Physiological response of grazing horses to seasonal fluctuations in pasture nonstructural carbohydrates

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Abstract

Forage is an essential part of the equine diet for health and performance. Pasture nonstructural carbohydrates (NSC) have been shown to fluctuate diurnally and seasonally throughout the year due to various factors including environmental conditions and plant stress. The intake of elevated NSC content is linked with metabolic and digestive diseases, including colic and laminitis. A yearlong grazing study was conducted at the Virginia Tech Middleburg Agricultural Research and Extension Center from October 2016 through September 2017 to investigate fluctuations in pasture NSC concentrations as well as the metabolic and digestive response of grazing horses. Twelve sporthorse mares (15 ± 3.4 yrs) were maintained on an 8.5-ha cool season mixed grass pasture with water, mineral (Buckeye Nutrition, Dalton, OH), and white salt *ad libitum*. Weekly pasture samples (200 g wet weight) were clipped at random 2.5 cm from the plant base at 0800 (AM) and 1600 h (PM) on d 1 and 0800 (AM) and 1300 h (PM) on d 2. Samples were weighed and dried at 70º C and submitted to a commercial laboratory (Equi-analytical, Ithaca, NY) to determine NSC content (water soluble carbohydrates [WSC] + starch). Environmental conditions were measured including ambient temperature, relative humidity, solar radiation, rainfall, soil and canopy temperature, and photosynthetically active radiation (PAR). Corresponding weekly blood samples were collected at 1300 h on d 2 via jugular venipuncture into 4 mL potassium oxalate, and 7 mL EDTA vacutainer tubes and analyzed for glucose (mg/dL), insulin (µIU/mL), and L-lactate (mg/dL). Each

month, fecal grab samples were collected from the midrectum to measure pH and D-lactate (µM). Additionally, monthly glucose and insulin dynamics (% Δ) were assessed via a modified oral sugar test. Pasture nutrients including DE (2.35 ± 0.12 Mcal/kg), NSC ($25.45 \pm 4.02\%$ DM), WSC ($19.65 \pm 3.47\%$ DM), and starch ($7.25 \pm 1.29\%$ DM) were higher in the afternoon hours (PM) compared to morning measurements (AM). Pasture CP and carbohydrate fractions were higher in the spring and fall months compared to summer and winter months with NSC concentrations being highest in May (wk 19) at 25.45% DM. Pasture NSC content was correlated ($P \leq 0.05$) with relative humidity (r = 0.38), solar radiation (r = 0.32), and PAR (r = 0.51) and tended ($P \leq 0.1$) to have a relationship with ambient temperature (r = 0.23) and rainfall (r = 0.23). There was seasonal variation in all morphometric measures in grazing horses. BW was highest in the spring ($P < 0.0001$), while BCS and CNS were highest in the fall ($P = 0.0021$ and $P < 0.0001$, respectively). Metabolic responses in grazing horses also fluctuated seasonally with glucose and insulin concentrations being most elevated in the spring ($P < 0.0001$). There was also seasonal variation in digestive measures in grazing horses. Plasma L-lactate and fecal D-lactate means differed by month ($P < 0.05$) with the highest concentrations in April (11.8 ± 0.91 mg/dL and 4220.4 ± 185.5 µM, respectively). Fecal pH was most acidic in April (6.52 ± 0.08). Pasture NSC content was correlated with weight (r = 0.35), glucose (r = 0.21), and insulin (0.26) in grazing horses and tended to have a relationship with CNS (r = 0.14). There was also a relationship between NSC and plasma L-lactate (r = 0.33), fecal D-lactate (r = 0.48) and pH (r = -0.27). Lastly, glucose and insulin % Δ ($P < 0.0001$) were greatest during spring months, but there was no effect of fasting insulin ($P < 0.2787$) or fasting glucose ($P < 0.2055$) on glucose % Δ. These

data indicate a relationship between seasonal changes in pasture NSC content and the physiological response in grazing horses. Future aims include evaluating possible seasonal fluctuations in the hindgut microbiome of grazing horses to better understand the link between the equine microbiome and nutritionally-related disturbances. Improved grazing management strategies are needed to reduce the risk of metabolic and gastrointestinal disorders in horses, which may lead to subsequent colic and pasture-associated laminitis.

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General Audience Abstract

Lush pastures are an important part of the equine diet for overall health and performance. However, there are several nutrition-related diseases that can occur when environmental conditions favor starch and sugar (nonstructural carbohydrates, NSC) accumulation in pasture grasses. Environmental conditions such as air temperature, intensity of sunlight, frost, and drought can all lead to increased accumulation of NSC in pasture grasses, especially in spring and fall months. When horses graze pastures with elevated NSC concentrations they can develop several conditions such as obesity, insulin resistance, and gastrointestinal upset. One of the most common but least understood equine diseases is pasture-associated laminitis, in which inflammation causes pain and damage to the structure of the equine hoof. The objectives of our research were to measure seasonal changes in pasture NSC concentrations as well as the metabolic and digestive response in grazing horses to better understand how the intake of pasture NSC content may lead to disturbances or disease in the horse. A yearlong grazing study was conducted at the Virginia Tech Middleburg Agricultural Research and Extension Center from October 2016 through September 2017 to investigate the relationship between pasture NSC and grazing horses. Twelve sporthorse mares were maintained on a 21-acre mixed grass pasture with water, mineral (Buckeye Nutrition, Dalton, OH), and white salt *ad libitum*. Weekly pasture samples collected to determine NSC content of the grasses. Weekly blood samples were collected from the horses to measure glucose, insulin, and L-

lactate concentrations. Each month, fecal samples were collected to measure pH and D-lactate. Additionally, monthly glucose and insulin dynamics (% Δ) were assessed via a modified oral sugar test. Pasture NSC content fluctuated throughout the year and was most elevated in the spring and fall months. There was seasonal variation in the metabolic response of grazing horses with glucose and insulin concentrations being highest in the spring months. There was also seasonal variation in digestive measures in grazing horses. Plasma L-lactate and fecal D-lactate were most elevated in the spring and fecal pH was most acidic in the spring. These results indicate a relationship between seasonal changes in pasture NSC content and the physiological response in grazing horses. Future aims include evaluating possible seasonal fluctuations in the hindgut microbiota of grazing horses to better understand the link between the equine gastrointestinal bacteria and nutritionally-related diseases. Improved grazing management strategies are needed to reduce the risk of metabolic and gastrointestinal disorders in horses, which may lead to diseases such as colic and pasture-associated laminitis.

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List of Abbreviations

ADF   Acid Detergent Fiber

AIRg   Acute Insulin Response to Glucose

AUC   Area Under the Curve

BCS   Body Condition Score

CLIA   Chemiluminescent Immunoassay

CGIT   Combined Glucose and Insulin Test

CNS   Cresty Neck Score

DE   Digestible Energy

DM   Dry Matter

DNA   Deoxyribonucleic Acid

ELISA   Enzyme-Linked Immunosorbent Assay

EMS   Equine Metabolic Syndrome

ESC   Ethanol Soluble Carbohydrates

EHC   Euglycemic-Hyperinsulinemic Clamp

FSIGTT   Frequently Sampled Intravenous Glucose Tolerance Test

GLUT   Glucose Transporter

LPS   Lipopolysaccharide

NDF   Neutral Detergent Fiber

NIR   Near Infrared Spectroscopy

NSAIDS   Nonsteroidal Anti-Inflammatory Drugs

NSC   Nonstructural Carbohydrates

OF   Oligofructose

OST   Oral Sugar Test

PAL   Pasture-Associated Laminitis

PCR   Polymerase Chain Reaction

RIA   Radioimmunoassay

SC   Structural Carbohydrates

VFA   Volatile Fatty Acids

WSC   Water Soluble Carbohydrates

Chapter One

Introduction

Forage is essential to the equine diet for normal digestion and overall health. Cool season pasture forages contain varying amounts of nonstructural carbohydrates (NSC) which fluctuate throughout the year and are typically most elevated in spring and fall [1]. Metabolic and digestive dysfunction has been linked with the intake of elevated NSC content and can lead to laminitis in predisposed animals [2]. Laminitis is a costly and debilitating disease that is associated with obesity, inflammation, metabolic and digestive disorders, which manifests itself in the hoof.

Obesity is a growing concern in many species including horses. Obesity can result when horses consume additional calories above the bodies maintenance and energy demands and can be linked with the intake of elevated NSC. Diets rich in NSC can also result in decreased insulin sensitivity [3]. Insulin dysregulation can also result from obesity, and in combination, both conditions predispose a horse for developing laminitis [4–6].

Digestive disturbances have been implicated with the onset of laminitis when NSC are rapidly fermented in the hindgut [1,2]. When horses consume a meal with elevated NSC content, the small intestine can become overwhelmed. Undigested starch spills into the hindgut causing starch utilizing bacteria to increase the production of lactic acid [7–10]. Subsequently, the hindgut pH decreases, disrupting the integrity of the gut epithelial barrier allowing compounds in the gastrointestinal contents to enter the bloodstream and systemic circulation.

Another consequence of the acidic pH is the die off of indigenous gram-negative bacteria causing their outer cell membranes to lyse and release endotoxins, specifically lipopolysaccharide (LPS), into the blood. This is thought to lead to inflammation and damage to

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the lamina of the hoof, as well as colic (Shirazi-Beechey, 2008; Toth et al., 2009). The increase in abundance in lactate producing bacterial populations has also been shown to lead to the production of vasoactive amines which is thought to cause digital vasoconstriction and consequent laminitis [13,14].

Prevention and avoidance of laminitis and inflammation are key, since structural damage to the hoof is irreversible and horses are often euthanized due to the debilitating nature of the disease [15]. Current avoidance strategies are focused on nutritional management by reducing the intake of elevated NSC content [16]. Although NSC have been implicated in acute digestive disturbances due to their rapid fermentation in the hindgut, there is a need for further research in grazing horses. The development of optimized nutritional management strategies will aid in the prevention and avoidance of metabolic and gastrointestinal disruption, including pasture-associated laminitis.

Objectives

1. Determine the seasonal and circadian variation of pasture nonstructural carbohydrates in a cool season mixed grass pasture in Virginia.

2. Evaluate the seasonal fluctuations in the metabolic and morphometric response of grazing horses.

3. Determine the seasonal fluxes in the digestive response of grazing horses.

4. Evaluate seasonal fluctuations in glucose and insulin dynamics during an oral sugar test in grazing horses.

Study timeline for chapters 3-6: October 2016 – September 2017

Tuesdays   Wednesdays   Thursdays

0800   1600   0800   1300   2200   0700   0845
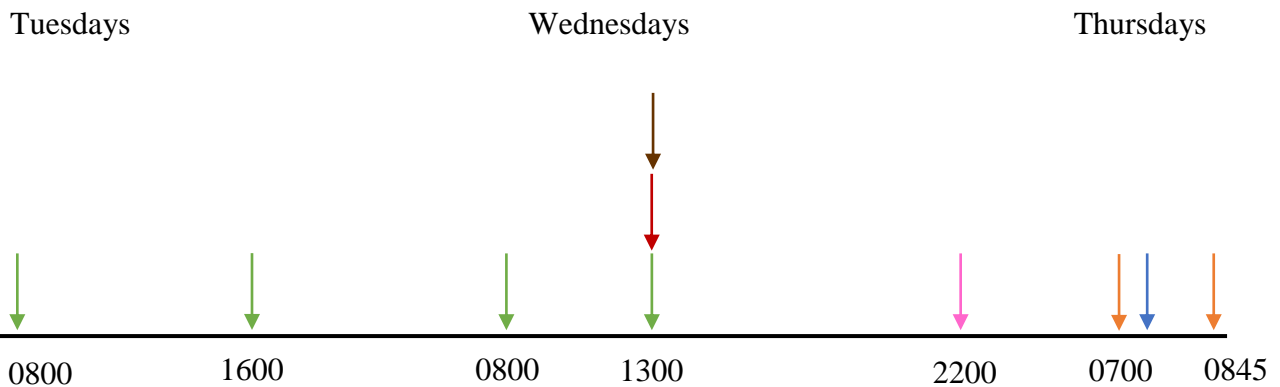
↓ = Weekly pasture collection & weather measurements

↓ = Weekly blood collection

↓ = Weekly sterile fecal collection

↓ = Monthly overnight fasting for oral sugar test

↓ = Monthly blood collection for oral sugar test

↓ = Monthly Karo syrup administration for oral sugar test

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Literature Review

2.1. Introduction

Pastures are an important part of the horse's diet for their overall health and wellbeing by offering nutrition and exercise. Horses in moderate to heavy work may need additional calories supplemented in the form of grain-based concentrates to meet their increased energy demands. In some cases, these high starch diets can prove to be too ideal for some horses, especially those that are overweight and predisposed to metabolic dysfunction as they can lead to negative consequences on horse health [2]. Diseases that can result from starch overload include acidosis, colic, and laminitis [17]. Laminitis is a painful and costly disease associated with insulin resistance, the production of inflammatory cytokines, and gastrointestinal disturbances that manifest in the hoof. Damage occurs in the basement membrane of the hoof's lamina, which can lead to laminar separation causing the coffin bone to rotate downward in the hoof capsule. It is estimated that laminitis represents 7.5% to 15.7% of lameness problems [18]. Pasture-associated laminitis is thought to account for nearly half of laminitis cases and has been associated with the intake of nonstructural carbohydrates (NSC) [18]. However, limited information is available on the seasonal changes of pasture NSC and how it affects the metabolic and digestive profile of grazing horses. Researchers are uncertain of the exact mechanism of laminitis and how inflammation is associated with disruption to the lamina and eventual rotation of the coffin bone. A better understanding of the link between gastrointestinal and metabolic disturbances and onset of laminitis is needed to avoid disease. Investigating the seasonal physiological response of grazing horses will allow for optimization of equine health and nutrition to reduce the morbidity and mortality of horses.

2.2. Nonstructural Carbohydrates in Forages

Plants are comprised of both structural carbohydrates (SC) and NSC. The SC include cellulose, hemicellulose, and lignin, which together make up the plant cell wall. Starch and water soluble carbohydrates make up the NSC components. Fructan and simple sugars (glucose, sucrose, and fructose) make up the WSC fraction. The production of sugar is vital for plant growth and development. This process of carbohydrate metabolism occurs through photosynthesis when plants utilize carbon dioxide and water in the presence of sunlight to produce sugar. Following photosynthesis, the plant will undergo respiration to utilize the sugar produced during the day as energy. This process of respiration occurs mainly at night. When the production of sugar exceeds the needs of the plant the carbohydrates are stored as reserve carbohydrates. Cool season grasses store fructan in the vacuoles of the leaves, which transfer to the stem; while warm season grasses accumulate starch in the chloroplasts of the plants leaves. Warm season plants have a self-limiting process when the chloroplasts become saturated with starch and the maximum storage has been achieved. However, cool season grasses that build up fructan have no self-limiting process and concentrations can accumulate rapidly [6,19].

The NSC content of pasture forages can differ between individual species and varieties of grasses, especially comparing warm and cool season grasses [19]. Examples of cool season grasses are bluegrasses (*Poa sp.*), fescue (*Schedonorous sp.*), orchardgrass (*Dactylis glomerata*), and timothy (*Phleum pratense*). Examples of warm season grasses include bermudagrass (*Cynodon dactylon*), bluestem (*Andropogon sp.*), crabgrass (*Digitaria sp.*), and switchgrass (*Panicum virgatum*). Typically, cool season grasses will have higher levels of NSC as compared to warm season grasses [20]. Cool season species such as perennial ryegrass (*Lolium perenne*), orchardgrass and timothy have been shown to accumulate NSC as high as 200 g/kg dry matter (DM). Perennial ryegrass varieties selected for high sugar content contained 330 g WSC/kg DM

[21]. In addition to species of plant, various other factors can cause the amount of NSC to fluctuate including environmental conditions, and seasonal and circadian variations.

2.2.1. Environmental Factors Affecting Forage NSC

There are various environmental factors that can alter the amount of NSC in forages [22]. Previous research has shown that the production of sugar through photosynthesis is directly related to light intensity. When pastures containing bulbous canarygrass (*Phalaris aquatica*), a cool season perennial, were shaded for an average of 42.5 h the concentration of starch and WSC (excluding sucrose) decreased. Once the shade cover was removed the concentration of NSC increased to the equivalent of unshaded pasture [23].

Temperature influences pasture NSC concentrations. A study conducted by Chatterton and others discovered that cool season grasses had increased levels of NSC when grown at 10°C during the day and 5°C during the night as compared to concentrations when grown at 25°C during the day and 15°C during the night [20]. Another study found similar results in temperate climates. They suggested the daily temperature range during the spring and fall months allows for increased NSC accumulation which exceeds the rate of respiration, unlike summer and winter months [22].

In many circumstances, vegetative and rapidly growing pasture grasses will have elevated NSC content. However, an increase in starch and sugar can occur when plants undergo stress such as drought. When drought conditions were simulated over a period of five days, the concentration of sucrose in the base of fescue leaves increased by 258%, hexose concentrations increased 187%, while fructan decreased by 69% [24]. Another study investigated the effects of severe drought on 21 orchardgrass populations. After three months of drought, WSC and fructan increased reaching 35-40% DM in the stem bases [25].

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A second form of plant stressor that increases NSC content is frost [26]. Freezing

temperatures lead to a phenomenon of plant growth called cold acclimation to minimize damages

caused by low temperature. Plants in temperate regions experience cold stress at temperatures of

0-15°C [27]. During cold stress, accumulation of sugars occurs in order to stabilize the plant's

biological components; thus leading to increased NSC content [28].

2.2.2. Seasonal and Circadian Variation of Forage NSC

Previous research demonstrated seasonal and diurnal variations in pasture NSC. When

perennial ryegrass was sampled in the spring throughout the day, the WSC content increased

from 250 g/kg at 0500 h to 310 g/kg DM between 1300 and 1500 h followed by a decrease to

180 g/kg at 2300 h. However, when WSC was measured in October the perennial ryegrass had

only 150 g/kg at 0600, 1300, and 2000 h decreasing as low as 120 g/kg at 1400 h [21]. Research

by Morin and others investigated diurnal fluctuations of NSC content in alfalfa *(Medicago*

sativa) in which maximum NSC content occurred between 1600 and 1800 h [29]. These studies

support previous literature that NSC content is greatest in the later daylight hours.

Cool season pasture NSC content will reach the highest concentrations during the spring

and fall with the lowest amounts during the summer and winter [30]. When NSC levels in

Virginia pastures were monitored during 2006 and 2007, April and May demonstrated the

greatest amounts at more than 150-200 g NSC/kg DM. Winter and summer months had the

lowest amounts of NSC at less than 5 to 7% DM, while fall months fell intermediately [1]. In a

study measuring seasonal trends of grasses in Utah, the sugar concentration in cool season

grasses was highest in early May. Sugar content then decreased in the summer (July), increased

in the fall (September/October), and decreased again going into the winter (November).

Although the WSC and total nonstructural carbohydrates (TNC) followed the same seasonal

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trend as the sugar concentration of the grass species, the highest average concentration of WSC occurred in October [31]. While previous research demonstrates seasonal variation in NSC, especially elevated in the spring and fall, environmental conditions can cause NSC to rise during unexpected times of the year.

2.2.3. Laboratory Analyses of Forage NSC

Forage analysis, of both hay and pasture, is important for optimizing horse health and reducing the NSC in the diet of horses at risk for laminitis. Commercial forage testing laboratories utilize different methods and terminology when measuring and reporting NSC fractions. This can make understanding analyses of NSC fractions confusing for horse owners to understand. When analyzing NSC in forage samples, monosaccharides, disaccharides, oligosaccharides, fructan, and starch should ideally be measured. There are two approaches utilized to measure nutritive value (including NSC) of forages and feed: wet chemistry and near infrared reflectance spectroscopy (NIR). Wet chemistry uses heat and chemicals to break down the feedstuffs. When using NIR, a spectrophotometer analyzes the light spectrum reflected off the sample (700 – 2,500 nm). This light spectrum is compared to samples that have been analyzed by wet chemistry. When comparing the two methods wet chemistry is a more accurate measure than NIR, but NIR requires less labor and produces faster results. However, NIR technique has been perfected to accurately measure nutrient content in common forages and feeds [32].

Using traditional wet chemistry, NSC fractions can be broken down into ethanol soluble carbohydrates (ESC), water soluble carbohydrates (WSC), and starch. The carbohydrate fraction ESC includes mainly simple sugars and is measured through the process of ethanol extraction. Water soluble carbohydrates (WSC) includes both sugars (ESC) and fructan when extractions are

performed with water [33]. When evaluating total NSC, both starch and WSC should be included. Knowledge of starch and sugar analyses from commercial labs will allow horse owners and caretakers to better provide an appropriate diet for horses predisposed to metabolic disease and laminitis and to better nutritionally manage their horse.

2.3. Implications of NSC and Grazing Horses

During times of elevated levels of NSC, specifically spring and fall months, horses may be at an increased risk for pasture-associated laminitis and digestive upset [19,34]. Diets high in NSC increase risks for digestive, metabolic, and inflammatory disorders and may ultimately culminate laminitis [2]. Although nearly half of the laminitis cases are associated with pasture, there is minimal research evaluating the seasonal effects of pasture NSC on the metabolic and gastrointestinal physiology of horses [18]. In Virginia, nearly 70% of horses live on pasture at least part of the time, so pasture NSC analysis is crucial for the health and performance of horses [35].

2.4. Metabolic Response

Although some animals may have nutrient demands that require greater amounts of NSC in forages and concentrates, horses can be sensitive to elevated amounts of NSC content. The intake of elevated NSC content may exceed the energy requirements of the horse leading to obesity. A scoring system was developed by Henneke and others to assess fat deposition in horses on a scale of 1 to 9, with a 1 representing an emaciated horse and 9 being a morbidly obese horse [36]. When using the Henneke system, a score of ≥ 7 is considered obese [5,36]. Obesity is of growing concern in many species including horses, with the prevalence of equine obesity estimated at approximately half of the horse population [3,37].

The incidence of obesity can lead to an increased risk of many diseases such as insulin dysregulation, laminitis, equine metabolic syndrome, lipomas, and hyperlipidemia [38,39]. Horses are considered to have Equine Metabolic Syndrome (EMS) when they experience insulin dysregulation, obesity and/or regional adiposity, and laminitis [40]. Regarding regional adiposity, instead of having uniform fat deposition over their entire body, the affected animal will have localized fat deposits, which defines the metabolic phenotype. Ponies and certain breeds of horses, including warmbloods, Quarter horses, Andalusians, Morgans, and Saddlebreds, are predisposed to these metabolic conditions, suggesting there may be a genetic link to metabolic disorders [41].

The intake of elevated NSC content has been linked with metabolic dysfunction, especially in horses with obesity and metabolic tendencies [2]. Previous research has demonstrated that overweight horses had decreased insulin sensitivity compared to nonobese horses [4,42]. Although studies have shown a correlation with insulin resistance and obesity in horses, it is important to note that not all obese horses are insulin resistant [43]. Additionally, Pratt and colleagues discovered that insulin sensitivity can also be decreased due to the intake of a diet rich in NSC [3].

2.4.1. Insulin Dysregulation

Glucose serves as an important energy source and powers certain metabolic actions in the body. When glucose levels are elevated in the blood, insulin is secreted via pancreatic β cells to maintain glucose homeostasis by taking up the glucose to be stored as glycogen or fat. Additionally, glycogen synthesis in the muscle and liver are stimulated which inhibits gluconeogenesis in the liver to maintain glucose homeostasis [44]. Insulin mediated glucose dispersal may be reduced in some horses leading to insulin resistance. When the cells become

less sensitive to insulin action more insulin is needed to keep blood glucose levels within normal limits, especially following the ingestion of a high NSC meal. Insulin resistance primarily affects insulin receptors in skeletal muscle, fat tissues, and the liver [45,46].

Insulin receptors are located in the cell membrane and involve tyrosine kinase. Tyrosine kinase is activated when insulin binds to the receptor which begins a cascade of intracellular signaling. Following receptor activation, glucose transporters (i.e., GLUT-4) translocate to the cell membrane to allow glucose to move into the cells of adipose and skeletal muscle tissues. Glucose transporters are necessary regardless if insulin is needed to regulate glucose, as lipid bilayers in the cell are otherwise impermeable. The liver is typically responsible for clearing approximately 70% of the insulin secreted from β cells in portal blood before it reaches circulation in horses [47]. GLUT-2 is the transporter that aids glucose transport in the liver, small intestine, kidney, and pancreatic β cells. If liver insulin receptors are downregulated and impaired, this can lead to the incidence of a compensated insulin response due to decreased tissue sensitivity which plays a role in insulin resistance and hyperinsulinemia. Downregulation of skeletal muscle and adipose tissue receptors can also occur. The combination of both insulin resistance and hyperinsulinemia is termed insulin dysregulation [48].

2.4.2. Assessment of Insulin Dynamics

There are a variety of testing measures that can assess insulin dynamics in horses. The most basic measure is to fast the horse overnight and collect a basal blood sample the following morning. Most researchers and veterinarians use a value of $> 20\mu IU/mL$ as a measure of insulin resistance. Gold standard laboratory tests for insulin dynamics include the euglycemic-hyperinsulinemic clamp (EHC) and the frequently sampled insulin-modified intravenous glucose tolerance test (FSIGTT).

The EHC test involves continuous intravenous (IV) infusion of insulin to achieve a hyperinsulinemic state. Simultaneously, glucose is infused to maintain a euglycemic level. The purpose for maintaining the steady euglycemic state is to demonstrate insulin sensitivity in adipose and muscle tissue. Calculations derived from the EHC procedure include glucose uptake by the body (M) and steady-state insulin concentration (I), which can be used to measure insulin sensitivity (M/I ratio).

The FSIGTT, with minimal model analysis, also uses an infusion of IV glucose and insulin to measure insulin dynamics. The procedure requires a strenuous protocol of frequent and numerous blood samples. Twenty minutes following glucose administration, insulin is administered and frequent sampling resumes. The minimal model calculates three variables from the frequent sampling protocol. One variable is glucose effectiveness (Sg) which represents the ability to inhibit hepatic production of glucose as well as the capacity of glucose to mediate its own disposal. A second calculation is acute insulin response to glucose (AIRg) which represents pancreatic insulin secretion in response to circulating glucose. A third measure is insulin sensitivity (SI) which is suggestive of the rate of glucose dispersal via insulin. Although the EHC and FSIGTT have been deemed the gold standards in testing insulin dynamics, the labor-intensive protocols and materials needed to complete these procedures may restrict their use in some research settings.

More recently, additional tests have been developed to give estimates of insulin dynamics in horses and ponies, including the combined glucose and insulin test (CGIT) and oral sugar test (OST). The CGIT also uses IV infusion of dextrose closely followed by insulin to measure the body's resistance to insulin. This is evaluated based on the time it takes for the body's blood

glucose concentrations to return to normal via clearance by insulin at 45 min. Insulin resistance may also be present if insulin concentrations exceed 100 µIU/mL at 45 min.

The OST is different than the aforementioned testing procedures, due to the measure of postprandial hyperinsulinemia in response to oral glucose administration. Light corn syrup is orally dosed and much less frequent blood samples are collected. Sampling protocols vary from blood collection at min 0, 30, 60, 75, 90, and 120 min to look at area under the curve (AUC) values, to a more conservative protocol of 0, and 60 or 75 min [48–51]. Insulin concentrations of ≥ 45 or 60 µIU/mL at either time points, 60 or 75 min, are suggestive of insulin dysregulation [48,50,52].

2.4.3. Insulin Laboratory Analyses

Once a preferred testing method has been selected to evaluate insulin dynamics in a horse, the next step is to determine which laboratory analysis will be used to measure insulin concentrations. The gold standard for measuring equine insulin in blood samples was the Siemens Coat-A-Count Radioimmunoassay (RIA) (Siemens Medical Solutions Diagnostics, Los Angeles, CA). However, this assay is no longer available. Currently, the available methods for evaluation of insulin include the Mercodia Equine Insulin enzyme-linked immunosorbent assay (ELISA) (Mercodia AB, Uppsala, Sweden) and Immulite insulin solid-phase chemiluminescent immunoassay (CLIA) (Siemens Healthcare, Malvern, PA, USA). Previous research comparing all three methods (RIA, ELISA, and CLIA) found all methods differed when measuring insulin concentrations in basal blood samples. The CLIA insulin values in this study were much lower than results from RIA or ELISA [53]. Results from the various insulin assays should be interpreted carefully and not compared across testing measures. This gives rise to further

question appropriate dosing recommendations for various insulin dynamics tests as well as cut off values for diagnosis of insulin dysregulation and hyperinsulinemia.

2.5. Digestive Response

The hindgut microbiome is a diverse community of bacteria, archaea, protozoa, and fungi that has many important functions, which play a role in the overall health and performance of horses. These features include digestion of feedstuffs for energy, protection against pathogenic organisms and disease, maintenance of the gut epithelial barrier, role in metabolic functions, and modulating the immune system. The microbiome plays a critical mutualist role in forage breakdown and utilization for the horse, which the horse could not otherwise use, making their relationship essential. During this fermentative process, volatile fatty acids (VFAs) such as acetate, butyrate, and propionate are produced in the hindgut and used as energy.

Acetate is the predominant VFA produced by microbial fermentation in horses and is primarily utilized as an energy source for many tissues in the body [54,55]. Propionate is largely responsible for aiding in gluconeogenesis, while butyrate provides energy for the gut epithelium [56]. When horses consume a largely forage diet, acetate constitutes 70% of VFA production, while propionate is 17% and butyrate is 6%. When horses consume a diet rich in concentrates, there is less acetate production and more propionate production which decreases the energy available to the body via acetate [54].

Lactate is also a product of starch fermentation via bacteria and host enzymatic digestion. Two different isomers of lactate are produced: D- and L-lactate. Both host enzymatic digestion and microbial fermentation of starch can produce L-lactate. However, microbial fermentation of starch is the only way to produce the D- isomer, as horses do not express D-lactate dehydrogenase which converts pyruvate to D-Lactate. Horses also do not express lactate

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racemase which catalyzes the conversion of L-lactate to D-lactate. In cases of laminitis, elevated blood D-lactate concentrations have been observed [17,57]. Research has shown peak concentrations of D-lactate around 20 h post oligofructose (OF) administration when the OF laminitis induction model was used [58].

The gastrointestinal anatomy and physiology of the horse is adapted for grazing or browsing 14-17 hours a day for continuous forage intake [59,60]. However, under modernized management of horses, often diets are supplemented with grain-based concentrates containing elevated amounts of NSC content. Higher NSC content is usually offered to horses in the form of meal feeding rather than trickle feeding as horses were evolutionally adapted. The intake of NSC can cause a host of gastrointestinal dysfunction when rapidly fermented in the hindgut, and diseases affecting the gastrointestinal system are one of the leading causes of death in horses [17,61].

2.5.1. The Hindgut Microbiome

Although the importance of the hindgut microbiome has been noted, there is still much to learn about the resident microbial communities in the horse, especially compared to other livestock species. A core microbiome in the horse has been observed, despite there being much evidence of individual variation. The three major phyla described in the horse gastrointestinal system include Firmicutes, Bacteroidetes, and Proteobacteria [17,61–64]. Within these phyla, the species are generally classified according to their substrate utilization. The major fiber digesting bacteria in the horse include *Ruminococcus albus*, *Ruminococcus flavefacians*, and *Fibrobacter succinogenes*. These bacteria are the predominant species of the equine cecum which is the primary site for forage fermentation [65]. Cellulolytic bacteria prefer a hindgut pH of 6.0 – 7.0 to thrive.

Amylolytic bacteria utilize starch as an energy source to produce lactic acid and can tolerate more acidic pH levels of 4.5 – 5.5. Predominant lactic acid producing bacteria include some *Clostridium* spp., *Streptococcus* spp. and *Lactobacillus* spp. and largely inhabit the small intestine which is the primary site for starch breakdown and utilization [17]. *Mitsuokella* have also been identified in the horse as a major D-lactate producer [66]. Microbial populations that utilize lactate include *Megasphaera* and *Veillonella* [67].

2.5.2. Factors that Influence the Microbiome

The hindgut microbiome is a dynamic community of organisms that are beneficial to the horse when in a homeostatic state. Horses experience dysbiosis when shifts occur in the balance of the microbiome community. Shifts in the hindgut microbiome can lead to gastrointestinal disease, which is one of the leading causes of death in horses [61]. Changes in the hindgut microbiome can lead to the production of toxic compounds including bacterial-derived endotoxins, exotoxins, and amines which may subsequently lead to inflammation [13,68,69]. There are many factors that can influence the hindgut microbiome including disease, medications, and diet [70–72].

2.5.3. Influence of Diet on the Microbiome

The diet of the horse largely determines what bacteria will be present and their abundance in the gastrointestinal tract. When horses were maintained on forage only diets, lower counts of lactic acid bacteria were observed in mature geldings. In this same study, when horses were switched to concentrate diets, *Lactobacillus ruminis* was present, but not when horses were on the forage only diet. Additionally, horses on the concentrate diet had 10 times more lactic acid bacteria [73]. Research by Daly and others found horses consuming diets with increased

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hydrolysable carbohydrates had increased counts of Bacteroidetes and decreased counts of *Fibrobacter* and *Ruminococcus* spp. compared to pasture-fed horses [74].

Sudden changes in the diet of horses can have a profound impact on the hindgut bacterial community. Research by Potter and colleagues estimates the capacity for starch digestion in the equine hindgut at approximately 0.35 – 0.40% BW/meal [75]. If horses consume a meal containing NSC content above that capacity, the small intestine may become overwhelmed. During this event, undigested starch will spill into the hindgut leading to the proliferation of starch-utilizing bacteria, increasing the concentration of lactic acid in the hindgut [7–10]. The increase in lactic acid production causes a decrease in pH which is unfavorable to indigenous fiber-utilizing bacteria causing their death and release of the outer cell membrane contents, specifically lipopolysaccharide (LPS). Research by Biddle and others found an increase in *Streptococcaceae*, while *Ruminococcaceae* and *Lachnospiraceae* decreased during starch inclusion *in vitro*. This was followed by an increase in *Lactobacillalceae* as a decrease in *Streptococcaceae* was observed [67]. Additionally, an increased abundance in lactate-utilizers, *Megasphaera elsdenii* was observed following the inclusion of both starch and lactate.

An increase in starch-utilizing spp. has also been shown to lead to the production of vasoactive amines. These bacteria can decarboxylate amino groups creating vasoactive compounds. Bailey and others found higher concentrations of vasoactive amines in vitro when excess starch or fructan was added to cecal contents [13]. One consequence of the acidic pH in the hindgut is the decrease in integrity of the gut epithelium. Due to weakened tight junctions in the epithelium, gastrointestinal contents, LPS, and vasoactive amines are then able to cross the protective barrier and enter the bloodstream. This infusion of vasoactive amines is suggested to cause vasoconstriction of the equine digit. LPS can also cause negative effects when they enter

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circulation, leading to subsequent inflammation. Both vasoactive amines and LPS may lead to consequent laminitis in horses [13,76].

2.5.4. Influence of Disease on the Microbiome

The incidence of disease influences the hindgut microbiome in horses. Researchers have suggested a rapid proliferation of *Streptococcus* spp. occurs following the onset of laminitis. As lactate production increases and the pH becomes more acidic, *Lactobacillus* spp. increase and subsequently *Streptococcus* spp. decrease due to the further reduction in pH [7–9,77]. Research by Steelman and colleagues observed an increased abundance of *Clostridiales* species in horses with a previous history of laminitis. The horses also had a greater representation of Verrucomicrobia and lower Firmicutes representation compared to control horses that had no previous history of laminitis [10].

More recently, studies have focused on microbial changes in horses with EMS. Microbial analyses from EMS horses demonstrated a decrease in fecal microbial diversity compared to non-EMS horses. Horses with EMS also had higher relative abundance of Verrucomicrobia while non-EMS horses had higher *Fibrobacter* counts [78]. Decreased diversity may be of concern, as it has been associated with obesity, diet changes, and in response to antimicrobial treatment [7,8,10,61,79].

Bacteria in the gut are suggested to play a role in weight gain, glucose and insulin dynamics, and predisposition to cardiovascular and other metabolic diseases in humans [80,81]. The Human Microbiome Project focuses research efforts on understanding the relationship with the microbiome's role in health and disease [82]. In humans and mice, research has shown that obese subjects had an increased abundance of Firmicutes and decreased abundance of Bacteroidetes [81,83]. This altered ratio is thought to increase energy harvest, especially higher

concentrations of acetate and butyrate in cecal contents, thereby further exacerbating obesity. When the microbiota of obese mice was transplanted into lean germ-free mice, it resulted in weight gain compared to the lean germ-free mice that did not receive obese mouse microbial contents. Similarly, insulin sensitivity decreased in germ-free mice that had received fecal transplantation from obese metabolic type mice [84].

2.5.5. Influence of Medications on the Microbiome
        Medications are another factor that can alter the hindgut microbiome of horses. Antibiotics are commonly administered to horses for a variety of illness and infections. Similarly, with humans, the administration of antibiotics has been shown to cause dysbiosis in resident microbial populations. Costa and colleagues discovered antibiotic use in horses affected the microbiome, ultimately reducing bacterial diversity [85]. Previous research demonstrated the prevalence for horses to develop colitis following antibiotic administration [86]. Like in humans, the use of antibiotics may lead to the colonization of pathogenic bacteria and incidence of diarrhea in horses. Research by Harlow and others found horses that received antibiotic treatment had decreased counts of cellulolytic bacteria and increased abundance of *Salmonella* compared to control horses. *Clostridium difficile* was not detectable in control horses, while antibiotic treated horses had colonization of 10^4 cfu/g [87].

        Nonsteroidal anti-inflammatory drugs (NSAIDS) are another drug commonly administered to horses which influence the microbiome. Research by McConnico and others administered Phenylbutazone (8.8 mg/kg) to 12 healthy adult horses for 3 wks. Two of the horses developed acute enterocolitis following Phenylbutazone administration and were hospitalized for supportive care. Additionally, horses receiving the phenylbutazone treatment had increased concentrations of acetic acid which can alter microbial populations via decreasing pH

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[88]. The administration of NSAIDS and other medications to grazing horses may further increase the risk for pasture-associated laminitis by causing hindgut dysbiosis.

2.6. Laminitis

Arguably the greatest concern with insulin dysregulation and fluctuating pasture NSC concentrations is the link with metabolic and digestive dysfunction, specifically pasture-associated laminitis. Laminitis is a devastating and painful disease that occurs as a result of metabolic and/or digestive disturbance and inflammation which manifests itself in the hoof. Laminitis is one of the leading causes of veterinary care for horses [18]. In cases of severe laminitis and coffin bone rotation, horses are often euthanized to prevent further pain and suffering as there is no cure for laminitis [15]. There are several types of laminitis including endocrinopathic laminitis and gastrointestinal-associated laminitis. Endocrinopathic laminitis is associated with insulin dysregulation and metabolic syndrome in horses. While gastrointestinal-associated laminitis is liked with digestive disease, carbohydrate overload, and other forms of systemic inflammation in the body.

2.6.1. Endocrinopathic Laminitis

While the exact mechanisms of laminitis are not fully understood, insulin dysregulation is associated with laminitis in horses [89–92]. One theory behind endocrinopathic laminitis suggests in times of insulin resistance the tissues are being deprived of glucose causing damage and cell death, specifically in the hoof [93,94]. Additionally, vascular dysfunction and inflammation are also thought to play a role in endocrinopathic laminitis. This occurrence is often encountered in humans with Type 2 diabetes. Insulin has been shown to influence blood flow and invoke vasodilation in both animals and humans [95–97]. During an insulin resistant state, impaired insulin function leads to vasoconstriction and endothelial damage. Further

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supporting insulin's role in laminitis, research by Asplin and colleagues successfully induced laminitis in 100% of ponies on their study by maintaining a state of hyperinsulinemia [98]. The researchers utilized the EHC protocol and ponies developed laminitis in all four limbs.

2.6.2. Gastrointestinal-associated Laminitis

Although this mechanism is not fully understood, this pathophysiological cause of laminitis is thought to occur due to a shift in the hindgut microbiome. Dysbiosis may occur for a variety of reasons, including rapid fermentation of carbohydrates in the hindgut. Subsequently, endotoxins and other toxic compounds cross the gastrointestinal-epithelial barrier and enter systemic blood circulation causing an inflammatory response that may ultimately lead to rotation of the coffin bone.

2.7. Treatment of Laminitis

If a horse is suspected to have laminitis, immediate attention is needed. It is imperative to find the underlying cause of laminitis and remove it or confiscate the horse from that situation (i.e. removal of grain or horse from pasture). It will be necessary to determine if coffin bone rotation has occurred via radiographs. Then a treatment plan can be decided, and prognosis determined. Treatments may include the administration of Metformin, Levothyroxine, NSAIDS, cold therapy, and hoof care.

Levothyroxine is a synthetic drug used to correct low circulating thyroid hormone that has been used as treatment for laminitis. Research by Frank and others found Levothyroxine treatment improved insulin sensitivity and caused weight loss in treated horses [99]. Metformin is another drug that may be administered to laminitis horses, but there are conflicting results on the effectiveness of this drug. Research by Durham and others found Metformin to increase insulin sensitivity in metabolic type horses and ponies [100]. Another study at the same dosage

rate did not find improvement in insulin sensitivity in insulin resistant ponies [101]. A third type

of medication given to laminitic horses is NSAIDS to reduce pain and inflammation. The

inability to control pain associated with laminitis is what ultimately leads to euthanasia of the

affected horse. Phenylbutazone is one of the most commonly administered NSAIDs to laminitic

horses [102].

Cryotherapy is an effective tool in reducing inflammation and the severity of laminitis

[103]. For effective cryotherapy a protocol of $3.5°C – 7.1°C$ for 48-72 hours is recommended

[103,104]. Lastly, providing cushion or stability for the coffin bone and hoof lamina may be

helpful therapies. This could include the use of deep bedding in a stall, applying pads or rubber

inserts for sole support, removing shoes, or adding corrective shoes [105]. Although the

previously mentioned tools and treatments can help to reduce the pain and suffering of laminitic

horses, the best course of action is preventing laminitis from occurring.

2.8. Strategies to Avoid Pasture-Associated Laminitis

Prevention and avoidance of inflammation and pasture-associated laminitis are key since

horses are often euthanized due to the debilitating nature of this disease [15,61]. Current

prophylactic strategies are focused on nutritional management by reducing the intake of elevated

NSC content through various grazing strategies such as: the use of grazing muzzles, feeding a

low NSC diet, grazing when NSC content is the lowest, or simply reducing the hours a horse is

allowed on pasture [16].

Restrictive grazing devices, such as grazing muzzles, are one effective way to limit intake

of NSC content. Previous research by Longland and colleagues found that when ponies wore

grazing muzzles while out on pasture during four three-hour grazing events throughout three

seasons, dry matter intake was reduced by 77, 77, and 83% for spring, summer and autumn

pastures, respectively [106]. Some limitations of using grazing muzzles include increased labor

to keep the muzzles on the horses and treatment of rubs and sores from muzzle wear.

Feeding horses and ponies who are at an increased risk of insulin dysregulation and

laminitis a low NSC diet is key in preventing disease. A low NSC diet would be void of grain-

based concentrate rations. Feeding hay that is low in NSC content is a good way to control how

much NSC is consumed, while still allowing for gut motility and health. Current

recommendations of a low NSC forage are below 10-12% DM [16,107]. If additional calories are

needed, research has shown that feeding high fat and fiber diets would benefit at risk horses

[108].

Another way to restrict intake of elevated NSC content is to allow horses to graze during

times when NSC may be lowest. This may include grazing when environmental conditions lend

to lower NSC content accumulation or during times of the day and year when NSC is lowest.

Grazing in the summer and winter months, or during the overnight and early morning hours prior

to when the plant would begin photosynthesis can reduce the intake of NSC. It is also

recommended that grazing on overcast days or in shaded areas can decrease the ingestion of

elevated NSC as a result of reduced photosynthetically active radiation. However, NSC content

may still be too high (> 10-12% NSC) during these times for at-risk equines.

Lastly, it has been recommended that reducing the amount of time horses can graze

pasture will aid in reducing pasture intake and subsequent NSC content. Research by Ince and

others investigated the effects of reduced turnout time on pasture intake [109]. Ponies were

allowed three hours of turnout a day over the course of the six-week study. During the first week

of the study, ponies consumed 0.49% of their liveweight as pasture. That amount increased to

0.91% of their liveweight by week six, suggesting that reducing turnout time may not be an effective management strategy in reducing the intake of pasture NSC by equines.

2.9. Nutritional Supplements and Laminitis

Limited research has been conducted on the use of nutritional supplements to aid in preventing laminitis [110]. Feeding a hindgut buffer product may be useful in mitigating the effects of pasture-associated or carbohydrate overload laminitis [111]. Research by Suagee-Bedore and colleagues found that supplementing horses with bicarbonate buffer prevented an increase in inflammatory marker interleukin-1β and reduced plasma D-lactate and pH compared to controls [112]. Thus, it may provide benefit in reducing metabolic and digestive dysfunction associated with the rapid fermentation of NSC and subsequently laminitis.

Probiotics are another nutritional supplement that may prove beneficial in preventing laminitis and reducing inflammation and EMS. To the author's knowledge there is no scientific literature using probiotics as a preventative for EMS or laminitis. Probiotics are live microorganisms that provide health promoting effects to the host when administered in an adequate dose. Probiotics have been used therapeutically in humans with metabolic dysfunction [113]. Probiotics containing *Lactobacillus* and *Bifidobacterium* have been shown to influence glucose metabolism and reduce inflammation [114]. In humans, a decrease in *A. muciniphila* has been associated with the onset of obesity and Type 2 diabetes [115–117]. Further research investigated the effects of administering *A. muciniphila* daily for four weeks to obese diabetic mice. Treatment with this bacteria resulted in reversed diet-induced obesity, insulin resistance, Type 2 diabetes, and decreased inflammation [116]. Thus, probiotics may have prophylactic and therapeutic action with laminitis in horses.

2.10. Summary

With gastrointestinal disruption being a leading cause of morbidity and mortality of horses, it is important to understand how to manage animals to prevent such instances. Additional nutritional management strategies are needed to prevent and reduce the risk of metabolic and gastrointestinal disease. Most studies have investigated the cascade of events following grain-overload in horses, even though more than half of laminitis cases are pasture-associated. There is a need to further understand the metabolic and digestive responses of grazing horses to prevent such devastating diseases, especially during times of the year when NSC content is typically most elevated. Future directions may include the use of probiotic supplements, specifically *A. muciniphila*, to combat and prevent pasture-associated laminitis in horses. Diligent nutritional management is important to optimizing the health and performance of horses.

Chapter Three

Circadian and seasonal variation of pasture nonstructural carbohydrates

Abstract: Forage is essential to the equine diet for overall health and performance. However, the intake of pasture nonstructural carbohydrates (pNSC) is associated with metabolic and gastrointestinal diseases in horses. Nutrient content including pasture NSC (pNSC) was monitored on an 8.5-ha cool season mixed grass pasture for 52 wks beginning October 2016. Weekly pasture samples (200 g wet weight) were clipped at random 2.5 cm from the plant base at 0800 (AM) and 1600 h (PM) on d 1 and 0800 (AM) and 1300 h (PM) on d 2. Samples were weighed, dried at 70° C, and submitted to a commercial laboratory (Equi-analytical, Ithaca, NY) for nutrient analysis, including pNSC content (water soluble carbohydrates [WSC] + starch), digestible energy (DE), and crude protein (CP). Corresponding environmental variables were measured. Macroclimate conditions included high ambient temperature, low ambient temperature, relative humidity, and solar radiation. Microclimate conditions included soil and canopy temperature, and photosynthetically active radiation (PAR). Data were analyzed using a repeated measures ANOVA in the Mixed procedure in SAS (v. 9.4, SAS Institute Inc.; Cary, NC). Data are summarized as means ± SEM with a $P \leq 0.05$ considered statistically significant. Pasture nutrients including DE (2.35 ± 0.12 Mcal/kg), NSC (25.45 ± 4.02% DM), WSC (19.65 ± 3.47% DM), and starch (7.25 ± 1.29% DM) were higher in the afternoon hours (PM) compared to morning measurements (AM). Pasture CP and carbohydrate fractions were higher in the spring and fall months compared to summer and winter months. There were no diurnal differences in CP. Pasture NSC content was correlated ($P \leq 0.05$) with relative humidity (r = 0.38), solar radiation (r = 0.32), and PAR (r = 0.51) and tended ($P \leq 0.1$) to have a relationship with ambient temperature (r = 0.23) and rainfall (r = 0.23). These data support the observation that pasture

nutrients, specifically carbohydrate fractions, can fluctuate rapidly in cool season pastures. These findings have implications for the nutritional management of grazing horses and will lead to optimized grazing strategies to reduce the risk of pasture-associated laminitis.

Introduction

During times of elevated pasture nonstructural carbohydrate (pNSC) content, specifically spring and fall months, horses may be at an increased risk for pasture-associated laminitis and digestive upset. Laminitis is a devastating disease, linked with metabolic and digestive dysfunction due to intake of elevated pNSC, that manifests in hoof. Approximately half of all laminitis cases are pasture associated and it is a leading cause of veterinary treatment [18]. The effects of NSC content on the physiological response of horses has been most studied using a grain-based concentrate model in horses. It is important to consider the effects of pNSC content on grazing horses since approximately half of all laminitis cases are pasture-associated. There is a need for further research on pNSC content to optimize nutritional management of horses to reduce the risk of NSC-associated diseases.

The pNSC content is comprised of starch and water soluble carbohydrates (WSC) which includes fructan and ethanol soluble carbohydrates (ESC). The plant accumulates NSC during photosynthesis, in the presence of sunlight and water. The NSC are used as energy for the plant for growth and reproductive processes. When NSC production exceeds the needs of the plant, NSC are stored as reserve carbohydrates and can accumulate rapidly.

There are many factors that can influence the carbohydrate profile in forage leading to the risk of increased pNSC content and subsequent intake of elevated NSC. Variables such as temperature, sunlight, and plant stress due to drought or frost can lead to seasonal and circadian fluctuations in pNSC concentrations [1,19,23]. Individual species and varieties of grasses can have differing levels of NSC content, especially comparing warm and cool season grasses, but typically cool season grasses have elevated NSC content compared to warm season grasses [19]. This is due to the ability of cool season grasses to store fructan in the leaf vacuoles of the plant, while warm season grasses store starch in the chloroplasts of the leaves. Warm season forages

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have the ability to limit starch accumulation when the plant becomes saturated; however, cool season plants do not have this ability to limit fructan accumulation.

Although pNSC content is typically most elevated in the spring and fall, varying weather patterns can lead to fluctuating patterns of pNSC throughout the year. The pNSC of cool season grasses have been implicated in the development of laminitis due to their rapid fermentation in the hindgut and ability to cause insulin dysregulation [2]. More research is needed to understand the patterns of fluctuating pNSC content in order to predict when grazing horses are at an increased risk for metabolic and gastrointestinal disturbances. The objective of this study was to characterize seasonal and circadian fluctuations in pasture nutrient composition, specifically NSC, in a Virginia horse pasture.

Materials and Methods

Weekly pasture samples were collected for 52 wks beginning in October 2016 to investigate the seasonal and circadian fluctuations of forage nutrients as well as macro- and microclimate conditions of an 8.5-ha horse pasture at the Middleburg Agricultural Research and Extension Center in Virginia.

Forage sample collection and analysis. Forage samples were collected four times per wk at 0800 and 1600 h on d 1 and 0800 and 1300 h on d 2. Approximately 200 g (wet weight) of clipped forage was collected (no more than 2.5 cm from the base) every 5 meters at random along a "W" pattern throughout the pasture to obtain a representative sample. Samples were weighed and dried at 70ºC in an oven to determine DM. Dried forage samples were submitted to a commercial laboratory (Equi-analytical, Ithaca, NY) to determine nutrient content including digestible energy (DE), crude protein (CP), starch, ethanol soluble carbohydrates (ESC), water

soluble carbohydrates (WSC), and NSC (starch + WSC). All sample analyses were performed in duplicate.

Macro- and microclimate. Environmental conditions were also measured four times per wk at 0800 and 1600 h on d 1 and 0800 and 1300 h on d 2. The macroclimate conditions included high and low ambient temperature (ºC), relative humidity (%), and solar radiation (watts/m^2). These measurements were recorded by a local weather station (BestForecast™). Microclimate conditions were measured at 5 random points on the "W" pattern and averaged for a composite reading at 0800 and 1600 on d 1 and 0800 and 1300 h on d 2. Forage canopy temperature (ºC) was measured using an infrared thermometer (Mikron Infrared Inc., Oakland, NJ), soil temperature (ºC) was measured using a digital thermometer (Bradshaw International Inc., Rancho Cucamonga, CA), and photosynthetically active radiation (PAR) (μmolm^{-2}s^{-1}) was measured using an AccuPAR Model LP-80 point sensor below the canopy (Decagon Inc., Pullman, WA).

Statistical analysis. A linear mixed effects repeated measures ANOVA was used to assess the effects of week to examine seasonal changes in the nutrient profile and environmental measurements. All data were analyzed using The SAS System (v. 9.4.; SAS Institute, Cary, NC 27513) with $\alpha = 0.05$ defined as statistically significant. Model adequacy was assessed graphically using plots of studentized residuals and quantile-quantile plots. Paired t-tests were performed to investigate differences between AM and PM samples.

To estimate correlations between pasture measurements and environmental variables a multivariate repeated measures mixed effects linear model [118] was fit using the MIXED procedure of the SAS System. From the output of the MIXED procedure, within-subject correlation coefficients were hand calculated between each environmental and each pasture

variable along with *P*-values to test whether they were significantly different from zero [119]. Because there were unequal numbers of observations in the subclasses the Kenward-Roger adjustment method was used to calculate denominator degrees of freedom. We used a first-order autoregressive covariance structure to account for covariation across time within the same observational units.

Results

The macro- and microclimate variables of the environment and pasture are listed in Table 3.1 (macroclimate) and Table 3.2 (microclimate). The highest temperatures were recorded in July when the temperature peaked at 33.8˚C. The lowest ambient temperature during the yearlong study occurred in December at -9.5˚C. Relative humidity fluctuated throughout the year with the lowest average humidity in February (63.1%) and the highest average humidity in September (82.6%). Mean solar radiation also varied by month with the lowest radiation in December (114.8 watts/m^2) and the highest radiation levels in June (515.7 watts/m^2). Soil and canopy temperature of the pasture were lowest in the winter months (January and December, respectively) and highest in July (27.7˚C and 26.3˚C, respectively). Photosynthetically active radiation (PAR) on average was most elevated in June (828.5 mol m^{-2}s^{-1}) and lowest in November (72.8 mol m^{-2}s^{-1}).

The pasture nutrient profile also fluctuated seasonally over the 52 wk study (Figure 3.1 and Figure 3.2). Diurnal fluctuations (AM vs PM) of pasture nutrients and environmental conditions are listed in Table 3.3. Digestible energy fluctuated seasonally and diurnally with higher concentrations typically in the PM hours. In addition, DE was highest during May at 2.35 ± 0.09 Mcal/kg and lowest in March at 1.81 ± 0.12 Mcal/kg. Crude protein was most elevated at the end of the study in September 2017 at 23.35 %DM. There were no apparent diurnal effects

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on crude protein. Pasture NSC concentrations were most elevated in the PM hours as well as during May (wk 32; 25.45% DM). NSC was lowest in the AM hrs at 5.3% DM and in January (wk 17). Pasture WSC concentrations were also highest in the PM hours and in May (wk 33; 19.65% DM). WSC content was lowest in the AM hrs and in January (3.65% DM). Starch also fluctuated seasonally and diurnally, with the highest concentrations during PM hours and in May (wk 32; 7.25% DM).

Correlation coefficients were calculated (Table 3.3) for pasture carbohydrate variables (nonstructural carbohydrates [NSC], starch, and water soluble carbohydrates [WSC]) and environmental variables (ambient temperature, relative humidity, solar radiation, rainfall, soil temperature, canopy temperature, and photosynthetically active radiation [PAR]). Overall NSC and starch were positively correlated with environmental variables. PAR had the strongest correlation with NSC ($r=0.51$; $P \leq 0.05$) and starch ($r=0.84$; $P \leq 0.05$). Relative humidity and solar radiation were moderately correlated with NSC ($r=0.38$ and $r=0.32$, respectively), while strongly correlated with starch in the pasture ($r=0.71$ and $r=0.74$, respectively).

Discussion

Fluctuations in pasture nutrients, specifically nonstructural carbohydrates, are of special interest to equine nutritionists, veterinarians, and horse owners due to the potential health risks associated with metabolic and digestive disturbances from ingestion of elevated NSC content. Anecdotally, more cases of pasture-associated laminitis are noted in the spring and fall months but there is limited research on the seasonal and diurnal fluctuations in cool season pasture NSC concentrations. This study aimed to describe seasonal and diurnal fluctuations in environmental conditions as well as the nutrient profile of a cool season Virginia horse pasture. As expected, pasture NSC concentrations fluctuated seasonally being highest in the spring and fall months.

The NSC content was also most elevated in the afternoon, especially on days which had cooler temperatures in the morning, followed by increased PAR measurements. Another study recorded similar seasonal and diurnal variations in NSC content in a cool season horse pasture, with NSC being most elevated in spring months (April) and late afternoon hours [120].

Additionally, Prince and others evaluated differences in WSC content in various cool season grasses in Kentucky. They concluded WSC concentrations were also highest in the afternoon hours. This occurrence is likely due to increased PAR during the daylight hours. When environmental conditions favor chilly temperatures but sunny days, NSC content has been shown to be most elevated. These studies support the findings in our seasonal grazing study in which spring and fall months had the most elevated NSC concentrations. These data support the common observation that more cases of laminitis occur in the spring and fall months, with approximately half of all laminitis cases occurring on pasture [18].

Environmental factors play a large role in the accumulation of starch and sugar in forages. Cool season plants accumulate more NSC content under cooler ambient temperatures during the day (10°C) and night (5°C) compared to warmer temperatures during the day (25°C) and night (15°C) [20]. This is one factor that likely contributes to higher spring and fall pasture NSC concentrations. Another important process for the plant is transpiration which is the act of moving water into the atmosphere. Plants do well at relative humidity up to 80%. As humidity levels rise, the plant may slow the rate of photosynthesis to direct further efforts towards transpiration. This study reported humidity levels greater than 80% during summer months. This, in combination with higher temperatures (27-30°C), may be two reasons summer months had decreased pasture NSC content.

The threshold for pasture NSC content is 10-12% DM for equines at risk of developing insulin resistance or laminitis [16]. It is advised to avoid grazing when NSC concentrations are above that limit. Based on the diurnal variation of pasture NSC content, predisposed horses and ponies should graze in the early hours of the morning when NSC concentrations are the lowest before photosynthesis has begun. However, the current study measured NSC concentrations above the threshold limit during spring and fall months even in the early morning. Limiting grazing to only the early morning hours during spring and fall months may still result in excessive NSC intake for overweight or insulin dysregulated horses depending on environmental conditions and forage quality.

Conclusions

The present study found the greatest NSC concentrations during the spring and fall months as well as late afternoon hours. These seasonal and diurnal fluctuations may be influenced by temperature, solar radiation, humidity, and PAR. Environmental interactions may impact the level of NSC content grazing horses are consuming which could lead to metabolic and digestive disturbances, especially in predisposed equine. Horses at-risk of developing obesity, insulin resistance, or laminitis may benefit from avoiding grazing lush spring and fall cool season pastures. The present study found NSC content greater than 12% during spring and fall months which is above the recommended threshold for animals predisposed to metabolic and gastrointestinal disease. Environmental factors may further exacerbate the levels of NSC content during spring and fall months which may overwhelm the equine small intestine digestive capacity. Understanding how to manage horse pastures based on the nutritional needs of the animals may aid in reducing the risk of metabolic and digestive-linked diseases that manifest from the intake of elevated pasture NSC concentrations.

Table 3.1. Summary of macroclimate variables by month over the 52 wks. Data are summarized as mean ± SE, minimum (Min) and maximum (Max) (n = 208).

Month	High Temperature (˚C)			Low Temperature (˚C)			Humidity (%)			Solar Radiation (watts/m^2)		
	Mean ± SE	Max	Min	Mean ± SE	Max	Min	Mean ± SE	Max	Min	Mean ± SE	Max	Min
Oct '16	21.3±1.8	28.9	14.2	7.6±1.4	16.3	0.3	73.6±2.5	83.0	58.0	226.3±60.5	793.0	16.0
Nov	16.2±1.5	26.8	8.9	3.2±1.1	9.7	-5.8	73.5±4.8	97.0	46.0	130.2±31.1	476.0	0.0
Dec	8.9±1.7	19.9	2.7	-1.5±1.2	7.8	-9.5	70.5±4.3	91.0	48.0	114.8±37.1	455.0	5.0
Jan '17	11.2±1.4	17.8	3.9	0.9±1.1	6.4	-8.2	73.8±5.1	96.0	53.0	148.2±48.4	694.0	0.0
Feb	15.3±1.6	21.5	9.7	3.0±1.1	10.0	-1.5	63.1±1.9	74.0	57.0	151.6±43.9	596.0	0.0
Mar	14.5±2.5	22.5	-0.2	4.2±1.5	11.9	-6.9	66.0±5.6	87.0	41.0	327.9±82	851.0	0.0
Apr	23.7±1.4	29.1	15.7	11.1±0.7	15.6	5.6	65.9±4.7	86.0	48.0	319.9±75.2	933.0	28.0
May	22.3 ± 1.5	32.3	16.5	10.5±1.1	17.4	2.3	71.9±4.4	93.0	57.0	421.7±79.7	1044.0	25.0
Jun	27.5 ± 1.4	33.1	16.5	16.2±0.9	22.3	2.3	69.8±2.6	85.0	57.0	515.7±74.3	1058.0	25.0
Jul	30.9 ± 1.0	33.8	25.2	18.9±0.5	21.6	14.3	76.5±1.7	87.0	70.0	357.6±59.1	872.0	70.0
Aug	28.1 ± 1.3	32.8	18.1	17.2±0.5	19.9	12.7	80.1±2.3	93.0	71.0	418.1±77.2	1065.0	19.0
Sep	27.0 ± 1.3	31.0	19.1	15.9±0.4	18.1	12.8	82.6±2.1	94.0	74.0	274.6±63.0	729.0	19.0

Table 3.2. Summary of microclimate variables by month over the 52 wks. Data are summarized as mean ± SE, minimum (Min) and maximum (Max) (n = 208).

Month	Soil Temperature (˚C)			Canopy Temperature (˚C)			PAR (mol m^{-2}s^{-1})		
	Mean ± SE	Max	Min	Mean ± SE	Max	Min	Mean ± SE	Max	Min
Oct '16	13.0±2.1	19.3	-17.8	17.1±1.8	26.8	4.1	84.1±20.0	259.0	7.0
Nov	10.1±0.7	15.8	4.5	13.1±1.0	23.6	3.4	72.8±18.7	307.0	4.0
Dec	5.3±0.5	8.5	2.0	8.5±1.2	18.7	-0.3	125.9±40.7	560.0	3.0
Jan '17	4.7±0.6	7.4	0.6	10.1±0.9	18.7	2.1	148.4±47.2	793.0	4.0
Feb	6.4±0.6	10.1	2.8	12.2±1.2	23.3	5.8	187.7±44.2	601.2	29.0
Mar	7.9±1.6	15.0	-17.8	12.3±2.1	21.3	-17.8	464.9±129.3	1570.0	18.0
Apr	15.6±0.6	21.1	12.6	18.8±1.0	25.1	13.9	531.4±126.9	1606.2	49.6
May	19.2±0.6	23.7	12.6	20.1±0.9	30.1	13.4	639.7±137.7	1902.2	57.8
Jun	25.2±1.0	33.8	12.6	24.3±0.9	30.1	13.4	828.5±140.4	1959.0	57.8
Jul	27.7±0.7	33.1	22.8	26.3±0.8	34.4	21.8	598.8±118.1	1826.0	49.6
Aug	24.7±0.6	30.6	20.3	23.9±0.7	29.5	17.5	406.7±77.8	1303.4	9.8
Sep	22.8±0.6	27.6	18.6	22.4±1.1	29.9	13.9	331.2±79.8	1155.6	6.0

Table 3.3. Summary of the diurnal variation (AM vs PM) in pasture nutrients and environmental variables over the 52 wks.

Hour	Variable	N	Minimum	Median	Maximum	Mean	Std Dev
AM	Digestible energy (MCal/kg)	103	1.81	2.01	2.23	2.01	0.09
	Crude protein (% DM)	103	7.25	11.85	23.10	12.78	3.91
	Nonstructural carbohydrate (% DM)	103	5.30	10.50	19.45	11.17	3.25
	Starch (% DM)	103	0.20	1.80	4.95	1.92	1.13
	Water soluble carbohydrates (% DM)	103	4.15	8.90	17.80	9.25	3.08
	Ethanol soluble carbohydrates (% DM)	103	1.90	7.25	12.95	6.88	2.24
	Solar radiation (watts/m^2)	103	0.00	44.00	369.00	94.27	102.39
	Soil temperature (˚C)	102	0.61	13.70	26.39	14.14	7.69
	Canopy temperature (˚C)	103	-0.33	14.67	26.95	14.51	6.78
	Photosynthetically active radiation (mol m^{-2}s^{-1})	103	4.00	72.00	522.40	145.12	142.55
PM	Digestible energy (MCal/kg)	104	1.86	2.07	2.35	2.07	0.12
	Crude protein (% DM)	102	6.90	11.93	23.35	12.58	3.92
	Nonstructural carbohydrate (% DM)	104	4.50	13.20	25.45	13.29	4.02
	Starch (% DM)	104	0.20	2.70	7.25	2.75	1.29
	Water soluble carbohydrates (% DM)	104	3.35	10.53	19.65	10.55	3.47
	Ethanol soluble carbohydrates (% DM)	104	1.60	8.40	14.50	7.92	2.66
	Solar radiation (watts/m^2)	103	5.00	455.00	1065.0	476.47	302.04
	Soil temperature (˚C)	104	0.56	16.56	33.78	16.89	8.94
	Canopy temperature (˚C)	104	4.94	20.72	34.39	20.60	6.36
	Photosynthetically active radiation (mol m^{-2}s^{-1})	104	3.00	384.30	1959.0	586.64	545.49

Table 3.4. Correlation coefficients[a] for pasture carbohydrate variables[b] (nonstructural carbohydrates [NSC][c], starch, and water soluble carbohydrates [WSC]) and environmental variables (ambient temperature, relative humidity, solar radiation, rainfall, soil temperature, canopy temperature, and photosynthetically active radiation [PAR] [d]).

Environmental Variables	Carbohydrate Variables[b]		
	NSC[c]	Starch	WSC
Ambient Temperature (°C)	0.23†	-0.33*	0.36*
Relative Humidity (%)	0.38*	0.71*	-0.26†
Solar Radiation (watts/m²)	0.32*	0.74*	-0.37*
Rainfall (cm)	0.23†	0.38*	-0.16
Soil Temperature (°C)	0.17	0.37*	-0.14
Canopy Temperature (°C)	0.22	0.29*	-0.20
PAR (μmol m^{-2}s^{-1}) [d]	0.51*	0.84*	-0.27†

[a] probability $P > |r|$ under H0: Rho = 0) ($P \leq 0.05$).
[b] Analysis (AOAC, 1990) performed by Dairy One, Ithaca, NY
[c] Nonstructural carbohydrate (NSC) = WSC + starch.
† $P \leq 0.10$, * $P \leq 0.05$
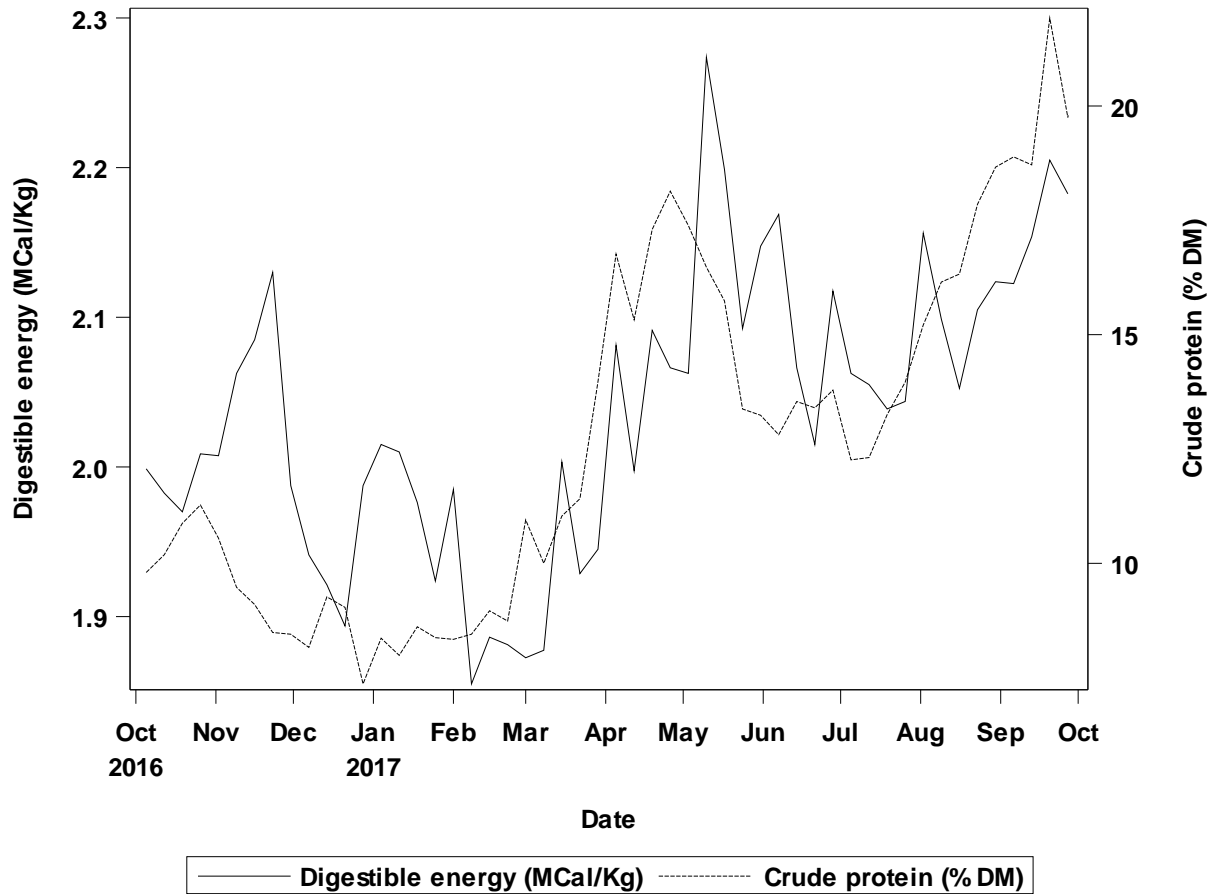
Figure 3.1. Digestible energy (DE; Mcal/kg) and crude protein (CP; % DM) on a dry matter (DM) basis of the pasture forage by week over the 52 wks.
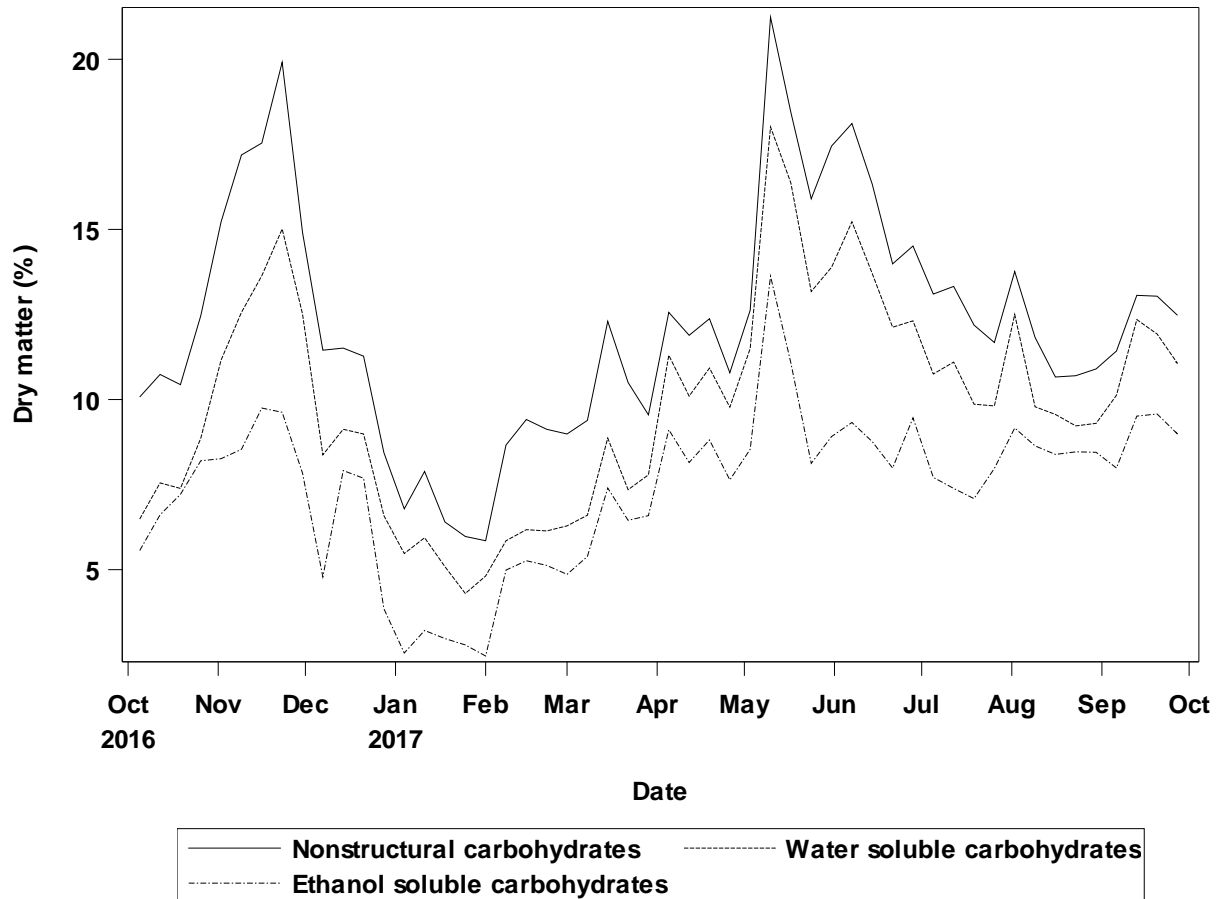
Figure 3.2. Nonstructural carbohydrates (NSC), water soluble carbohydrates (WSC), and ethanol soluble carbohydrates (ESC) on a dry matter (% DM) basis of the pasture forage by week over the 52 wks.
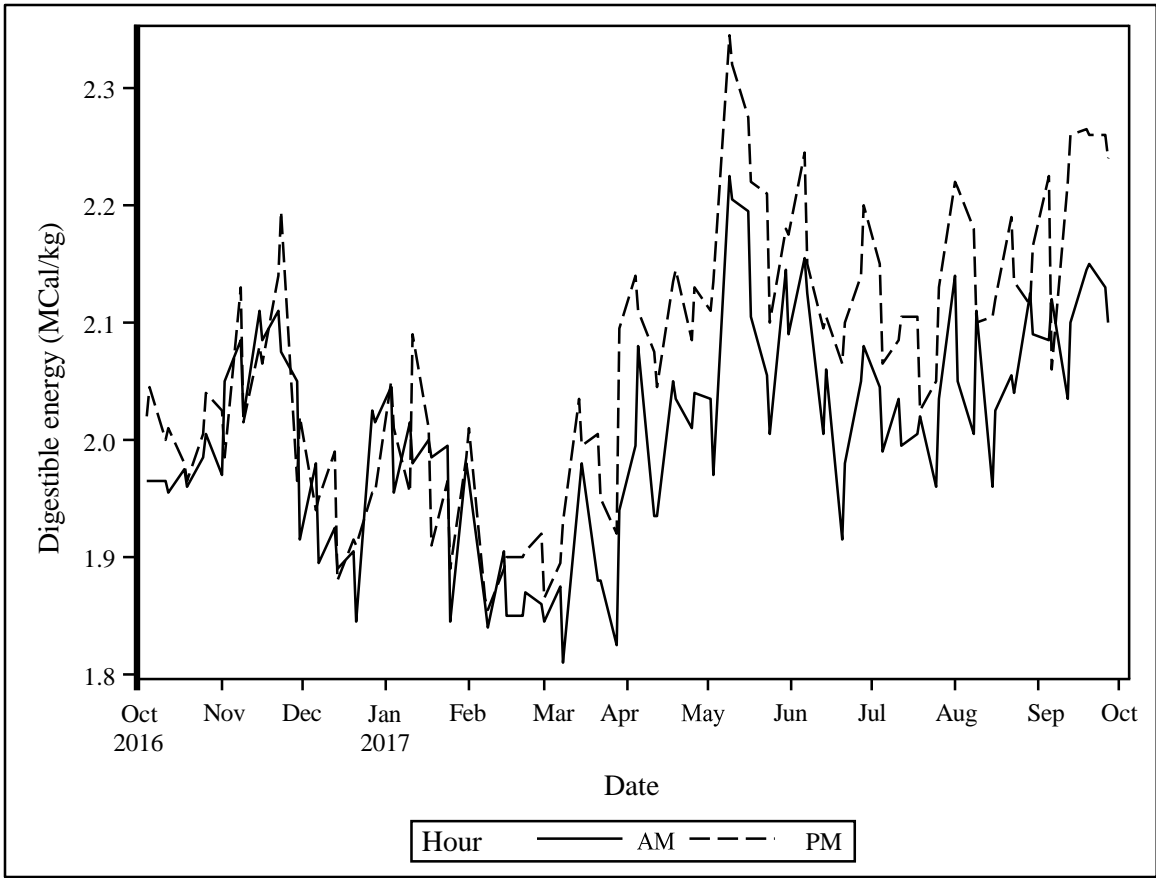
Figure 3.3. Diurnal variation (AM vs PM) in digestible energy (DE) over the 52 wks.
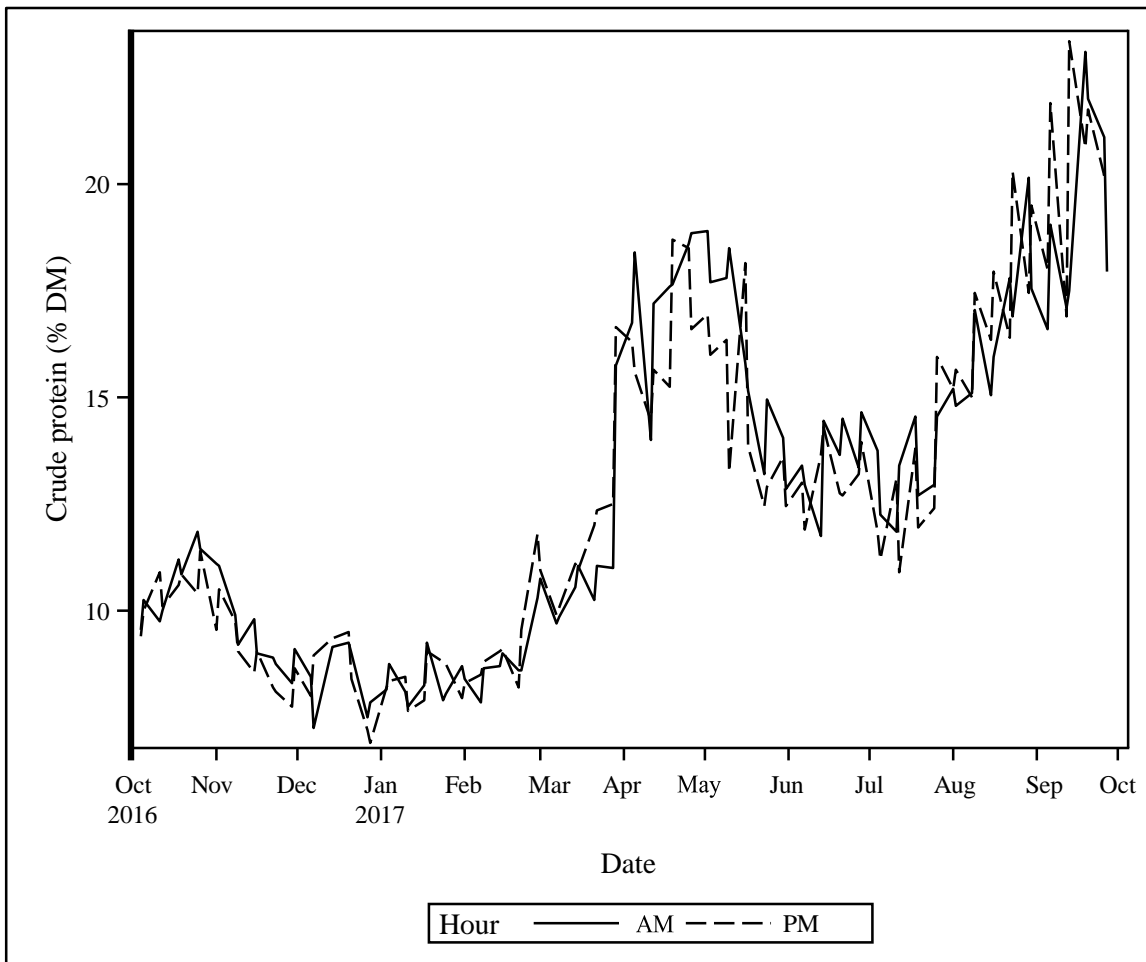
Figure 3.4. Diurnal variation (AM vs PM) in crude protein (CP) over the 52 wks.
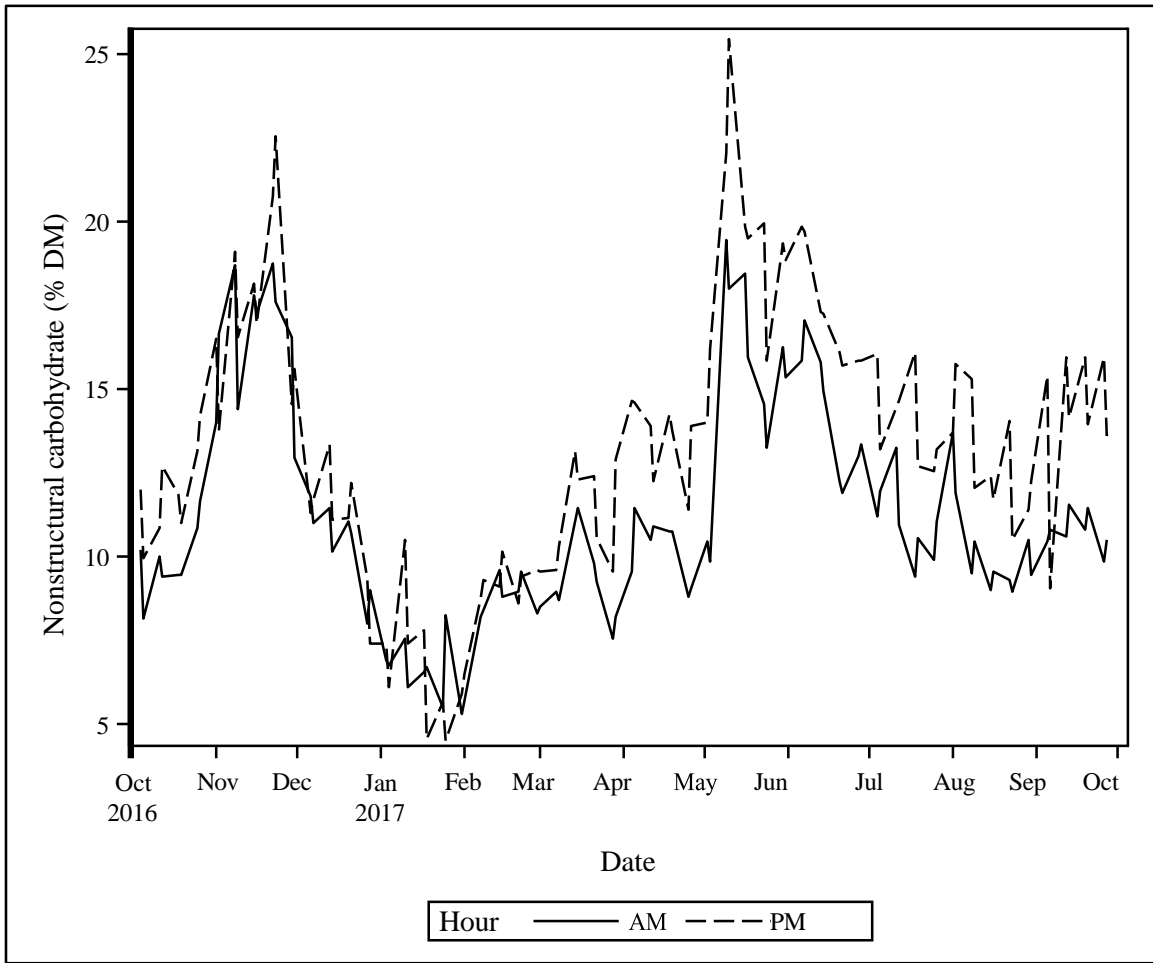
Figure 3.5. Diurnal variation (AM vs PM) in nonstructural carbohydrate (NSC) over the 52 wks.
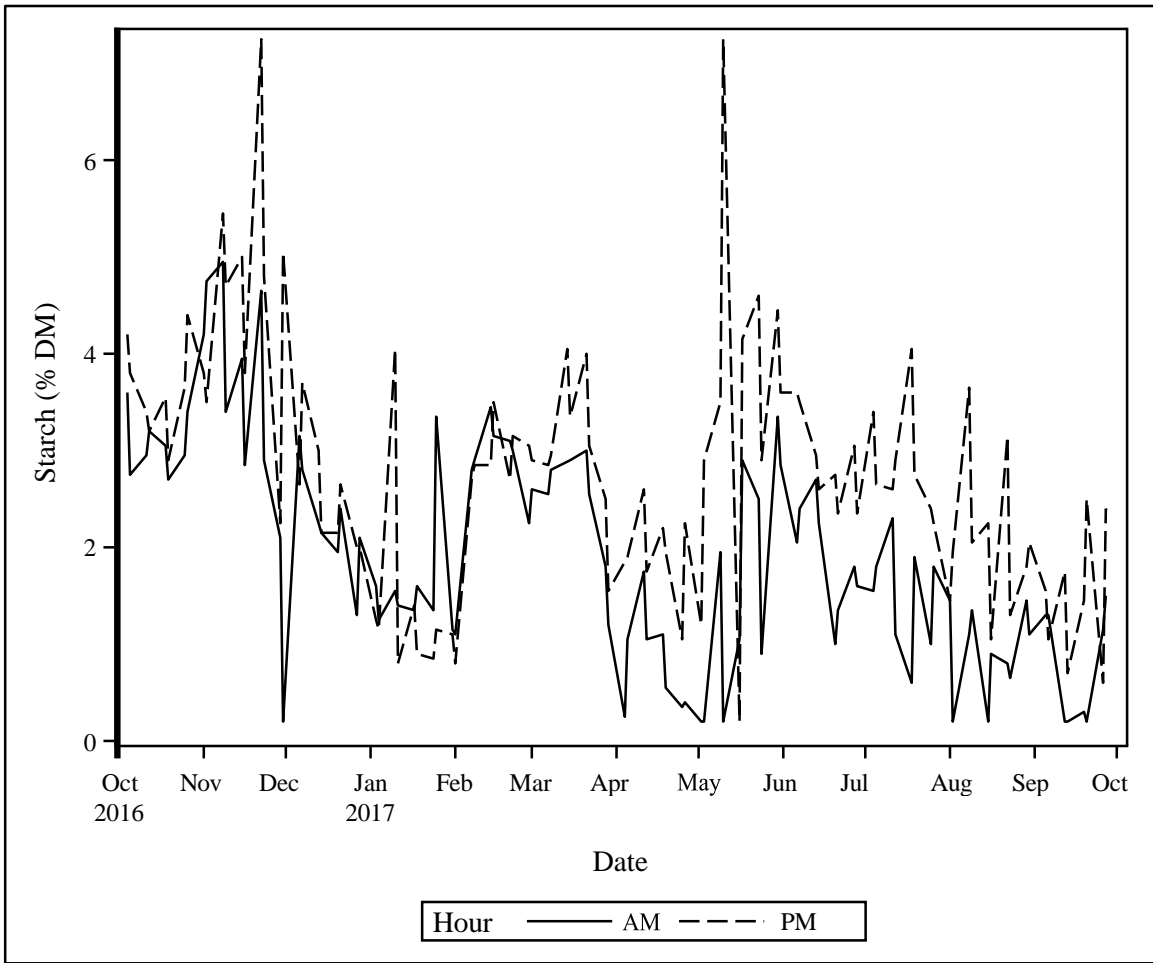
Figure 3.6. Diurnal variation (AM vs PM) in starch over the 52 wks.
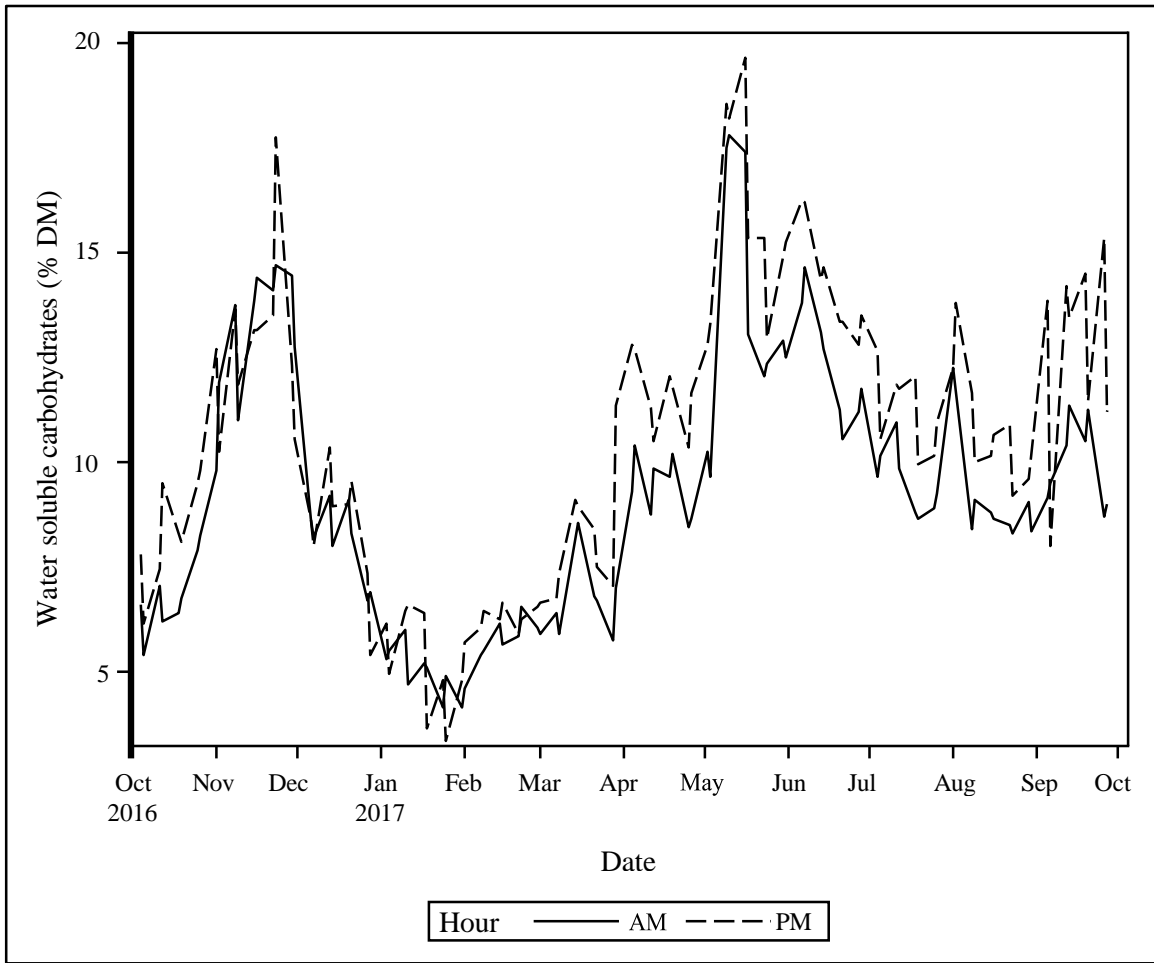
Figure 3.7. Diurnal variation (AM vs PM) in water soluble carbohydrates (WSC) over the 52 wks**.**
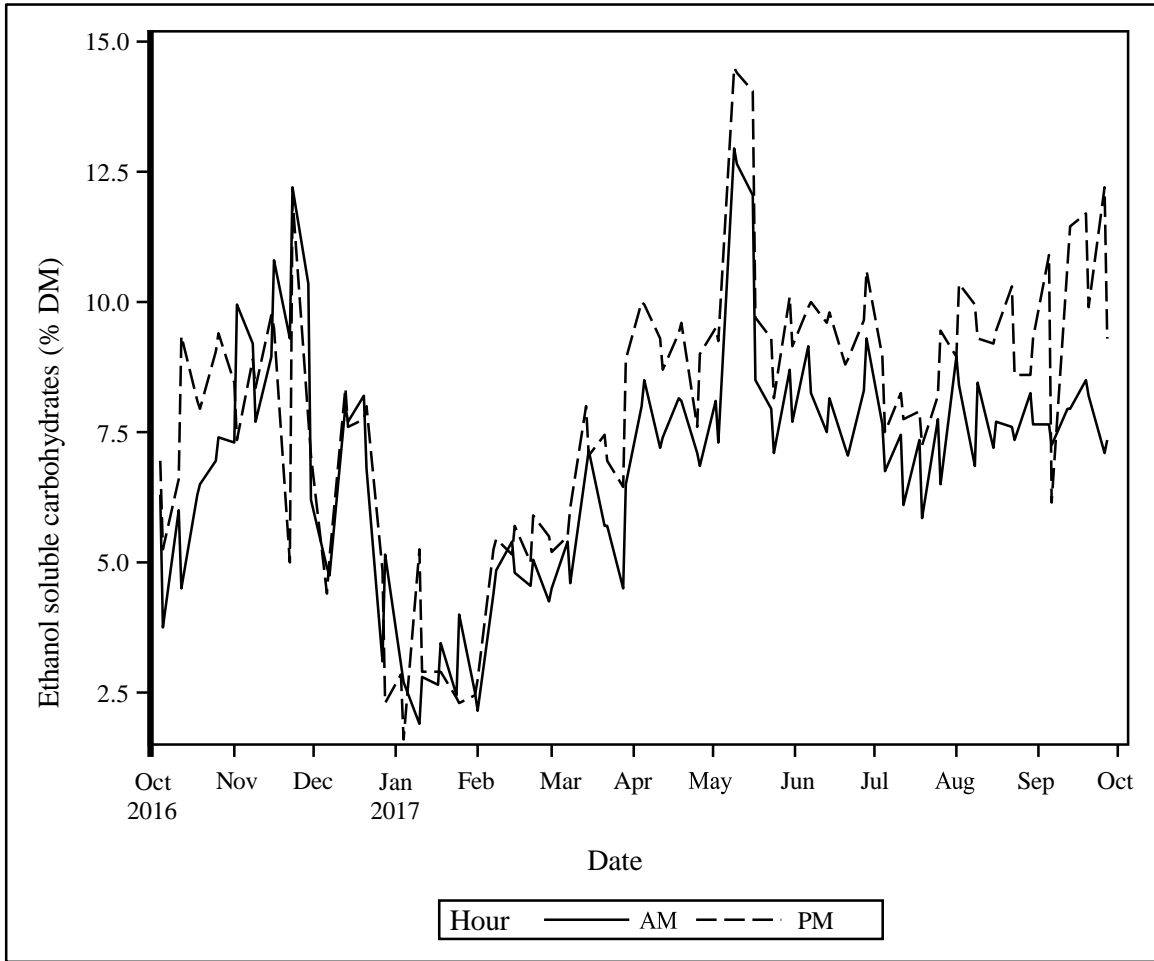
Figure 3.8. Diurnal variation (AM vs PM) in ethanol soluble carbohydrates (ESC) over the 52 wks**.**

Chapter Four

Seasonal fluctuations in the metabolic and morphometric response of grazing horses

Abstract: The intake of elevated pasture nonstructural carbohydrates (pNSC) can cause metabolic dysfunction in horses. This study aimed to investigate the influence of seasonal patterns of pNSC content on metabolic and morphometric responses in grazing horses. Twelve sporthorse mares (15 ± 3.4 yrs) were maintained together on an 8.5-ha cool season mixed grass pasture (free-choice mineral supplementation) for 52 wks beginning October 2016. Weekly pasture samples (200g wet weight) were clipped at random, 2.5 cm from plant base. Samples were weighed, dried at 70ºC, and analyzed to determine weekly pNSC content (Equi-analytical, Ithaca, NY). Corresponding weekly blood samples were collected via jugular venipuncture into 4 mL potassium oxalate and 7 mL EDTA vacutainer tubes and analyzed for glucose (mg/dL) and insulin (µIU/mL). Body weight (BW), body condition score (BCS), and cresty neck score (CNS) were also evaluated weekly. All sample analyses were performed in duplicate. Data were analyzed using a repeated measures ANOVA in the Mixed procedure and PROC Corr in SAS (v. 9.4, SAS Institute Inc.; Cary, NC). Data are summarized as means $\pm$ SEM with a $P \leq 0.05$ considered statistically significant. Pasture nutrients differed by week ($P < 0.05$) with NSC concentrations being highest in May (wk 19) at 25.45% DM. There was seasonal variation in all morphometric measures in grazing horses. BW was highest in the spring ($P < 0.0001$), while BCS and CNS were highest in the fall ($P = 0.0021$ and $P < 0.0001$, respectively). Metabolic responses in grazing horses also fluctuated seasonally with glucose and insulin concentrations being most elevated in the spring ($P < 0.0001$). Pasture NSC content was correlated with weight ($r = 0.35$), glucose ($r = 0.21$), and insulin (0.26) in grazing horses and tended to have a relationship with CNS ($r = 0.14$). These data support the common observation that the incidence

of pasture-associated laminitis is increased in the spring and fall months when NSC content is highest in cool season pastures. The findings from this research will lead to optimized grazing management strategies to reduce the risk of pasture-associated laminitis in horses predisposed to obesity and insulin dysregulation.

Keywords: Pasture, Seasonal, Insulin, Horses

Introduction

Obesity and insulin resistance are risk factors for developing laminitis and equine metabolic syndrome. Pasture-associated laminitis (PAL) is a devastating disease that accounts for nearly half of all reported laminitis cases, and is linked with the intake of elevated pNSC content [18]. Seasonal fluctuations in pNSC content are well documented, with spring and fall months typically having the highest accumulation of NSC. This pattern occurs due to the increased photosynthetic activity during the day and cooler temperatures at night that occur during the cool season forages growing period (spring and fall) in temperate climates [1,19].

Insulin sensitivity can be impacted by diet and condition in horses. When horses consumed a high starch diet (53% NSC), insulin sensitivity of glucose uptake was reduced by 30% [3]. Hoffman and colleagues investigated effects of diet and obesity on insulin and glucose dynamics in horses [4]. Obese horses had decreased insulin sensitivity compared to nonobese horses. Additionally, insulin sensitivity was decreased when horses consumed a high starch and sugar diet (46.2% NSC) compared to a high fat and fiber diet (14% NSC). The researchers suggested that insulin sensitivity was confounded by body condition when comparing the different diets.

Obesity is a growing concern in horses, with approximately half of the equine population being overweight or obese [121]. Generalized obesity and/or regional adiposity increases the risk of laminitis and equine metabolic syndrome. Specifically, the presence of a cresty neck could indicate a predisposition for these conditions [5]. Research by Carter and others found that together, BCS ($\geq$ 7) and CNS ($\geq$ 4) were useful predictors of laminitis in ponies [42].

Many horses are kept on pasture at least part of the time and may consume elevated NSC content, which could be dangerous for horses predisposed to metabolic conditions [35]. Glucose and insulin dynamics, as well as morphometric measures may fluctuate throughout the year

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further increasing the risk of developing PAL. However, there is limited research on the seasonal effects of fluctuating pasture NSC content on insulin and glucose dynamics, as well as morphometric measures in horses. Therefore, the objective of this study was to evaluate seasonal patterns of pasture NSC content, and to characterize seasonal fluctuations in the metabolic and morphometric response in grazing horses.

Materials and Methods

This study investigated the influence of fluctuating pasture nutrition on the metabolic and morphometric response of grazing horses. Twelve mares were selected for this study based on their previous history; half of the mares were previously laminitic (n=6) and the other half were non-laminitic (n=6). The previously laminitic mares exhibited a metabolic phenotype including regional and/or generalized obesity and a cresty neck greater than 2.5 on the scale of 0-5 [122]. None of the mares had active signs of laminitis at the beginning of the study.

The horses were maintained on an 8.5-ha cool season mixed grass pasture for 52 wks at the Middleburg Agricultural Research and Extension Center in Virginia. The pasture consisted of approximately 52% Tall fescue, 43% Kentucky bluegrass, 3% other grass, and 2% weed species. Corresponding blood and pasture samples were collected weekly throughout the year beginning in October 2016.

Pasture. Forage samples were collected two times the day prior to horse sampling at 0800 and 1600 h. Random pasture samples (200 g wet weight) were clipped every 5 meters throughout the pasture, 2.5 cm from the base of the plant. Samples were weighed, dried at 70º C, and then submitted to a commercial laboratory (Equi-analytical, Ithaca, NY) to determine nutrient content including digestible energy (DE), crude protein (CP), starch, water soluble

carbohydrates (WSC) and NSC (starch + WSC). All sample analyses were performed in duplicate and nutrient values at 0800 and 1600 h were averaged.

Horses. Twelve mares were maintained on an 8.5-ha cool season mixed grass pasture with water, mineral (Buckeye Nutrition, Dalton, OH) and white salt *ad libitum*. The horses were aged 15 ± 3.4 yrs (range 9-19), mean body weight (BW) of the horses was 621.8 ± 67.8 kg (range 466.3-770.7 kg), body condition score (BCS) averaged 6.19 ± 1.01 (range 4.0-8.5 on the 1-9 scale), and cresty neck score (CNS) averaged 2.39 ± 0.99 (range 1.0-4.7 on the 0-5 scale) [36,122]. The horses received supplemental hay during winter months due to reduced pasture nutrition and cold weather (5.7% NSC). The horses were acclimated to the pasture 30 d prior to sampling.

Plasma. Blood samples were collected via jugular venipuncture into 4 mL potassium oxalate, 7 mL EDTA, and 10 mL lithium heparin vacutainer tubes (Fisher Scientific, St. Louis, MO) and stored on ice. Plasma was separated by centrifugation at 3000 x g for 10 min and stored at -20˚C until analysis. Plasma was analyzed for glucose (mg/dL) via biochemical analyzer (YSI 2300D SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA) and insulin (μIU/mL) using an enzyme-linked immunosorbent assay (Mercodia Equine Insulin ELISA, Mercodia AB, Uppsala, Sweden).

Morphometric measurements. Horses were assessed for BW, BCS (scale 1-9), and CNS (scale 0-5) weekly by two experienced evaluators and averaged.

Statistical analysis. A linear mixed effects repeated measures ANOVA was used to assess the effects of month to examine seasonal changes in morphometric measurements, as well as glucose and insulin plasma concentrations. All data were analyzed using The SAS System (v. 9.4.; SAS Institute, Cary, NC 27513) with $\alpha = 0.05$ defined as statistically significant. Model

adequacy was assessed graphically using plots of studentized residuals and quantile-quantile plots. Insulin data were logarithmically transformed before statistical analysis. Means with 95% confidence limits were back transformed and are displayed in all figures.

To estimate correlations between pasture measurements and measurements of horse morphometry and physiology a multivariate repeated measures mixed effects linear model [118] was fit using the MIXED procedure of the SAS System. From the output of the MIXED procedure, within-subject correlation coefficients were hand calculated between each horse and each pasture variable along with *P*-values to test whether they were significantly different from zero [119]. Because there were unequal numbers of observations in the subclasses, the Kenward-Roger adjustment method was used to calculate denominator degrees of freedom. We used a first-order autoregressive covariance structure to account for covariation across time within the same observational units.

Results

Pasture nutrients varied by week throughout the study ($P < 0.05$). The DE concentration was highest in May (wk 19) at 2.32 Mcal/kg and lowest in February (wk 6) at 1.86 Mcal/kg (Figure 4.1). The CP content was highest in September (wk 37) at 23.35% DM and lowest in December (wk 52) at 7.85% DM (Figure 4.1). The NSC and WSC content was highest in May (wk 19) at 25.45% DM and 18.20% DM, respectively (Figure 4.2). Lowest concentrations for the carbohydrate fractions (NSC and WSC) were in January at 6.5% DM (wk5) and 4.9% DM (wk 4), respectively. Lastly, starch content was highest in November (wk 44) at 4.75% DM and lowest in September (wk 37) at 0.7% DM (Figure 4.2).

There was seasonal variation in all morphometric measures in grazing horses. BW means differed by month ($P < 0.0001$) with the highest BW in June (wk 23) and the lowest BW in

March (wk13) (figure 4.3). BCS means also differed by month ($P = 0.0021$) with the highest

BCS in September (wk 39) and the lowest BCS at the start of the study in October (wk 40)

(figure 4.4). CNS means were different between month ($P < 0.0001$) with the largest CNS in

September (wk 38) and the smallest CNS in January (wk 1) (figure 4.5).

Metabolic responses in grazing horses also fluctuated seasonally. Mean glucose

concentrations differed by month ($P < 0.0001$) being most elevated in May (wk 18) and lowest in

June (wk 25) (Figure 4.6). Insulin concentrations differed by month ($P < 0.0001$) and were also

highest in May (wk 18) but were lowest in December (wk 48) (figure 4.7).

Correlation coefficients used to describe relationships between the pasture nutrient profile

and morphometric and metabolic responses in grazing horses are shown in Table 4.1. The DE

content in the pasture was associated with most horse variables including weight, BCS, CNS,

and insulin (r = 0.49, 0.16, 0.34, and 0.38, respectively). The pasture CP concentrations were

related to all horse variables and tended to be associated with glucose. There was a relationship

between ESC and weight, CNS, glucose, and insulin (r = 0.37, 0.30, 0.24, and 0.38), but not with

BCS. Similarly, the WSC content in the pasture was associated with all horse variables except

BCS. There was only a relationship between starch content in the pasture and BCS (r = 0.24) and

tendency with CNS (r = -0.14). Lastly, pasture NSC content was related to weight, glucose, and

insulin (r = 0.35, 0.21, and 0.26, respectively) with a tendency to be related to CNS in the horses.

Relationships between horse variables are displayed in Table 4.2. Weight was related to

BCS (r = 0.31), CNS (r = 0.35), and insulin (r = 0.31) in grazing horses, but not glucose. The

BCS of horses was associated with CNS and insulin (r = 0.36 and 0.14). CNS was related to

insulin (r = 0.16), but not glucose. Lastly, glucose and insulin had a moderate relationship (r =

0.51) in grazing horses.

Discussion

The pasture carbohydrate profile and metabolic response of the horses followed similar trends throughout the year with the most elevated NSC, glucose, and insulin concentrations in the spring (May). The intake of elevated NSC content can lead to metabolic disorders, including PAL, due to increased insulinemic responses [2,89]. Research by de Laat and others found laminitis could be caused within 48 hrs following induced hyperinsulinemia in otherwise insulin sensitive horses [123]. Therefore, investigation into insulinemic responses in grazing horses is important to identify horses at risk for developing PAL. In Virginia, research has shown PAL develops in spring months (March – May) in ponies [90,122]. A study by Donaldson and others found PAL cases develop in both fall (September) and spring months (May) [124]. Cool season pastures experience two vegetative growing periods leading to increased NSC accumulation in the spring and fall in temperate climates. Thus, grazing horses may intake elevated amounts of NSC content which has been linked to increased insulinemic responses.

There is limited research on seasonal variation in glucose and insulin dynamics in grazing horses. Research by Williams and others observed seasonal variation in glucose and insulin dynamics in grazing Standardbred horses [125]. Plasma insulin was highest in October, while plasma glucose was highest in November. In the study by Frank and others with PPID horses, they also found peak glucose and insulin concentrations in the fall (September). In the present study, glucose and insulin were highest in the spring months. Differences between the two studies may have been a result of sampling frequency. In the current study, samples were collected weekly to capture the frequent shifts that occur in pasture NSC profile due to fluctuating environmental conditions. In the study by Williams and colleagues, sampling occurred every 4 hr over one 24 hr period in spring (June), summer (August), and fall (October). The study by Frank and others sampled 1 day per month. With limited pasture sampling

timepoints in both of the aforementioned studies, pasture carbohydrate content may not be accurately represented since it can fluctuate rapidly depending on the current weather conditions. For example. if it was cloudy during the day of sampling, pasture NSC content would be low compared to a sunny day due to decreased photosynthetic activity. Thus, the insulin and glucose response of the horses would also likely have also been lower due to grazing lower NSC content.

When investigating changes in morphometric measures of grazing horses in our study, horses had the greatest BW in June, but the greatest BCS and CNS in September. Similarly, pasture NSC content had two peaks, in the spring and in the fall. The intake of high NSC concentrations is associated with increased regional adiposity and generalized obesity. However, there is limited information on the seasonal fluctuations in morphometric measurements in grazing horses. One study by Frank and others investigated the association of season and pasture grazing with blood hormone and metabolite concentrations in horses with Pituitary Pars Intermedia Dysfunction (PPID) [126]. Seasonal fluctuations were observed in body weight, BCS, and neck circumference with BCS and neck circumference being the greatest in October. The present study also found the greatest BCS and CNS in the fall months (September). With the growing epidemic of equine obesity and the health concerns associated with being overweight, careful consideration should be given to fluctuations in BW, BCS, and CNS, since they aid in the prediction of increased risk of PAL.

One limitation of this study was the inability to measure pasture intake of the mares. There are limited ways to measure pasture intake in grazing horses. One method is to weigh the horses pre and post grazing and calculate intake. Challenges with this method include accounting for water intake, defecation, urination, and labor intensive nature of weighing the horses twice per day. Another way to measure pasture forage intake is to take pasture measurements pre and

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post grazing. Limitations of this methodology include inaccurate sampling due to the sheer size of the study pasture (8.5-ha). Additionally, horses may have been resting leading up to sampling. Since horses were maintained on pasture alone, it is impossible to coordinate the horses actually grazing leading up to sample collection (blood draws). This may have led to decreased insulinemic and glycemic responses if the horses were resting leading up to sampling.

Conclusions

In conclusion, pasture and horse measurements followed a similar seasonal fluctuation pattern throughout the 52 weeks. Morphometric responses in grazing horses fluctuated seasonally with BW most elevated in the spring and BCS and CNS most elevated in the fall. Glucose and insulin concentrations also varied by month with the highest concentrations observed in the spring. The findings from the present study support the common observation that the incidence of pasture-associated laminitis is increased in the spring and fall months when NSC content is highest in cool season pastures. The findings from this research will lead to optimized grazing management strategies to reduce the risk of pasture-associated laminitis in horses predisposed to obesity and metabolic abnormalities.

Table 4.1. Correlation coefficients[a] for pasture variables (digestible energy [DE], crude protein [CP], ethanol soluble carbohydrates [ESC], water soluble carbohydrates [WSC], starch, and nonstructural carbohydrates [NSC][b]) and horse variables (weight, body condition score [BCS], cresty neck score [CNS], glucose, and insulin).

	DE	CP	ESC	WSC	Starch	NSC
Weight	0.49*	0.30*	0.37*	0.41*	0.002	0.35*
BCS [c]	0.16*	0.19*	0.17	0.09	0.24*	0.06
CNS [d]	0.34*	0.47*	0.30*	0.22*	-0.14†	0.14†
Glucose	0.13	0.13†	0.24*	0.21*	0.116	0.21*
Insulin	0.38*	0.44*	0.38*	0.33*	-0.07	0.26*

[a] probability P > |r| under H0: Rho = 0) ($P \leq 0.05$).
[b] Nonstructural carbohydrate (NSC) = WSC + starch.
[c] The average of 2 scores by experienced assessors; 1 to 9 scale.
[d] The average of 2 scores by experienced assessors; 0 to 5 scale.
† $P \leq 0.10$, * $P \leq 0.05$

Table 4.2. Correlation coefficients[a] of horse variables (weight, body condition score [BCS], cresty neck score [CNS], glucose, and insulin) of grazing horses. Correlations were significant if $P \leq 0.05$.

	BCS	CNS	Glucose	Insulin
Weight	0.31*	0.35*	-0.04	0.31*
BCS [b]		0.36*	0.08	0.14*
CNS [c]			-0.05	0.16*
Glucose				0.51*

[a] probability P > |r| under H0: Rho = 0), n = 624 ($P \leq 0.05$).
[b] The average of 2 scores by experienced assessors; 1 to 9 scale.
[c] The average of 2 scores by experienced assessors; 0 to 5 scale.
† $P \leq 0.10$, * $P \leq 0.05$
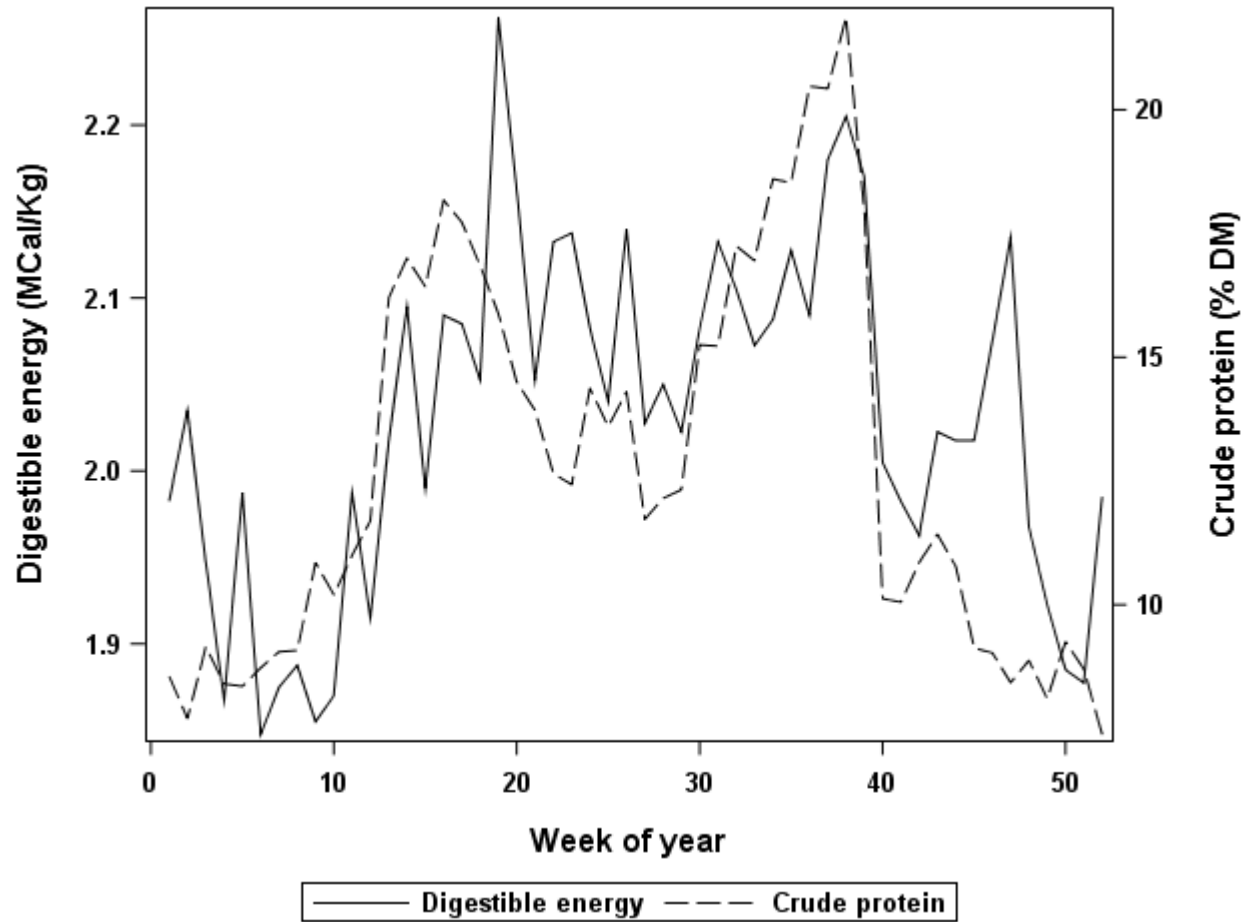
Figure 4.1. Seasonal variation in mean pasture digestible energy (Mcal/kg) and crude protein (%DM) over the 52 week study.
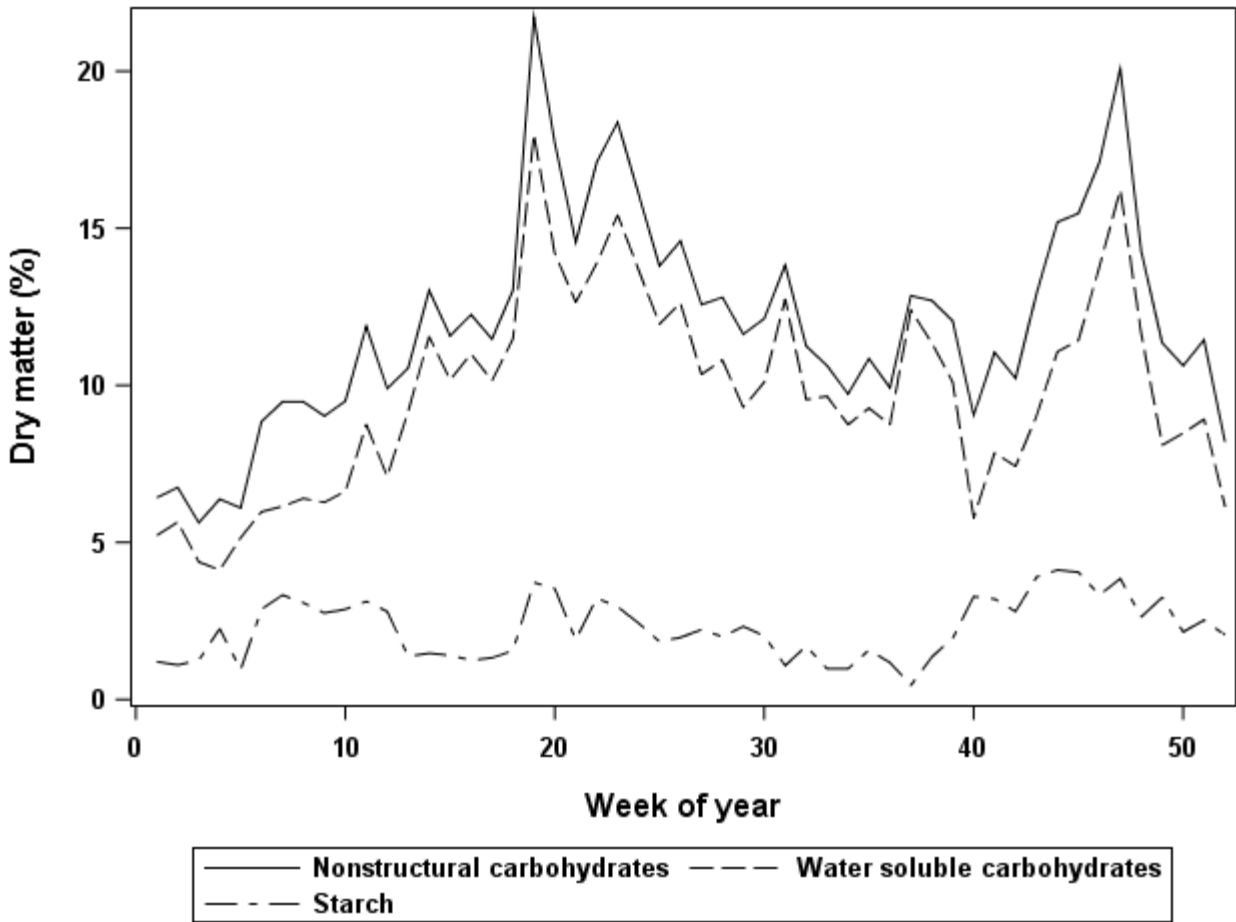
Figure 4.2. Seasonal variation in mean pasture percent dry matter of nonstructural carbohydrates, water soluble carbohydrates, and starch over the 52 week study.
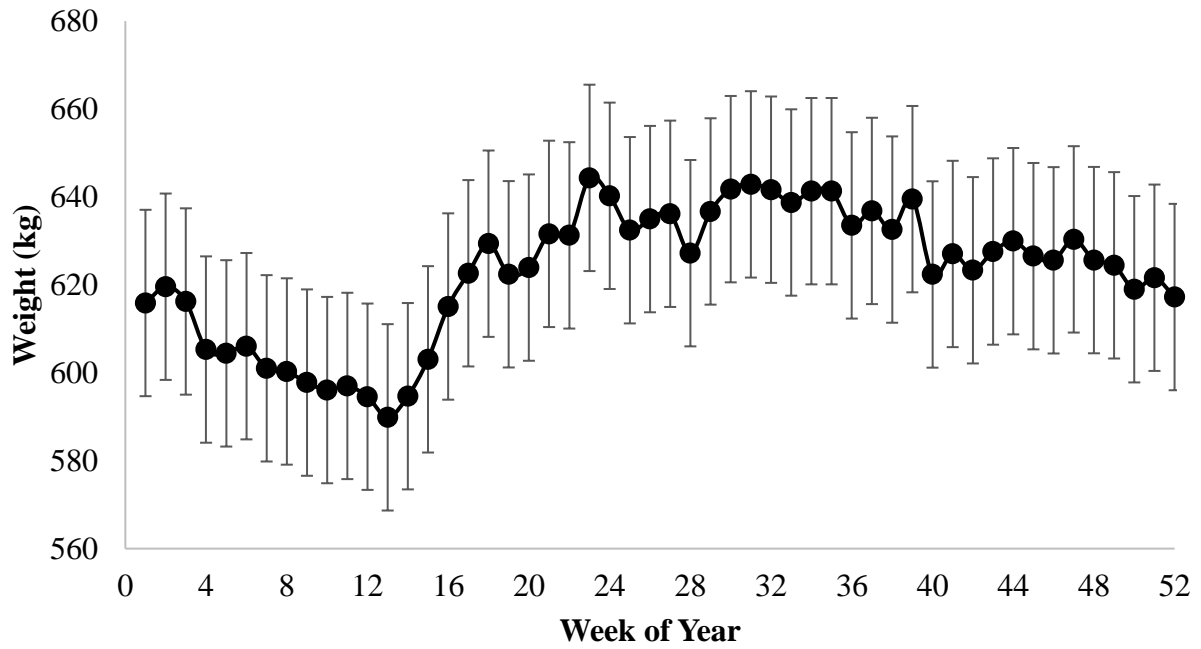
Figure 4.3. Seasonal variation in mean (± 95% confidence limits) body weight (BW) in horses maintained on a cool season pasture over 52 weeks.
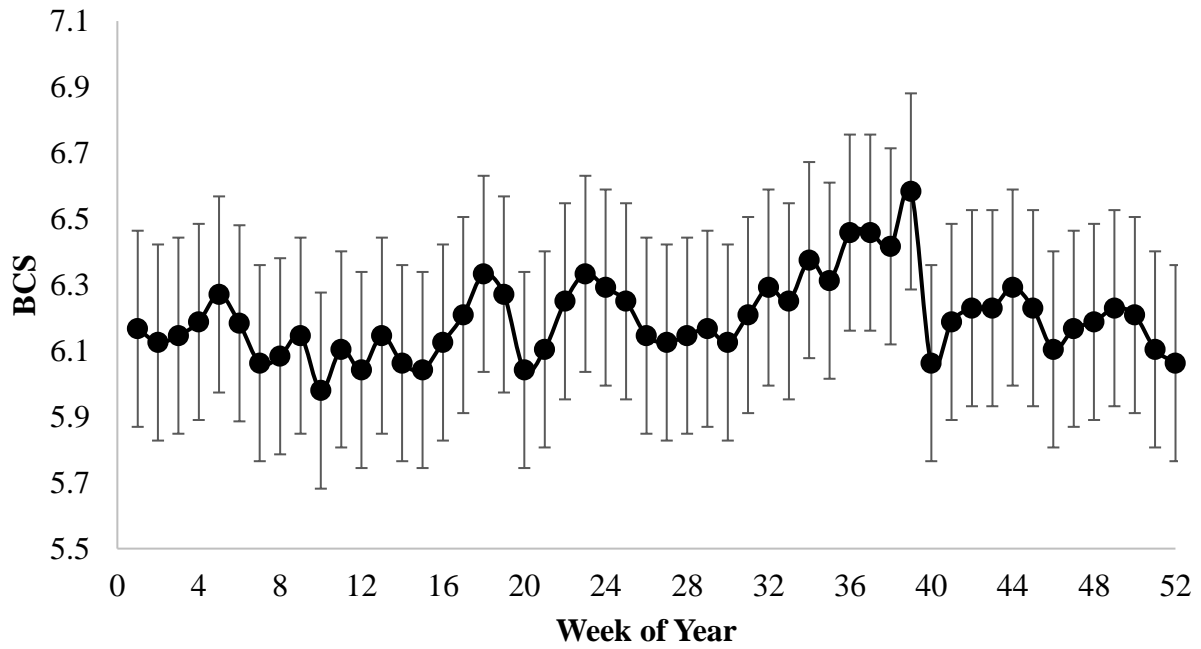
Figure 4.4. Seasonal variation in mean (± 95% confidence limits) body condition score (BCS; scale 1-9) in horses maintained on a cool season pasture over 52 weeks.
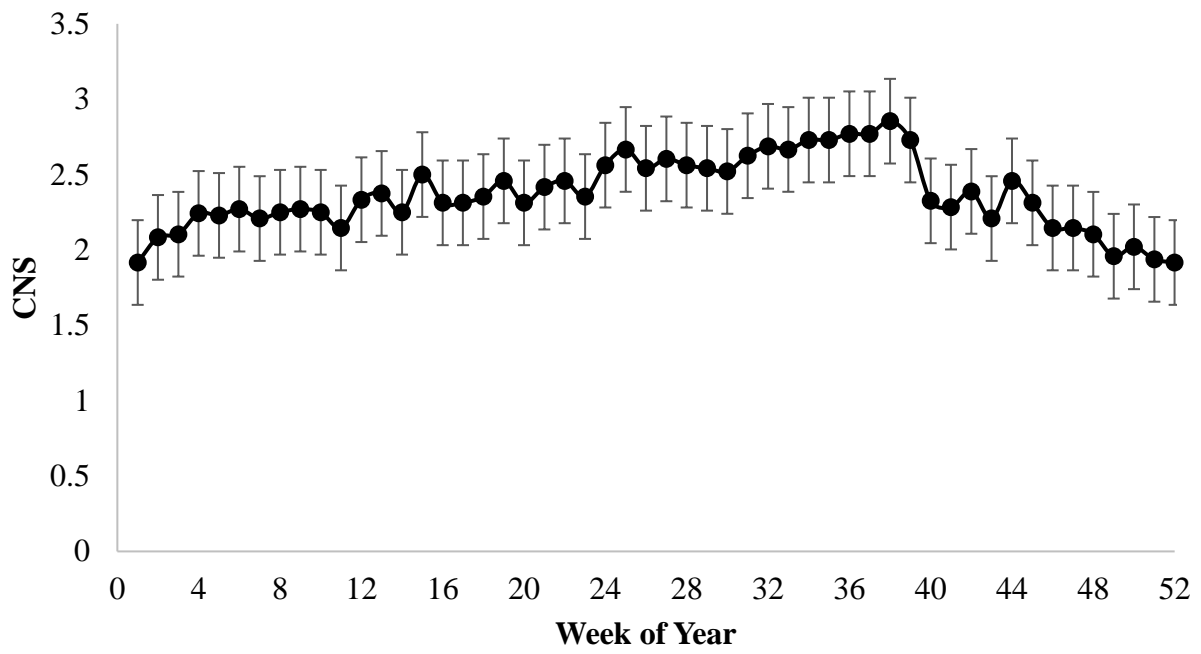
Figure 4.5. Seasonal variation in mean (± 95% confidence limits) cresty neck score (CNS; scale 0-5) in horses maintained on a cool season pasture over 52 weeks.
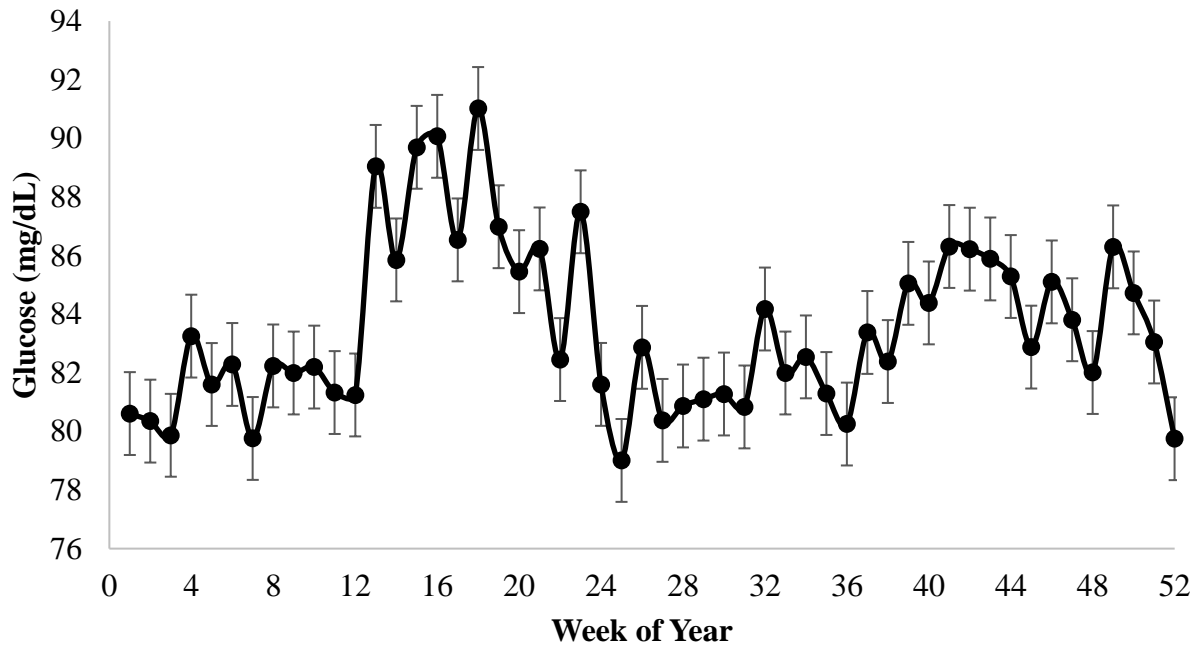
Figure 4.6. Seasonal variation in mean (± 95% confidence limits) glucose (mg/dL) in horses maintained on a cool season pasture over 52 weeks.
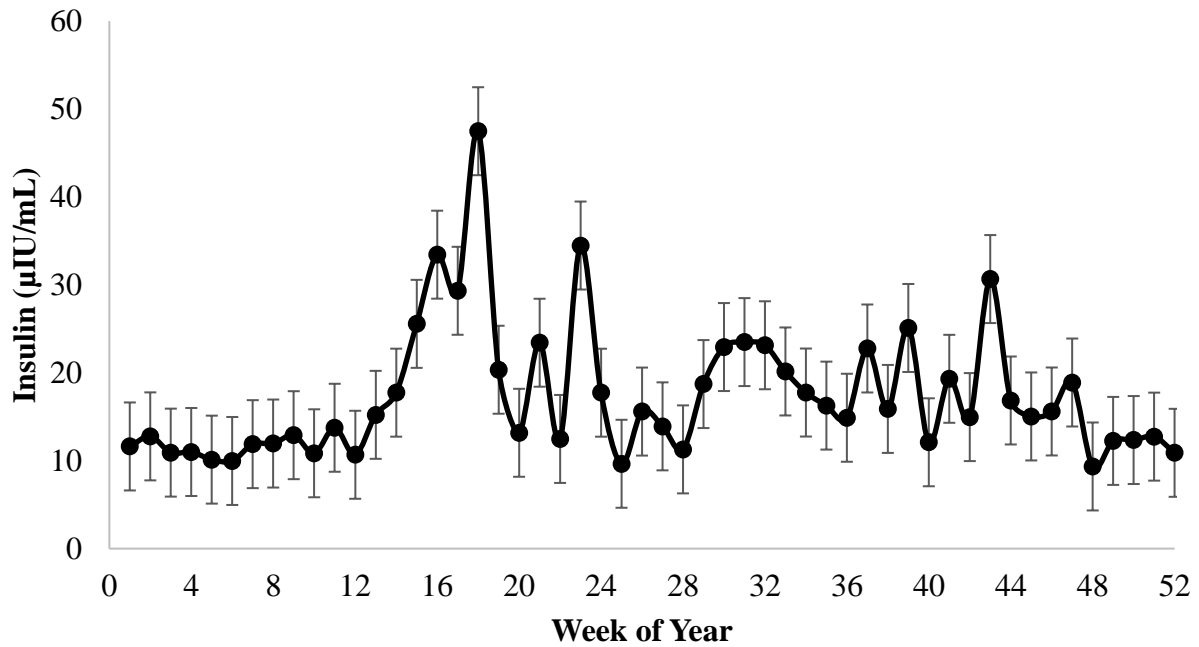
Figure 4.7. Seasonal variation in mean (± 95% confidence limits) insulin (µIU/mL) in horses maintained on a cool season pasture over 52 weeks. Data were log transformed prior to statistical analysis and are displayed as geometric means.

Chapter Five

Seasonal fluctuations in the digestive response of grazing horses

Abstract: The intake of elevated pasture nonstructural carbohydrates (pNSC) can cause digestive dysfunction due to the rapid fermentation of pNSC in the hindgut. This study aimed to investigate the influence of seasonal patterns of pNSC content on the digestive response of grazing horses. Twelve sporthorse mares (15 ± 3.4 yrs) were maintained together on an 8.5-ha cool season mixed grass pasture (free-choice mineral supplementation) for 12 mo beginning October 2016. Weekly pasture samples (200g wet weight) were clipped at random, 2.5 cm from plant base. Samples were weighed, dried at 70ºC, and analyzed to determine monthly pNSC content (Equi-analytical, Ithaca, NY). Monthly fecal grab samples were collected from the midrectum to measure pH and D-lactate (µM). Corresponding monthly blood samples were collected via jugular venipuncture into 4 mL potassium oxalate vacutainer tubes and analyzed for L-lactate (mg/dL). All sample analyses were performed in duplicate. Data were analyzed using a multivariate repeated measures mixed effects linear model in SAS (v. 9.4, SAS Institute Inc.; Cary, NC) with $P \le 0.05$ considered statistically significant. Monthly pNSC content was highest in May ($19.17 \pm 3.18\%$ DM) and November ($17.08 \pm 2.02\%$ DM). There was also seasonal variation in digestive parameters ($P < 0.0001$) with L- and D-lactate being highest in April (11.8 ± 0.91 mg/dL and 4220.4 ± 185.5 µM, respectively). Fecal pH was most acidic in April (6.52 ± 0.08). Pasture NSC was correlated ($P \le 0.05$) with plasma L-lactate (r = 0.33), fecal D-lactate (r = 0.48), and pH (r = -0.27). These findings suggest increased cool season pasture NSC concentrations in the spring and fall months may cause digestive disturbances which could increase the risk of pasture-associated laminitis in predisposed equines. Further understanding of the digestive fluctuations that occur following the consumption of elevated pasture NSC content

will lead to optimized grazing management strategies to reduce the risk of gastrointestinal disturbance and consequent disease.

Introduction

Laminitis is a devastating disease that manifests itself in the hoof and is commonly associated with digestive disruption. Pasture-associated laminitis accounts for nearly half of all laminitis cases and is linked with the intake of elevated pNSC [18]. Horses are at an increased risk of pasture-associated laminitis during the spring and fall months when pNSC is typically most elevated [1]. NSC have been implicated in acute digestive disturbances associated with their rapid fermentation in the hindgut [2].

The recommended limit for starch digestion in the small intestine is between 0.35 – 0.40% BW/meal. When that capacity is exceeded, undigested starch spills over into the hindgut allowing starch-utilizing bacteria to proliferate and produce D-lactate and volatile fatty acids (VFAs). These products of starch fermentation cause a decrease in pH, which ultimately leads to indigenous microbial death and the release of endotoxins into the bloodstream causing subsequent inflammation. This cascade of events is thought to be the digestive connection with laminitis, although the exact mechanism is not well understood [127,128].

The majority of previous research has investigated changes in the digestive response of horses to carbohydrate overload following a large concentrate meal or oligofructose administration. However, limited research has evaluated digestive changes in horses on pasture. Following oligofructose (OF) supplementation, a model known to induce laminitis, peak D-lactate concentrations occurred 20 h post administration [7]. An increase in cecal D-lactate concentrations was also observed by Milinovich and others, as well as a simultaneous decrease in cecal VFA concentrations following administration of OF at laminitis inducing amounts [77].

Administration of OF is meant to resemble fructan in pasture forages, but it is not an exact comparison of naturally occurring nutrients. Fructan in pasture grasses is β2,6-linked,

whereas OF polymers are β2,1-linked. It is unknown whether this discrepancy influences the hindgut microbial populations. Additionally, the manufacturing process of OF requires partial enzymatic hydrolysis to 10 degrees of polymerization (DP) or less [9]. Timothy grass (*Phleum pretense*) was found to have 260 DP in the base of the stem and 50 DP present in the leaves; much higher than OF DP [129,130]. Since approximately half of laminitis cases occur on pasture, research is needed to assess the seasonal fluctuations of pNSC and the effects on the digestive response of grazing horses. Therefore, the objective of this study was to evaluate seasonal patterns of pasture NSC and the investigate seasonal variation in the digestive response in grazing horses.

Materials and Methods

This study investigated the influence of fluctuating pasture nutrition on the digestive response of grazing horses. Twelve mares were selected for this study based on their previous history; half of the mares were previously laminitic (n=6) and the other half were non-laminitic (n=6). The previously laminitic mares exhibited a metabolic phenotype including regional and/or generalized obesity and a cresty neck greater than 2.5 on the scale of 0-5 [122]. None of the mares had active signs of laminitis at the beginning of the study.

The horses were maintained on an 8.5-ha cool season mixed grass pasture for 52 wks at the Middleburg Agricultural Research and Extension Center in Virginia. The pasture consisted of approximately 52% Tall fescue, 43% Kentucky bluegrass, 3% other grass, and 2% weed species. Corresponding pasture and fecal samples were collected monthly throughout the year at 1300 h beginning in October 2016.

Pasture. Pasture samples (200 g wet weight) were clipped every 5 meters at random 2.5 cm from the base of the plant. Samples were weighed, dried at 70° C, and then submitted to a

commercial laboratory (Equi-analytical, Ithaca, NY) to determine nutrient content including starch, ethanol soluble carbohydrates (ESC), water soluble carbohydrates (WSC), and NSC (starch + WSC). All sample analyses were performed in duplicate.

Horses. Twelve mares were maintained on an 8.5-ha cool season mixed grass pasture with water, mineral (Buckeye Nutrition, Dalton, OH), and white salt *ad libitum*. The horses were aged 15 ± 3.4 yrs (range 9-19), mean weight of the horses was 621.8 ± 67.8 kg (range 466.3-770.7 kg), body condition score (BCS) averaged 6.19 ± 1.01 (range 4.0-8.5 on the 1-9 scale), and cresty neck score (CNS) averaged 2.39 ± 0.99 (range 1.0-4.7 on the 0-5 scale) [36,122]. The horses received supplemental hay during winter months due to reduced pasture nutrition and cold weather (5.7% NSC). The horses were acclimated to the pasture 30 d prior to sampling.

Fecal. Fecal grab samples were collected from the midrectum of the horses. Fecal pH was measured using a hand-held pH meter (S20, Metler Toledo, Columbus, OH) and remaining feces was stored at -20ºC for later D-lactate and volatile fatty acid (VFA) analyses. D-lactate concentrations were determined by colorimetric assay (Eton Bioscience D-lactate Assay Kit, San Diego, CA).

Plasma. Blood samples were collected via jugular venipuncture into 4 mL potassium oxalate vacutainer tubes (Fisher Scientific, St. Louis, MO) and stored on ice. Plasma was separated by centrifugation at 3000 x g for 10 min and stored at -20˚C until analysis. Plasma was analyzed for L-lactate (mg/dL) via biochemical analyzer (YSI 2300D SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA).

Statistical analysis. A linear mixed effects repeated measures ANOVA was used to assess the effects of month to examine seasonal changes in digestive measurements in grazing horses. All data were analyzed using The SAS System (v. 9.4.; SAS Institute, Cary, NC 27513)

with α = 0.05 defined as statistically significant. Model adequacy was assessed graphically using plots of studentized residuals and quantile-quantile plots. Plasma L-lactate concentrations were logarithmically transformed before statistical analysis. Means with 95% confidence limits were back transformed and are displayed in all figures.

To estimate correlations between pasture measurements and measurements of horse digestive responses a multivariate repeated measures mixed effects linear model [118] was fit using the MIXED procedure of the SAS System. From the output of the MIXED procedure, within-subject correlation coefficients were hand calculated between each horse and each pasture variable along with P-values to test whether they were significantly different from zero [119]. Because there were unequal numbers of observations in the subclasses the Kenward-Roger adjustment method was used to calculate denominator degrees of freedom. We used a first-order autoregressive covariance structure to account for covariation across time within the same observational units.

Results

There were seasonal fluctuations in monthly pasture NSC concentrations. The greatest fluctuations occurred in the spring (May; 19.17 ± 3.18% DM) and fall (November; 17.08 ± 2.02% DM). There was also seasonal variation in digestive measures in grazing horses. Plasma L-lactate means differed by month ($P < 0.0001$) with the highest L-lactate in April (mo 4; 11.8 ± 0.91 mg/dL) and the lowest L-lactate in February (mo 2; 5.0 ± 0.91 mg/dL) (figure 5.1). Fecal D-lactate means also differed by month ($P < 0.0001$) with the highest D-lactate in April (mo 4; 4220.4 ± 185.5 µM) and the lowest D-lactate in January (mo 1; 847.6 ± 185.5 µM) (figure 5.2). Fecal pH means were different between month ($P < 0.0001$) with the lowest pH in April (mo 4; 6.52 ± 0.08) and the highest pH in February (mo 2; 7.2 ± 0.8) (figure 5.3).

Correlation coefficients used to describe relationships between the pasture nutrient profile and digestive responses in grazing horses are shown in Table 5.1. There was a positive relationship between NSC and plasma L-Lactate (r = 0.33) and fecal D-Lactate (r = 0.48), but a negative relationship with NSC and pH (r = -0.27). Pasture WSC content was associated with fecal D-Lactate (r = -0.59) but had a tendency with fecal pH (r = -0.24). Starch was associated with L- and D-Lactate (r = 0.38 and r = -0.40, respectively) but not fecal pH.

Discussion

During carbohydrate metabolism in the horse, starch and sugar are normally broken down in the small intestine to be absorbed as glucose in the blood. When starch and fructan escapes the small intestine, it enters the cecum where it is rapidly fermented by microbes, leading to the production of lactic acid [71,131]. The production of lactic acid reduces the pH of the gastrointestinal contents. Therefore, the goal of this study was to record fluctuations in pasture NSC concentrations and measure products of fermentation (D- and L-lactate) as well as fecal pH for signs of NSC fermentation in the hindgut. L-lactate is a by-product of starch fermentation via mammalian enzymes, while the D- isomer is not. We chose to measure fecal D-lactate because it is the isomer that is only produced via bacterial fermentation of starch to lactate [132]. The coincidence of D-lactate in the feces when NSC content is most elevated in the pasture would suggest that the NSC content may be overwhelming the small intestine and spilling into the hindgut leading to microbial fermentation. Additionally, we measured plasma L-lactate as D-lactate can be converted to the isomer L-lactate by racemase enzymes which are produced by intestinal microbes [133]. Following the rapid fermentation of NSC in the hindgut, gastrointestinal disturbance and subsequent disease such as laminitis may occur.

Laminitis is one of the leading causes of veterinary treatment and nearly half of all cases are pasture associated [18]. However, limited research has been conducted on the effects of pasture NSC on the digestive response of grazing horses. Pasture NSC concentrations have been shown to have a seasonal fluctuation. We hypothesized that NSC content would be most elevated in the spring and fall months in a cool season Virginia Horse pasture. The greatest pasture NSC accumulation occurred in May at $19.17 \pm 3.18\%$ DM. Pasture NSC content was measured as a sum of WSC and starch. Both WSC concentrations and starch followed similar seasonal trends with WSC making up a much greater portion of NSC than starch.

Similar seasonal patterns were observed in the pasture carbohydrate profile and digestive response in grazing horses. Pasture NSC content was highest in spring, which is when the peak concentrations of D- and L-lactate were recorded along with the lowest fecal pH. These findings support previous studies in which hindgut fermentation of NSC reduced fecal pH and increased fecal lactate [134,135]. These changes may lead to an increased risk of pasture associated laminitis due to the rapid fermentation of NSC in the hindgut. Gastrointestinal disturbance due to rapid hindgut fermentation can lead to the incidence of diarrhea, colic or laminitis and is one of the leading causes of morbidity and mortality in horses [34,61].

Conclusions

Although approximately half of laminitis cases are pasture associated and linked with the intake of elevated NSC content, most research efforts have focused on NSC from a concentrate meal. In the present study, alterations in digestive parameters in grazing horses followed similar patterns of fluctuation that were correlated with the carbohydrate profile of the pasture. While evidence suggests more cases of pasture-associated laminitis occur in the spring and fall, there is little scientific research, beyond our study, into the effects of pasture NSC on the digestive

response of grazing horses. More research is needed to elucidate the interplay of the digestive

response and pasture NSC concentrations.

Table 5.1. Correlation coefficients[a] for pasture carbohydrate variables[b] (nonstructural carbohydrates [NSC][c], starch, and water soluble carbohydrates [WSC]) and horse variables (plasma L-Lactate and fecal D-Lactate and pH).

Horse Variables	Carbohydrate Variables[b]		
	NSC[c]	Starch	WSC
L-Lactate	r = 0.33*	r = 0.38*	r = -0.150
D-Lactate	r = 0.48*	r = -0.40*	r = -0.59*
pH	r = -0.27*	r = -0.13	r = -0.24 †

[a] probability P > |r| under H0: Rho = 0) ($P \leq 0.05$).
[b] Analysis (AOAC, 1990) performed by Dairy One, Ithaca, NY
[c] Nonstructural carbohydrate (NSC) = WSC + starch.
† $P \leq 0.10$, * $P \leq 0.05$
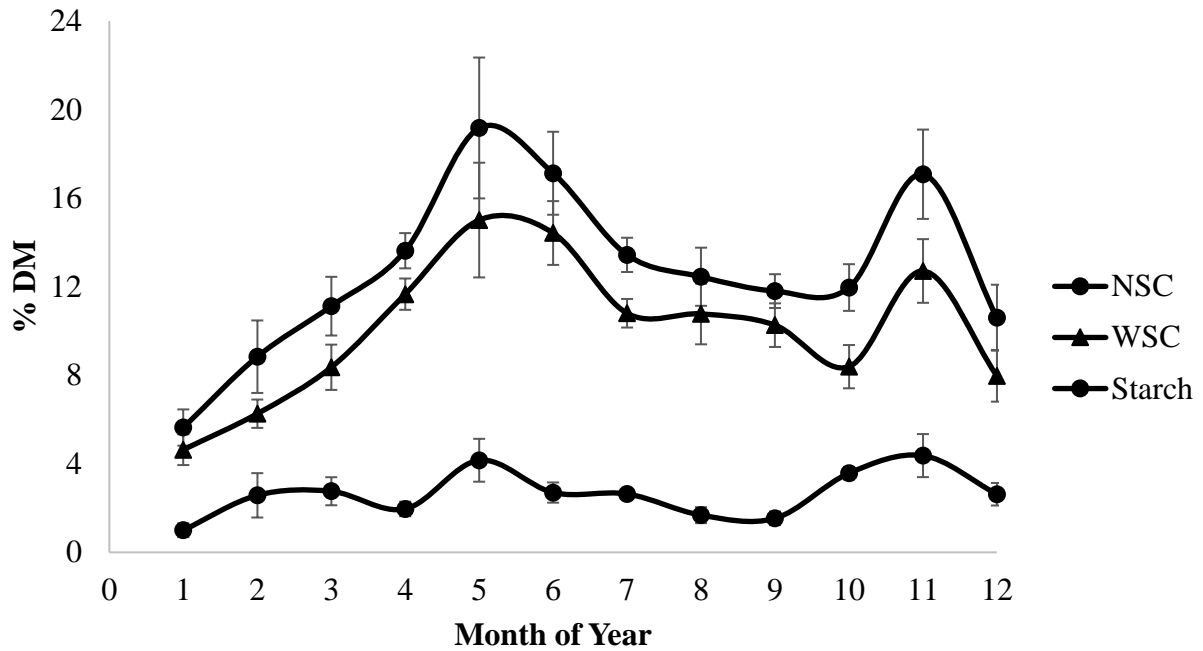
Figure 5.1. Seasonal variation in mean (± 95% confidence limits) nutrient composition on a dry matter basis (% DM) of the pasture forage by month over the 52 wks.
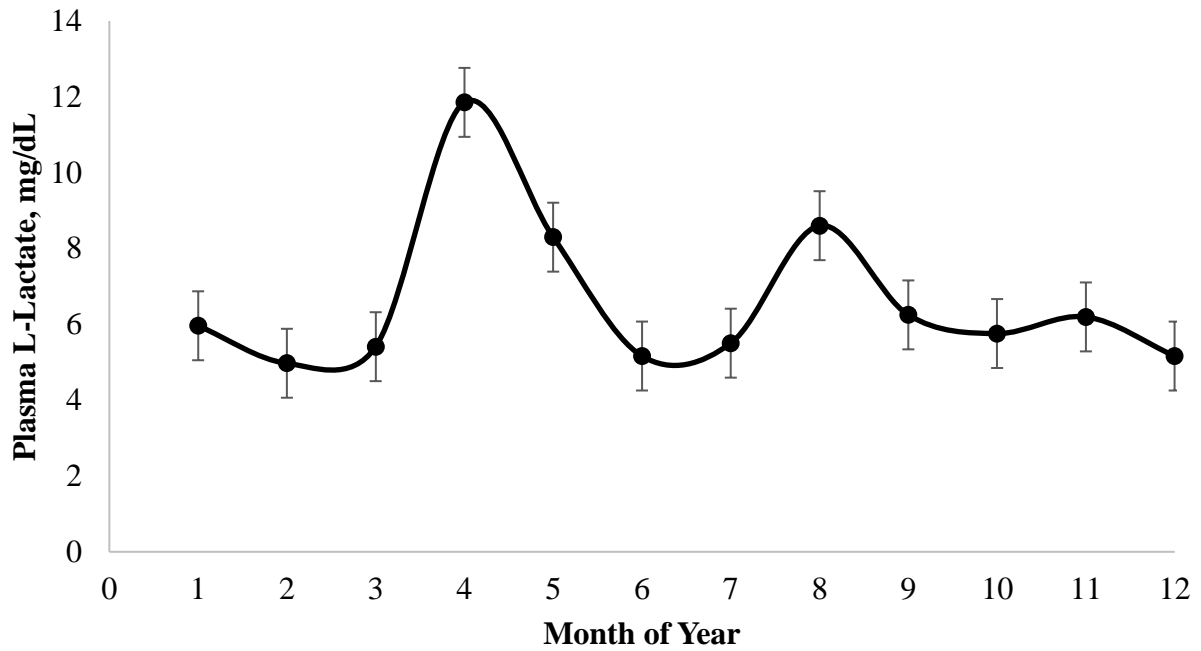
Figure 5.2. Seasonal variation in mean (± 95% confidence limits) plasma L-lactate (mg/dL) in horses maintained on a cool season pasture over 52 weeks.
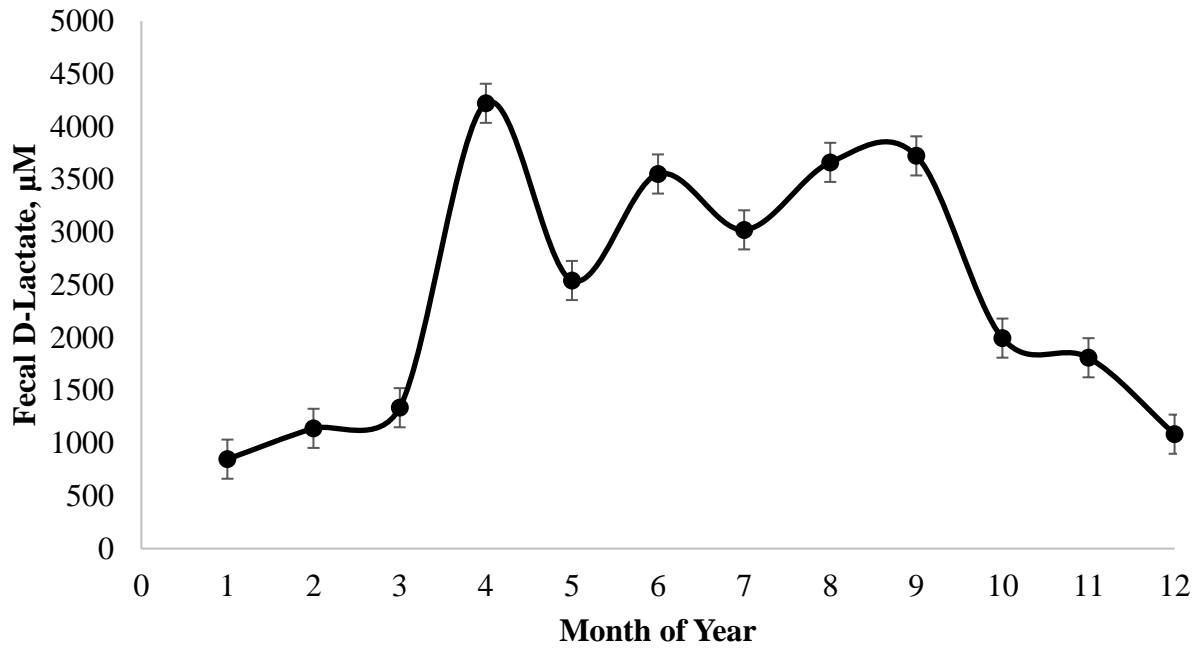
Figure 5.3. Seasonal variation in mean (± 95% confidence limits) fecal D-Lactate (µM) in horses maintained on a cool season pasture over 52 weeks.
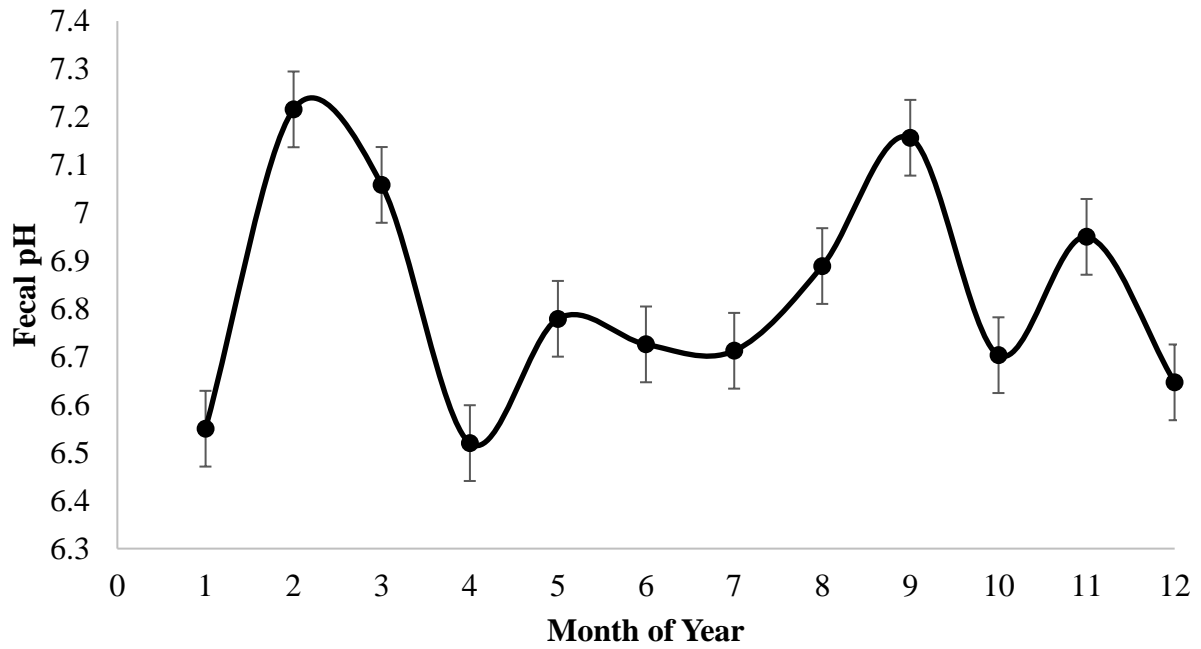
Figure 5.4. Seasonal variation in mean (± 95% confidence limits) fecal pH in horses maintained on a cool season pasture over 52 weeks.

Chapter Six

Seasonal fluctuations in glucose and insulin dynamics via oral sugar testing in grazing horses

Abstract: The intake of elevated pasture nonstructural carbohydrates (pNSC) can cause metabolic dysfunction in horses. This study investigated the influence of seasonal patterns of pNSC content on glucose and insulin dynamics using an oral sugar test in grazing horses. Twelve mares (15 ± 3.4 yrs) were maintained together on an 8.5-ha cool season mixed grass pasture (free-choice mineral supplementation) for 12 mo beginning October 2016. Monthly pasture samples (200g wet weight) were clipped at random, 2.5 cm from plant base. Samples were weighed, dried at 70ºC, and analyzed to determine monthly pNSC content (Equi-analytical, Ithaca, NY). Monthly blood glucose and insulin dynamics (% Δ) were assessed using an oral sugar test. All sample analyses were performed in duplicate. Data were analyzed using a repeated measures ANOVA in the Mixed procedure in SAS (v. 9.4, SAS Institute Inc.; Cary, NC). Data are summarized as means $\pm$ SEM with a $P \leq 0.05$ considered statistically significant. As expected, fasting affected insulin concentrations and subsequent % Δ insulin ($P = 0.0001$). Insulin (% Δ) was greatest ($P < 0.0001$) during spring months. The winter and summer months had the lowest % Δ insulin, while the fall months fell intermediately. Glucose (% Δ) was also the greatest ($P < 0.0001$) in the spring, but there was no effect of fasting insulin ($P < 0.2787$) or fasting glucose ($P < 0.2055$) on glucose % Δ. These results highlight seasonal changes in glucose and insulin dynamics in grazing horses. The results of this study will lead to improved grazing management strategies to reduce the risk of metabolic disorders in horses, including pasture-associated laminitis, especially in predisposed animals.

Keywords: Pasture, Seasonal, Insulin, Horses

Introduction

Pasture-associated laminitis is a painful and costly disease that is associated with the intake of elevated pNSC content and accounts for approximately half of all reported laminitis cases [18]. There is seasonal variation in pNSC content with spring and fall months typically having the highest accumulation of NSC. This pattern occurs in cool season grasses growing in temperate climates due to the increased photosynthetic activity during the day and cooler temperatures at night in the spring and fall months [1,19].

Similar seasonal fluctuations may occur in the metabolic response of grazing horses due to ingesting varying levels of pNSC content. It is important to monitor insulin dynamics in horses that are predisposed or at risk for developing laminitis [40]. Horses can experience both insulin resistance and hyperinsulinemia, defined as insulin dysregulation, which can play a role in laminitis [98,123]. Insulin dysregulation, obesity and/or regional adiposity, plus this incidence or predisposition to laminitis are the three characteristics of equine metabolic syndrome (EMS).

Oral sugar tests (OST) are one testing method used to identify insulin dysregulation [49]. This specific test builds uses the enteroinsular axis link between horses predisposed to laminitis and an increased insulinemic response to oral carbohydrates to identify insulin dysregulation [48]. The sugar dosage rate of 0.15 mL/kg is commonly used, but there has been limited research into other dosage levels [136]. Increased levels of Karo syrup may provide additional sugar comparable to the oral glucose test. It is recommended to perform OSTs the morning following an overnight fast through interpretation of blood samples collected pre and 60-90 min post corn syrup administration. Insulin concentrations of > 60 µIU/mL at 60 or 75 min are indicative of insulin dysregulation [48]. More recently, research has suggested insulin concentrations at > 45 µIU/mL at 60 or 75 min are indicative of insulin dysregulation when using the OST [137].

Repeatability of the OST has been investigated by several researchers. One group found good repeatability (κ = 0.7) when using a cutoff value of 60 µIU/mL at 60 or 90 min when ponies (n=10) were fasted prior to testing [50]. Frank and colleagues found 91 and 83% repeatability when the OST was performed between two groups of horses (n=53) tested in Tennessee and Missouri, respectively [137]. Research by Smith and others found there was 85% agreement between oral glucose test and OST (n=13) [138].

To the authors knowledge, there have been no studies to use the OST to investigate seasonal variation of insulin dynamics in grazing horses. Therefore, the objective of this study was to investigate seasonal variation in insulin and glucose dynamics via an OST in grazing horses.

Materials and Methods

This study investigated the influence of fluctuating pasture nutrition on glucose and insulin dynamics of grazing horses. Twelve mares were selected for this study based on their previous history; half of the mares were previously laminitic (n=6) and the other half were non-laminitic (n=6). The previously laminitic mares exhibited a metabolic phenotype including regional and/or generalized obesity and a cresty neck greater than 2.5 on the scale of 0-5 [122]. None of the mares had active signs of laminitis at the beginning of the study.

The mares were maintained on an 8.5-ha cool season mixed grass pasture containing approximately 52% Tall fescue, 43% Kentucky bluegrass, 3% other grass, and 2% weed species. Horses were supplied with water, mineral (Buckeye Nutrition, Dalton, OH) and white salt *ad libitum*. The horses were aged 15 ± 3.4 yrs (range 9-19), mean weight of the horses was 621.8 ± 67.8 kg (range 466.3-770.7 kg), body condition score (BCS) averaged 6.19 ± 1.01 (range 4.0-8.5

on the 1-9 scale), and cresty neck score (CNS) averaged 2.39 ± 0.99 (range 1.0-4.7 on the 0-5 scale) [36,122]. The horses were acclimated to the pasture 30 d prior to sampling.

Oral sugar testing. Blood glucose (mg/dL) and insulin (μIU/mL) dynamics (% Δ) were assessed monthly for 1-year beginning in October 2016 using a modified OST. Horses were fasted overnight beginning at 2200 h in 4x4 m stalls with *ad libitum* access to water. Basal blood samples were collected the following morning at 0700 h via jugular venipuncture into 4 mL potassium oxalate and 7 mL EDTA vacutainer tubes (Fisher Scientific, St. Louis, MO) and stored on ice. Light Karo corn syrup (0.3 mL/kg BW) was administered orally using a 60-cc dosing syringe. A second blood sample was collected 75 min post dosing. Plasma was separated by centrifugation at 3000 x g for 10 min and stored at -20˚C until analysis. Plasma was analyzed for glucose (mg/dL) via biochemical analyzer (YSI 2300D SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA) and insulin (μIU/mL) using an enzyme-linked immunosorbent assay (Mercodia Equine Insulin ELISA, Mercodia AB, Uppsala, Sweden). All laboratory analyses were performed in duplicate.

Pasture. Monthly pasture samples (200 g wet weight) were clipped every 5 meters at random 2.5 cm from the base of the plant. Samples were weighed, dried at 70º C, and then submitted to a commercial laboratory (Equi-analytical, Ithaca, NY) to determine nutrient content including starch, ethanol soluble carbohydrates (ESC), water soluble carbohydrates (WSC), and NSC (starch + WSC). All sample analyses were performed in duplicate.

Statistical analysis. All data were analyzed using SAS with $P \leq 0.05$ considered statistically significant (SAS v. 9.4.; SAS Institute, Cary, NC). Normal distribution was determined by inspection of residuals and fit statistics. A linear mixed model repeated measures ANOVA was used to assess the effects of month and fasting insulin to examine seasonal changes

in insulin response (% Δ). The % Δ measurement of glucose and insulin compared the difference between fasting and 75 min post syrup measurements by month. Following inspection of the relationship between fasting insulin and % Δ insulin, a negative association was observed. Therefore, fasting insulin was used as a covariate in the model. The outcome of each OST, insulin dysregulation or not, was defined as insulin concentrations of > 45 µIU/mL at 75 min post Karo syrup administration.

Results

There were seasonal fluctuations in monthly pasture NSC concentrations (Figure 6.1). The greatest fluctuations occurred in the spring (May; $19.17 \pm 3.18\%$ DM) and fall (November; $17.08 \pm 2.02\%$ DM). The lowest pasture NSC content occurred in winter (January; $5.64 \pm 0.82\%$ DM).

Both fasting insulin concentrations and 75 min post Karo syrup administration insulin concentrations varied by month ($P < 0.0001$ and $P = 0.0013$, respectively) (Figure 6.2). Glucose concentrations 75 min post Karo syrup also fluctuated by month ($P < 0.0001$), but fasting glucose concentrations did not ($P = 0.26$) (Figure 6.3).

Insulin concentrations (% Δ) varied by month ($P < 0.0001$) with spring months (April, May, and June) having the most elevated insulin response to OST (Figure 6.4). Winter months (January, February, and March) and summer months (July, August, and September) had the lowest insulin response (% Δ); while fall months (October, November, and December) had an intermediate % Δ insulin response. There was also an effect of fasting insulin concentration on % Δ insulin ($P = 0.0001$).

Glucose concentrations (% Δ) also varied by month ($P < 0.0001$) with spring having the most elevated glucose response (Figure 6.5). Winter months had the lowest glucose response (%

Δ). There was no effect of fasting glucose ($P < 0.21$) or fasting insulin ($P < 0.28$) on % Δ glucose.

Half of the horses were insulin sensitive throughout the year (< 45 µIU/mL at 75 min post Karo syrup administration) according to the results of the monthly OST. Horse 1 was in a state of insulin dysregulation during April, May, and June. Horse 2 was insulin resistant during May and October. Horse 7 experienced insulin dysregulation during the entire 12 mo study. Horse 9 was in a state of insulin dysregulation in February through June, August, and October. Horse 12 only experienced insulin resistance during May. Horses 1, 7, 9, and 12 exhibited the metabolic phenotype with generalized obesity and regional adiposity, specifically a cresty neck (CNS > 3).

Discussion

Testing for insulin dysregulation in horses may help to identify equine that are at an increased risk of metabolic type diseases, including inflammation and pasture-associated laminitis. Although frequently sampled intravenous glucose tolerance testing (FSIGTT) with minimal model analysis and euglycemic-hyperinsulinemic clamp (EHC) procedures have been the gold standard in assessment of insulin dynamics, other testing measures exist for both research and clinical settings to reduce labor and costs associated with FSIGTT and EHC procedures. Administering Karo syrup orally has been identified as a reliable method for assessment of insulin dysregulation in horses and ponies [49,138].

Typically, OST are performed using a dosage rate of 0.15 mL/kg BW. However, there has been interest to investigate differing levels of Karo syrup due to the limited research into varying doses [personal communication with Pat Harris; 136]. Following the current study, research has been published investigating varying doses of Karo syrup, comparing 0.15 mL/kg,

0.30 mL/kg, and 0.45 mL/kg BW in ponies [139]. Results from their study found the 0.45 mL/kg

BW dose reliably distinguished previously laminitic ponies from non-laminitic ponies. There

were no differences between the 0.15 mL/kg and 0.30 mL/kg doses. Although this study found

no differences between the 0.15 mL/kg and 0.30 mL/kg dose, their study was conducted in

ponies and not horses. Horses and ponies, although similar, are not physiologically the same in

terms of their metabolism and insulin sensitivity [40,140]. Further research is needed to

determine the appropriate dose of Karo syrup to identify insulin dysregulation in horses.

There are other benefits to using the OST compared to methods where glucose and

insulin are infused intravenously. The OST is representative of postprandial glucose and insulin

concentrations to be measured in a controlled setting. Since the Karo is administered orally

during the OST, it can stimulate incretin hormones in the gastrointestinal tract. Incretins are

hormones which stimulate insulin production. Incretins secreted from the intestines include

glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) [49].

Conclusions

In conclusion, the difference between fasting blood samples and samples collected 75

minutes post sugar administration (% Δ) in glucose and insulin concentrations fluctuated

seasonally with the greatest difference between samples in the spring months. When looking at

the horses individually, the spring months are when several of the horses experienced insulin

dysregulation. These data support the common observation that the incidence of pasture-

associated laminitis is increased in the spring and fall months when pNSC content is most

elevated. Further research is needed to determine the optimum Karo syrup dosage to identify

horses experiencing insulin dysregulation. This will allow for improved management strategies

of grazing horses to prevent disease, including pasture-associated laminitis, in horses

predisposed to obesity and metabolic diseases.
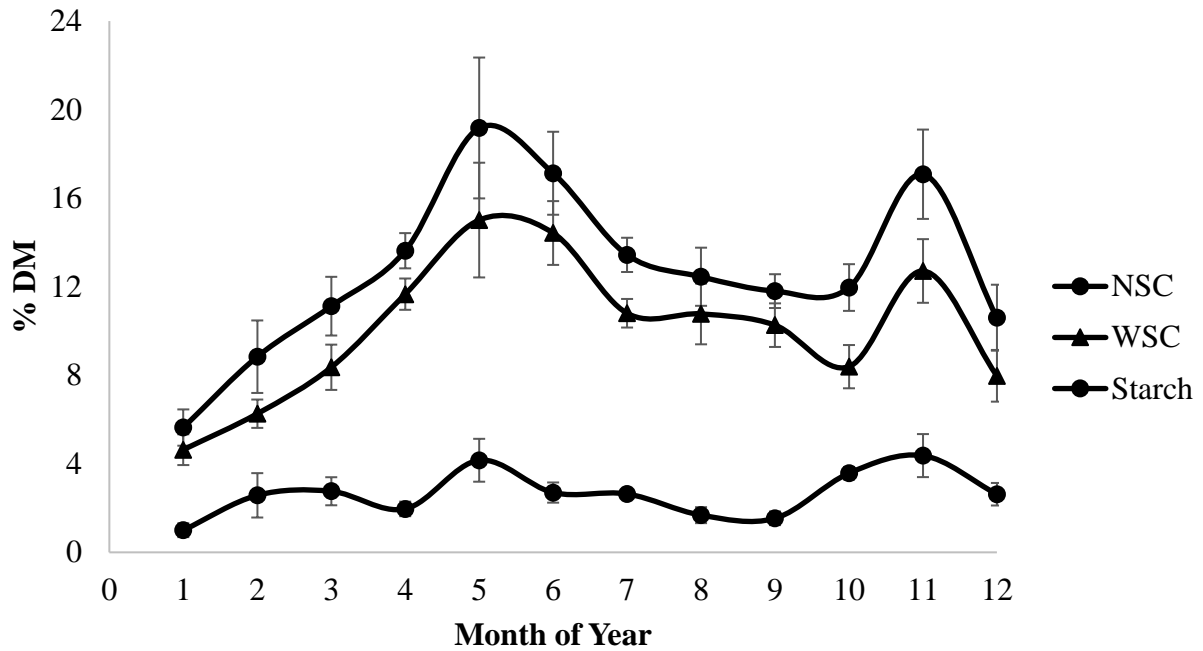
Figure 6.1. Seasonal variation in mean (± 95% confidence limits) nutrient composition on a dry matter basis (% DM) of the pasture forage by month over the 52 wks.
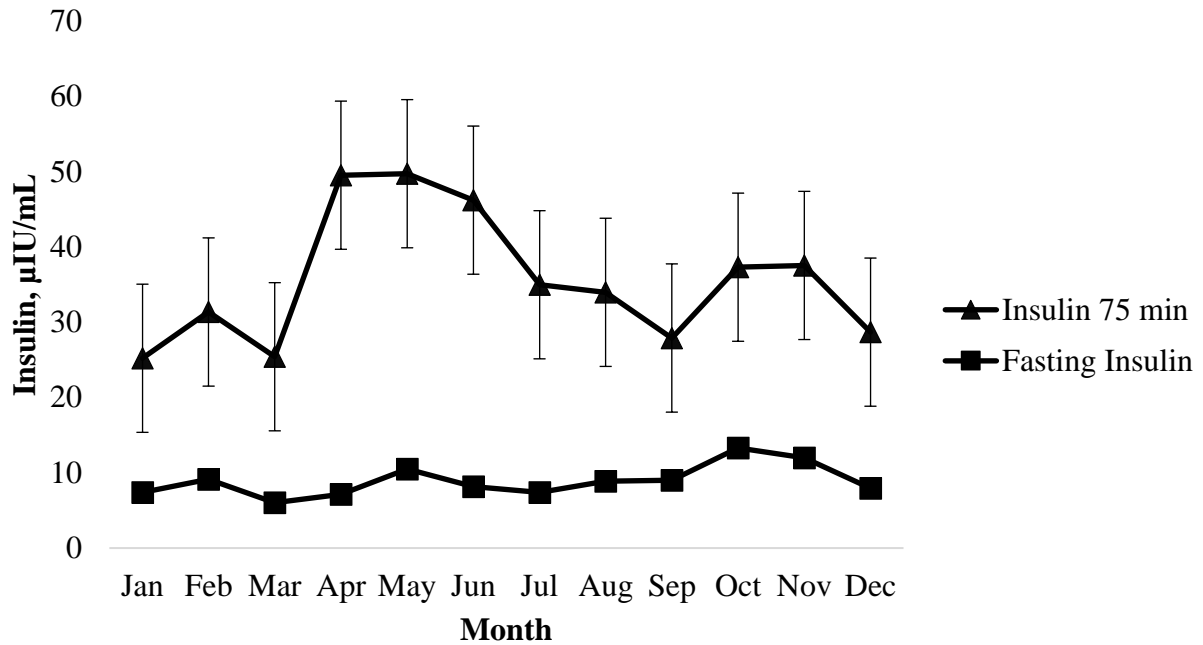
Figure 6.2. Seasonal variation in mean (± 95% confidence limits) plasma fasting insulin concentrations and insulin concentrations 75 min post Karo syrup administration during oral sugar tests (OST) in horses maintained on pasture over 12 months.
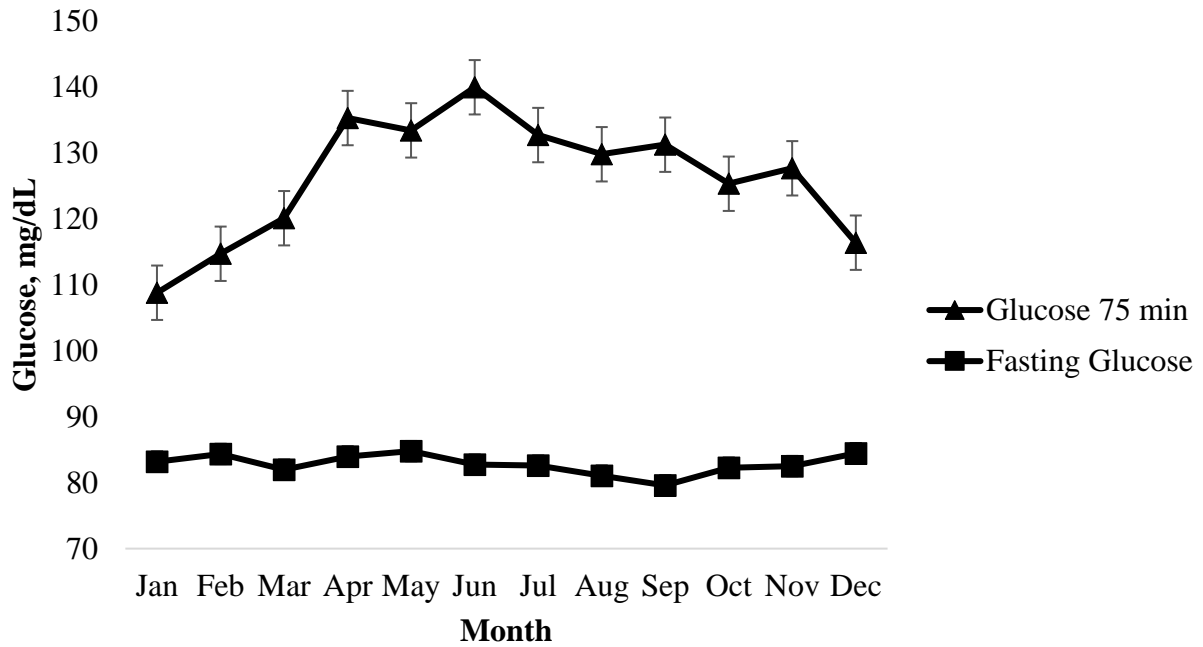
Figure 6.3. Seasonal variation in mean (± 95% confidence limits) plasma fasting glucose concentrations and glucose concentrations 75 min post Karo syrup administration during oral sugar tests (OST) in horses maintained on pasture over 12 months.

Figure 6.4. Seasonal variation in mean (± 95% confidence limits) plasma insulin concentrations (% Δ) during monthly oral sugar tests (OST) in horses maintained on pasture over 12 months.

Figure 6.5. Seasonal variation in mean (± 95% confidence limits) plasma glucose concentrations (% Δ) during monthly oral sugar tests (OST) in horses maintained on pasture over 12 months.

Chapter Seven

Summary and Implications

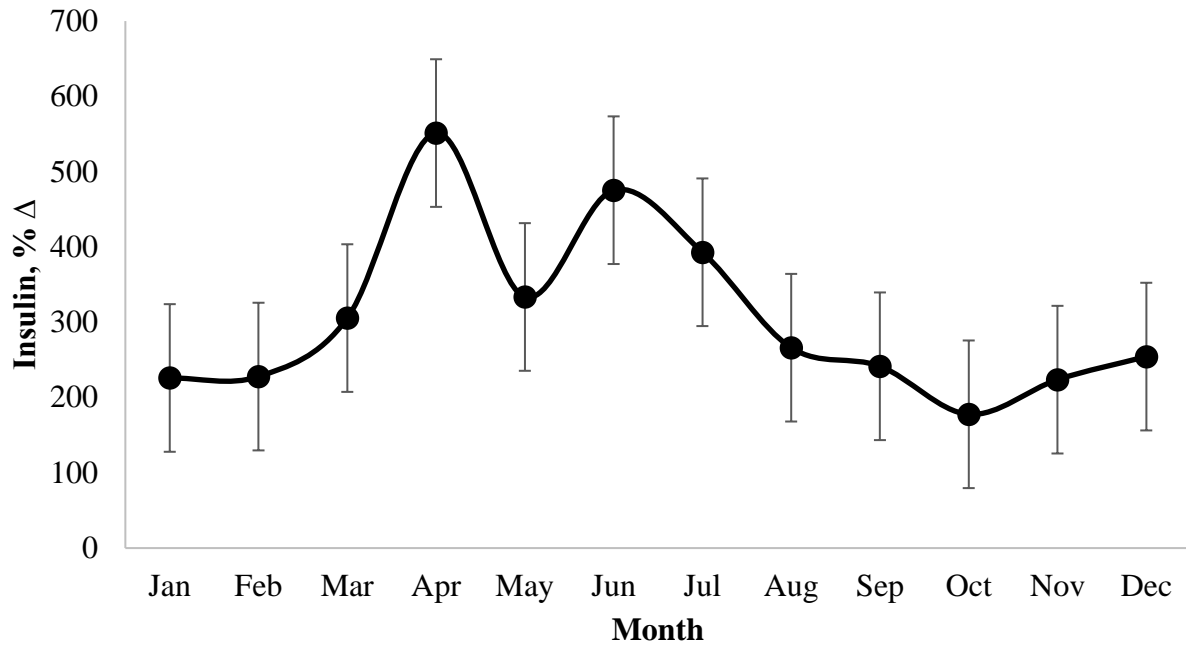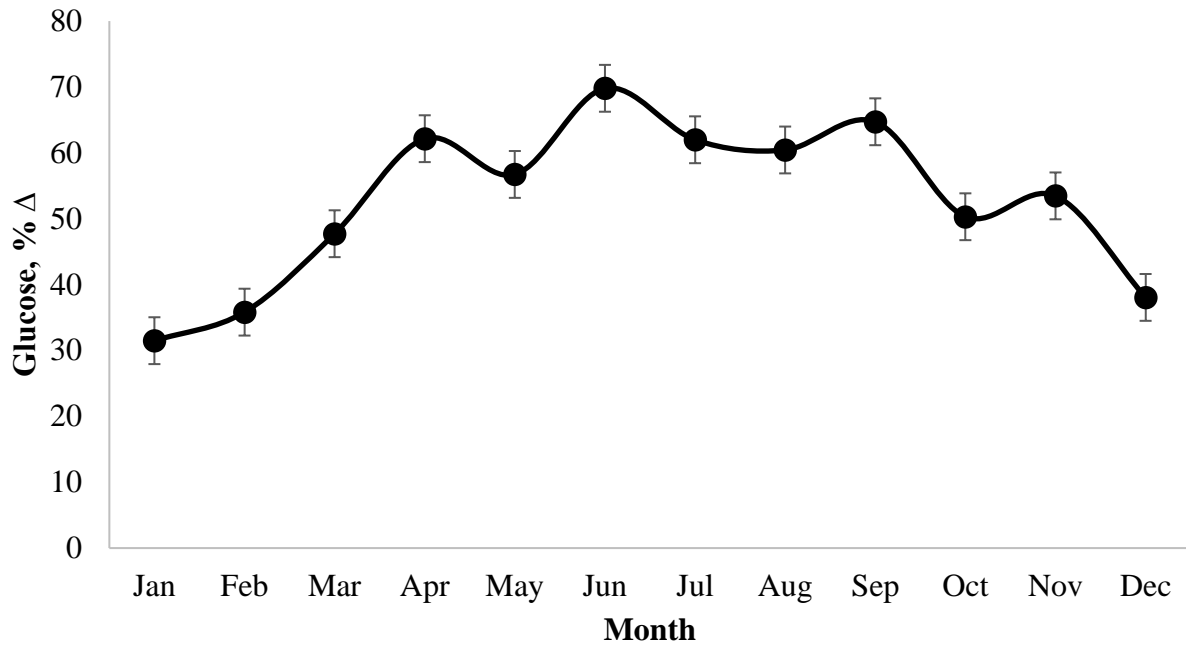The purpose of this research was to describe seasonal and circadian fluctuations in a cool season Virginia horse pasture and investigate the metabolic and digestive response of grazing horses. This study is one of few to characterize the relationship between plants, animals, and the environment and the implications with metabolic and digestive diseases. Half of the twelve mares selected on this study exhibited a metabolic phenotype (regional adiposity) while half exhibited non-metabolic phenotype. Our goal was to include horses of one gender and varying degrees of predisposition to metabolic disease.

Results from the pasture data demonstrated that carbohydrate fractions including NSC, WSC, ESC, and starch were most elevated in the spring (May) and fall (Nov) months. Circadian variation also occurred during the day with more elevated carbohydrate concentrations in the afternoon hours compared to the morning. These fluctuations are associated with environmental factors such as elevated PAR, ideal ambient temperature, and plant stress including frost during the cool season forage growing period in the spring and fall. This study was one of the first projects to investigate seasonal and circadian fluctuations in pasture nutrients with such intense sampling frequency over the course of one year. Understanding the relationship between plants and the environment will provide insight into implications for health and performance of grazing animals and guiding future research.

Limited research has been conducted on the metabolic and digestive response of grazing horses. Similar seasonal patterns were observed in both metabolic and digestive characteristics as compared to carbohydrate variation in the pasture. Glucose and insulin concentrations of grazing horses were most elevated in the spring. In addition to the insulinemic and glycemic responses

being affected by changes in the pasture nutrient profile, there were digestive responses that experienced alteration, likely due to hindgut fermentation of pasture NSC content. Fecal D-lactate and plasma L-lactate were most elevated in the spring, while fecal pH was lowest.

Identifying these trends in the metabolic and digestive characteristic of grazing horses will help to guide future research into the complicated factors that lead to metabolic and digestive disturbance and subsequent disease such as colic and laminitis. With additional insight into seasonal changes and the effects in grazing horses we can better target future research into potential risks for NSC-related diseases and ultimately improve and knowledge and nutritional management of grazing horses to reduce the risk of devastating diseases in predisposed animals. Prevention and avoidance of diseases such as laminitis is key since structural damage to the hoof lamina is irreversible and horses are often euthanized due to the painful and debilitating nature of coffin bone rotation.

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