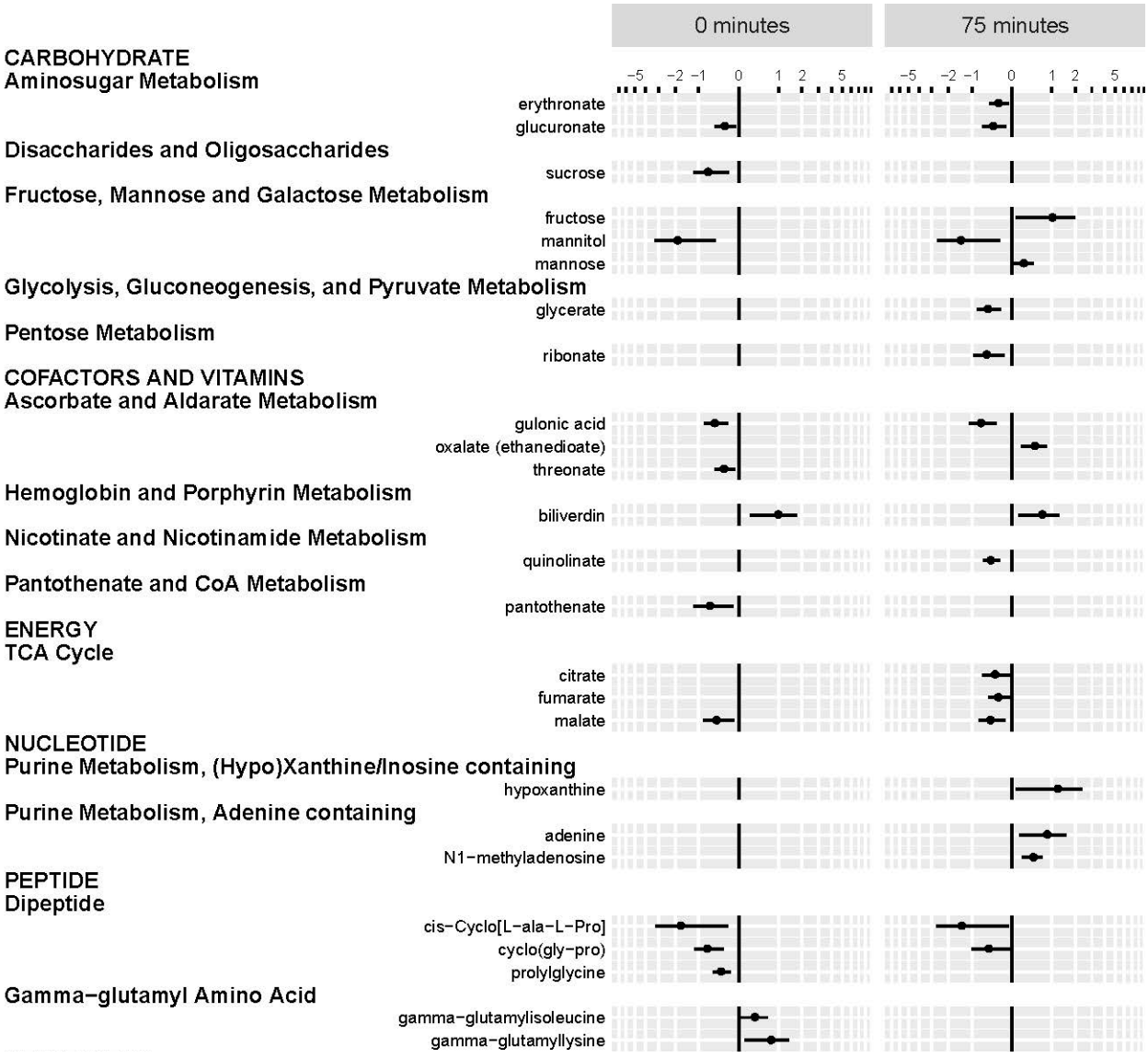


Figure 5.12 (cont'd)

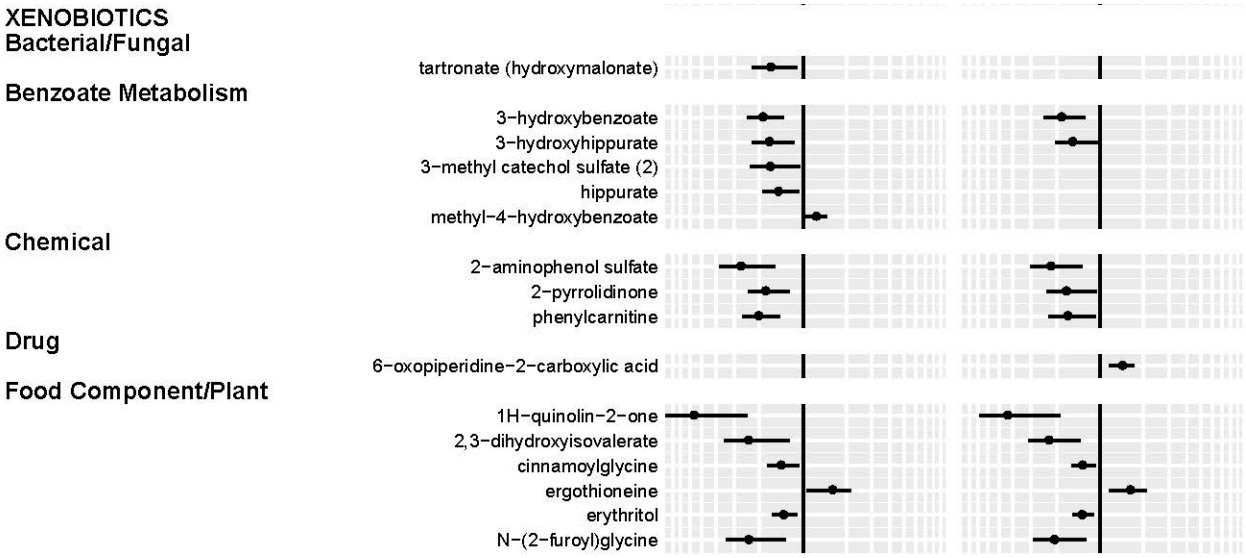


Figure 5.13 *The relationship between different phenotypes (insulin response, obesity status, laminitis history) and significant metabolites.*

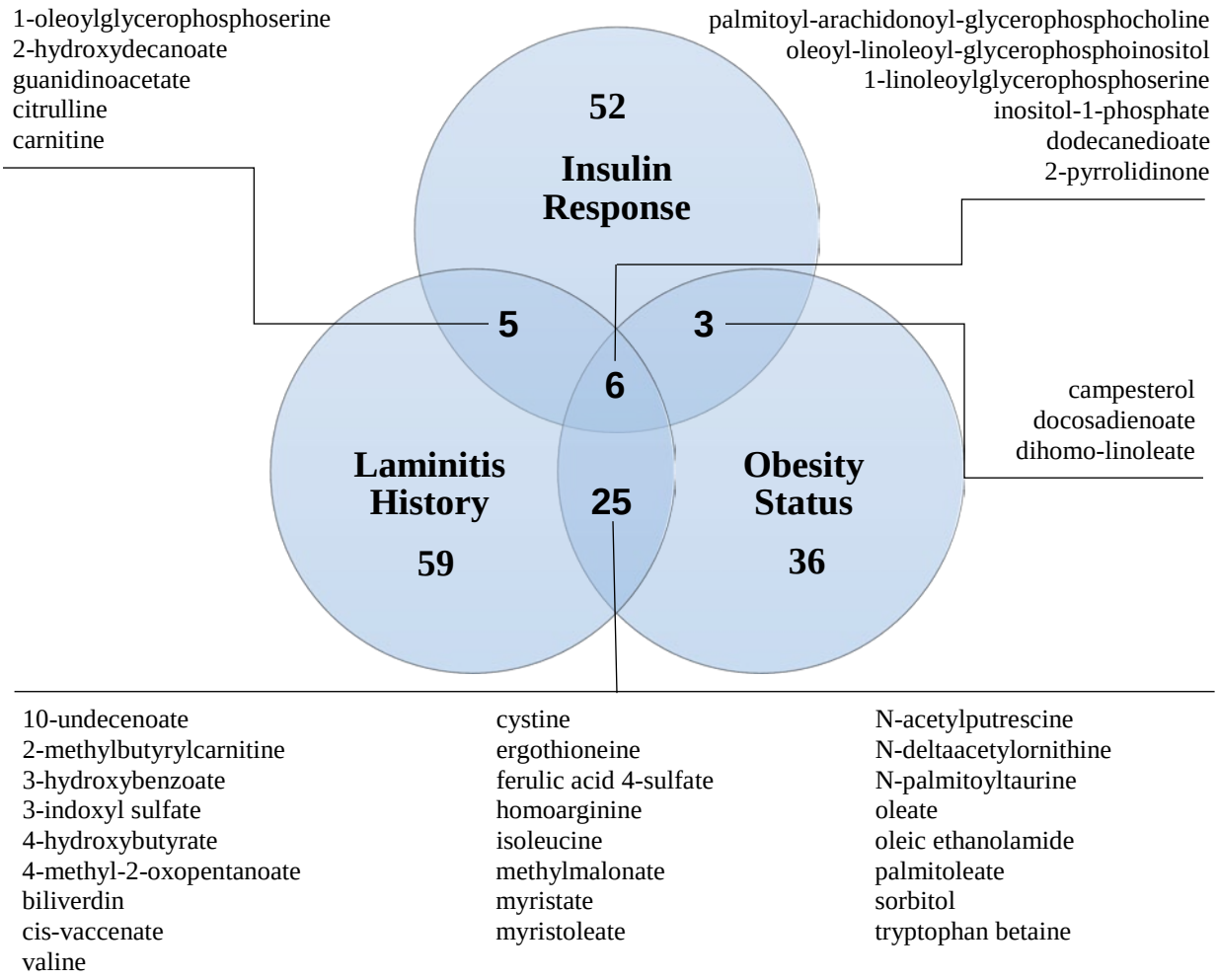


Figure 5.14a *Principal components analysis (PCA) plot of metabolic profiles for the insulin dysregulated (○) and non-insulin dysregulated (●) phenotype.*

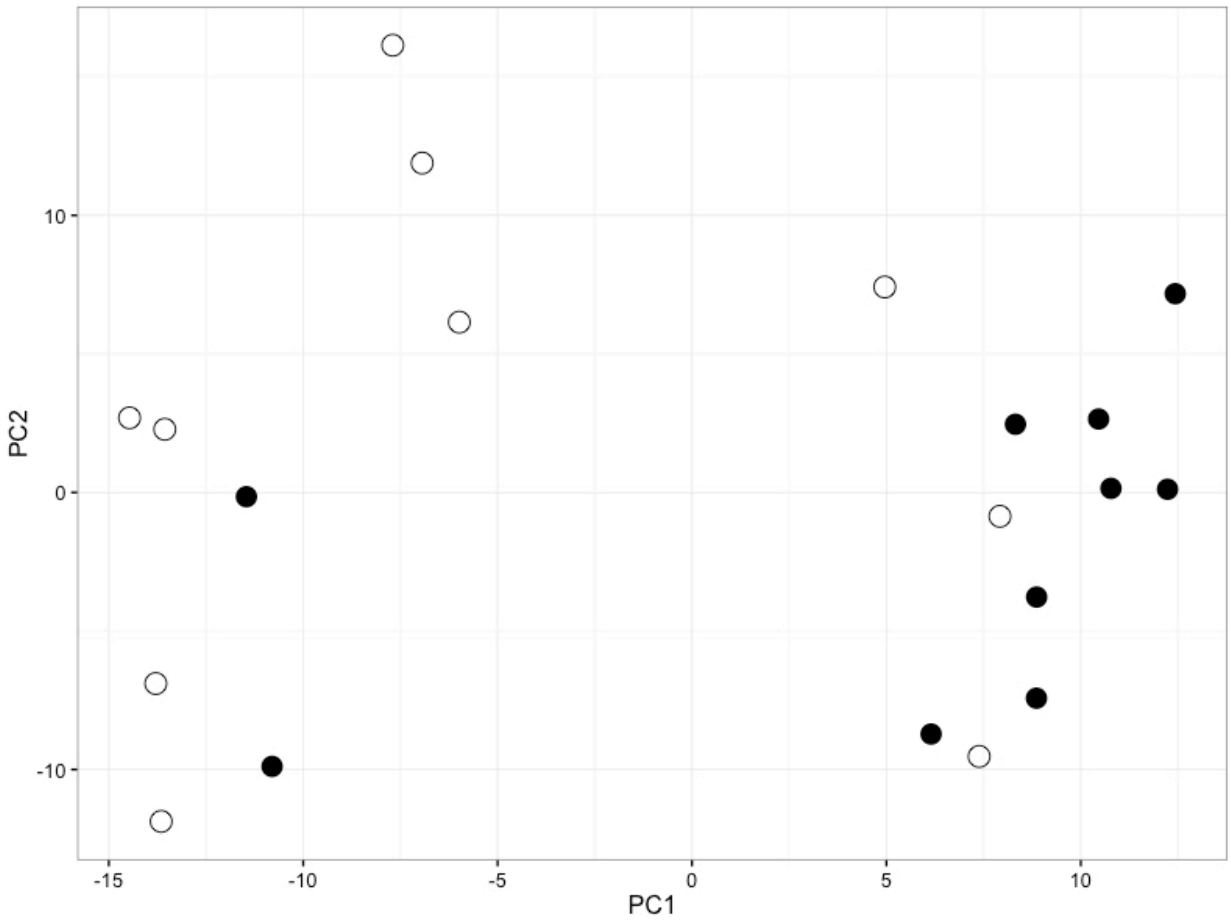


Figure 5.14b *Principal components analysis (PCA) plot of metabolic profiles for the obese (Δ) and non-obese (▲) phenotype.*

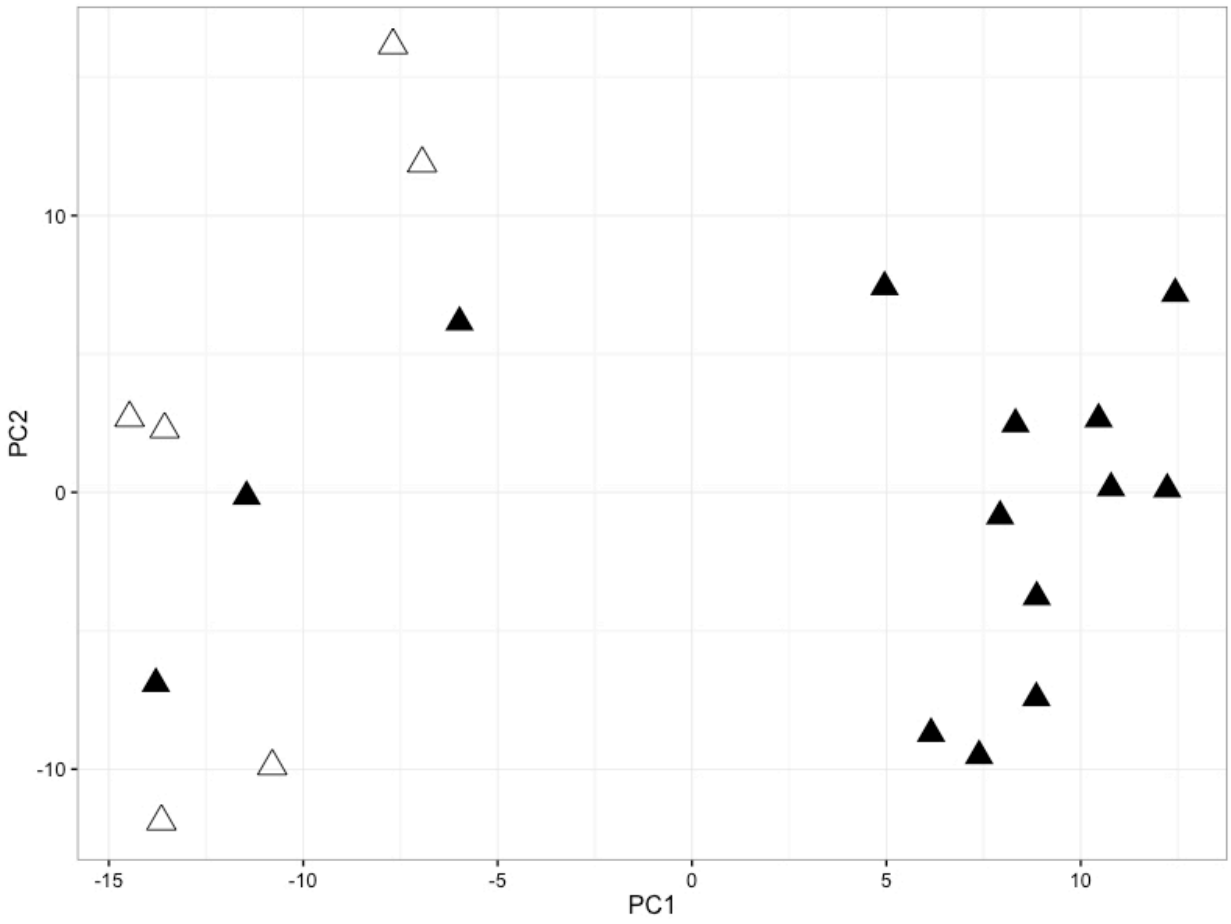
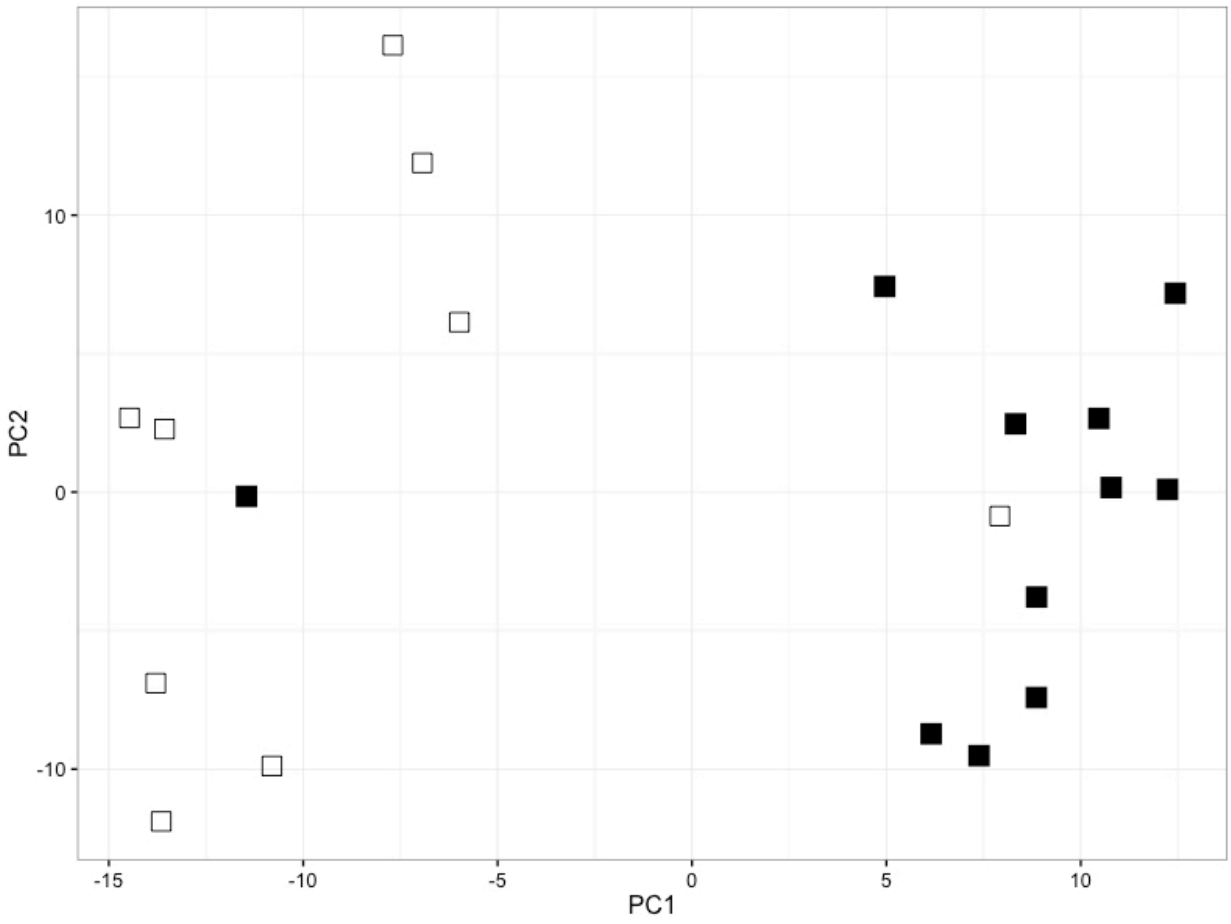


Figure 5.14c *Principal components analysis (PCA) plot of metabolic profiles for the history of laminitis (□) and no history of laminitis (■) phenotype.*



CHAPTER 6

Conclusions and Future Directions

CONCLUSIONS

Dietary adaptation to various carbohydrate profiles altered glucose and insulin dynamics in adult and aged horses; however, the response is variable depending on assessment. The insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT) showed that adult and aged horses had improved tissue insulin sensitivity (SI) following adaptation to the starch-rich and sugar-rich diet. However, a modified oral sugar test (OST) did not reveal significant changes in glucose and insulin dynamics. In contrast, the dietary meal challenge demonstrated enhanced postprandial hyperinsulinemia (AUCi) in both adult and aged horses following consumption of a single starch-rich or sugar-rich meal. These data would suggest that feeding a starch-rich or sugar-rich diet may be beneficial; however, enhanced postprandial hyperinsulinemia cannot be ignored given its causal role in the induction of laminitis. Further, it is important to note that the dynamic challenge tests were performed following adaptation to the respective diets over seven weeks and consumption of these diets long-term may yield different results.

Similar to previously reported studies, glucose and insulin dynamics vary between adult and aged horses. The insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT) showed that aged horses had a higher acute insulin response to glucose (AIRg) compared to adult horses. Further, a modified oral sugar test (OST) demonstrated that aged horses had higher peak insulin and area-under-the-curve insulin (AUCi). These data suggest that aged horses have higher insulin secretory responses; however, it is unknown whether this occurs due to increased uptake or decreased clearance.

Glucose and insulin dynamics also vary between breeds. The insulin-modified frequently sampled intravenous glucose tolerance test (FSIGTT) showed that Thoroughbreds had a higher basal (fasting) insulin compared to Standardbreds. Further, a modified oral sugar test (OST) demonstrated that Thoroughbreds had a higher area-under-the-curve insulin (AUCi) and area-under-the-curve glucose (AUCg). While differences in glucose and insulin dynamics have been previously reported between typically insulin-

sensitive breeds and relatively insulin-resistant breeds; these data suggest that insulin-sensitive breeds have important differences in glucose and insulin dynamics.

In addition to effects on insulin and glucose, dietary adaptation likely influences ACTH and cortisol concentrations in adult and aged horses. Aged horses had significantly higher baseline ACTH concentrations following adaptation to the starch-rich diet. However, ACTH concentrations following a TRH stimulation test were not affected by dietary adaptation. Further, baseline cortisol concentrations were not influenced by dietary adaption, but post-dexamethasone cortisol concentrations were significantly higher after adaptation to the starch-rich diet. These data suggest that diet is a potential confounder on ACTH and cortisol concentrations and should be considered when interpreting endocrine results.

Age influences ACTH and cortisol concentrations. The TRH stimulation test showed that aged horses had significantly higher baseline ACTH concentrations, but no statistical difference between age groups was appreciated for post-TRH ACTH concentrations. In addition, the overnight dexamethasone suppression test did not yield statistical differences between adult and aged horses for baseline cortisol concentrations or post-dexamethasone cortisol concentrations.

Time of year also influences ACTH concentrations and cortisol concentrations. Baseline and post-TRH ACTH concentrations were significantly higher in October. Post-dexamethasone cortisol was significantly higher in October compared to March, May, and August.

Previous studies have shown that ACTH and cortisol concentrations are influenced by age and time of year [121,134–136,138]; however, to the author's knowledge the finding that diet influences these endocrine parameters is novel information. A possible explanation for this finding may be the role of gastrointestinal microbes in the gut-brain communication pathway as studies in mice indicate that alterations in the gastrointestinal microbiome can affect the regulation of neuroendocrine hormones of the hypothalamic-pituitary-adrenocortical (HPA) axis [147,149–152,195]. Ideally, while none of the horses showed clinical signs (hypertrichosis, regional adiposity, skeletal muscle atrophy, lethargy) of pituitary pars intermedia dysfunction, a post-mortem examination would have been performed to evaluate the gross and histologic appearance of the pituitary gland.

Evaluation of the plasma metabolome between adult and aged horses and adaptation to dietary carbohydrate profiles provided initial information regarding molecular and cellular changes. Untargeted metabolomics provided an extensive and qualitative analysis as thousands of significant metabolite ion peaks were identified; however, a number of these peaks remain unknown. Examination of the plasma metabolome demonstrated significant differences in metabolites primarily derived from amino acids, lipids, and xenobiotics, and initial results show promise as aged horses show some differences in metabolites that mirror differences in aged humans. However, these results may change when additional metabolites are identified. Despite the limitations, the data to date suggest that metabolomics is a relevant approach for defining metabolic changes due to age and diet.

Evaluation of the serum metabolome between non-insulin dysregulated and insulin dysregulated ponies, obese and non-obese ponies, and ponies with and without a history of laminitis provided further evidence that metabolomic profiling is useful for further defining cellular and molecular physiology and pathophysiology. Comparison of ponies with and without insulin dysregulation primarily identified differences in lysolipids, TCA cycle intermediates, and urea cycle metabolites. Several glycerophosphocholines (oleoyl-linoleoyl-glycerophosphocholine) and TCA cycle intermediates (citrate, malate, fumarate) were decreased in insulin dysregulated ponies. Many of these findings are similar to humans with insulin resistance and type-II diabetes mellitus. Comparison of non-obese and obese ponies primarily identified differences in concentrations of long-chain fatty acids, acylcarnitines, and branched-chain amino acids (isoleucine, leucine, valine). These metabolites were increased in obese ponies. Metabolomic analysis showed similarities and differences between phenotypes with many of the metabolites derived from fatty acid metabolism and amino acid metabolism. The results presented here should be confirmed in a large cohort of animals that will allow for metabolite differences due to pathologic factors such as insulin dysregulation and obesity and physiologic factors such as age, gender, and breed to be differentiated. These data suggest that metabolomics is a relevant approach for understanding complex diseases that span multiple tissues by providing additional quantitative biologic information that may help decipher disease mechanisms and identify potentially useful disease biomarkers.

FUTURE DIRECTIONS

These studies provide evidence that the application of metabolomics to enable comprehensive metabolic profiling of horse serum and plasma samples is a relevant approach to gain deeper insight into metabolic adaptations and perturbations associated with dietary carbohydrate profiles, aging, and insulin dysregulation in horses. Further, the development of an equine-specific metabolite spectral library will allow for additional identification of biomarkers and development of a metabolomic signature for equine metabolic diseases that may eventually lead to early detection of affected animals.

Formulation of a nutrition plan for metabolically abnormal horses remains a challenge as there is a disconnect between tissue insulin sensitivity and postprandial insulin responses. Current dietary recommendations for horses at-risk for endocrinopathic laminitis include limiting dietary nonstructural carbohydrates (NSC) thereby reducing postprandial glucose and insulin responses because hyperinsulinemia has been shown to induce laminitis. The research presented in this dissertation demonstrated that a starch-rich diet and a sugar-rich diet fed for seven weeks *improved* tissue insulin sensitivity in non-obese horses. In a previous study, a sugar-rich diet fed for twenty weeks resulted in *improved* tissue insulin sensitivity; however, a starch-rich diet fed for twenty weeks resulted in tissue insulin resistance and induced obesity [128]. In both studies, despite changes in *tissue* insulin sensitivity, sugar-rich and starch-rich diets resulted in *greater* postprandial insulin concentrations compared to low nonstructural carbohydrate diets. These studies highlight the disconnect between the tissue insulin sensitivity/resistance and postprandial insulin responses; the two components of insulin dysregulation. Further, these findings complicate dietary recommendations for insulin dysregulation in horses -- improved tissue insulin sensitivity would be beneficial, whereas postprandial hyperinsulinemia would be detrimental. Currently, the mechanisms underlying improvements in tissue insulin sensitivity with sugar-rich diets are unknown, and it is unclear why starch-rich diets fed for different lengths of time had opposite effects on tissue insulin sensitivity. Understanding the mechanisms underlying the changes in tissue insulin sensitivity is key to understanding the consequences of different dietary components and to making appropriate dietary recommendations for horses with insulin dysregulation.

Changes in glucose and insulin dynamics due to dietary adaptation are likely in part due to alterations in the gut microbiome. The complex microbial population of the equine intestinal tract also plays a key role in health and disease, and the composition and complexity of this population are starting to be revealed. The availability of next-generation sequencing and bioinformatics methods offers an opportunity to improve understanding of the role of the gastrointestinal microbiome in the pathogenesis of metabolic disease and insulin dysregulation.

The emerging clinical importance of insulin dysregulation in horses justifies studies that advance understanding of underlying pathophysiology; knowledge that will lead to improved methods for identification of at-risk individuals before the onset of disease and will potentially identify new therapeutic targets. Metabolites identified in the cohort of Welsh Ponies provide new insight into the pathophysiology of insulin dysregulation. Additionally, dietary alterations in tissue metabolism measured through alterations in plasma metabolites provide information regarding the impact of dietary change at the molecular level. The ability to characterize the effects of diet and time on the gastrointestinal (fecal) microbiome and correlate the microbial changes with the metabolomic profile to determine the relative effects of diet and time on glucose and insulin dynamics are integral to the long-term goals to understand the mechanisms associated with metabolic adaptations and perturbations in horses. Advanced understanding of the physiology of insulin dynamics, the pathophysiology of insulin dysregulation, and the underlying molecular changes as it relates to tissue insulin sensitivity, gastrointestinal microbiome, and dietary adaptation will greatly advance our knowledge in moving towards additional opportunities for disease prevention through dietary manipulation and/or management as well as identification of reliable clinical diagnostic tests and potential pathways for therapeutic intervention.

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