CIRCADIAN AND SEASONAL VARIATION IN PASTURE NONSTRUCTURAL CARBOHYDRATES AND THE PHYSIOLOGICAL RESPONSE OF GRAZING HORSES

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(ABSTRACT)

Nonstructural carbohydrates (NSC), which includes sugars, starches and fructans in pasture forages, undergo circadian and seasonal variation which has direct effects on metabolism in grazing horses. Increased intake of NSC is implicated in the development of digestive and metabolic disorders, such as laminitis. A series of five studies at Virginia Tech's M.A.R.E. Center in April, May, August, and October 2005, and January 2006, examined circadian and seasonal variability in forage NSC content and metabolic and digestive variables in horses over a 36 h sampling period. Fourteen mares were randomly assigned to grazing (housed on a 5-ha predominantly tall fescue pasture; n = 10) or control (stabled within the pasture and fed timothy/alfalfa hay; n = 4) groups. Blood samples were collected hourly from the horses which corresponded to hourly pasture forage samples. In all five studies, plasma glucose and insulin were measured and proxies for insulin resistance were calculated. In the April study, plasma L-lactate and fecal pH, L-lactate, D-lactate and volatile fatty acids (VFAs) were also measured. Two approaches were used for the determination of carbohydrate profiles in pasture forage samples. For the first (LAB1), sugar was water soluble carbohydrates extracted prior to analysis for starch, and included fructans. The NSC was the sum of starch and sugar. For the second (LAB2), samples were analyzed for specific NSC fractions using hydrolytic enzymes, with the addition of HCL for the determination of fructans including graminans, the type of fructans in cool season grasses. Both the LAB1 and LAB2 analyses revealed circadian and seasonal patterns in forage NSC and its constituents. In general, pasture forage NSC content was lowest in the

morning and highest in the late afternoon. April had the highest NSC content which was comprised mostly of simple sugars. Forage NSC content (LAB1) was associated with environmental variables in all months with strongest correlations in April; ambient temperature ($r = 0.72$, $P < 0.001$), solar radiation ($r = 0.62$, $P < 0.001$), and humidity ($r = -0.84$, $P < 0.001$). In the animals, plasma insulin was highest in grazing horses in April ($P < 0.001$) followed by May ($P < 0.001$). Plasma insulin was higher in grazing compared to control horses at all sample points in April, and a circadian pattern was evident ($P = 0.012$). In grazing horses, plasma glucose was higher in April than all months except for May, and plasma glucose was higher in grazing horses compared to controls in April. In grazing horses, plasma insulin was significantly correlated with NSC and sugar in April ($r = 0.69$ and $r = 0.67$, respectively); May ($r = 0.46$ and $r = 0.47$, respectively); and January ($r = 0.44$ and $r = 0.46$, respectively). In April only, individual mean insulin response was proportional to the increase in insulin per increase in unit of NSC ($r^2 = 0.033$, $P < 0.001$). Sinusoidal circadian patterns in NSC ($r^2 = 0.51$, $P < 0.001$) and insulin in grazing horses ($r^2 = 0.12$, $P < 0.001$) had similar frequency ($P = 0.36$). Plasma L-lactate was higher in grazing horses (0.64 mmol/L) than control horses (0.40 mmol/L) ($P < 0.001$). Fecal pH was lower in grazing horses (pH 6.9) than control horses (pH 7.2) ($P = 0.008$). Fecal VFAs, including acetic acid, butyric acid, and D- and L-lactate were higher in grazing horses compared to control horses ($P < 0.05$). These studies identified a link between forage NSC content and alterations in carbohydrate metabolism and digestion that may increase risk of laminitis via exacerbation of insulin resistance. Strategies for management practices to decrease intakes of pasture NSC by horses at risk of developing metabolic disorders are needed.

Keywords: Horses, carbohydrates, glucose, insulin, laminitis

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Glossary of Terms

C_3 Plants- A C_3 plant is one that produces phosphoglyceric acid (a molecule that has three carbon atoms) as a stable intermediary in the first step in photosynthesis. Over 95% of the plants on earth are C_3, including cool-season grasses and legumes.

C_4 Plants- A C_4 plant is one that produces oxaloacetic acid (a molecule that has four carbon atoms) as a stable intermediary in the first step in photosynthesis. Photorespiration in C_4 plants is more efficient in strong light. C_4 plants include warm season grasses such as bermudagrass, pearl millet, or corn.

Cool Season Plants- Cool season species make most of their growth during the coolest months of the year, except for the coldest periods during winter. They are planted in the autumn or sometimes early spring. Also called C3 plants.

Fructan- Collective term for all oligo- and poly-fructosyl sucrose that consists of one or more fructosyl-fructose links. Inulin, levan and graminan are the three main types of fructans found in plants. Fructans are the primary reserve carbohydrate in grasses of temperate origin. Fructans are water soluble. Animals do not have the enzymes to digest fructans so they are fermented by microbes in the hindgut.

Graminan- Branched fructans linked by both β(2→1) and β(2→6) glycosidic bonds (mix of phlein and inulin linkages).

Grasses- Member of the *Poaceae* plant family. Grasses are monocots (produce one seed leaf), generally herbaceous (not woody), produce seed on an elongated seed stalk, they have parallel leaf veins, and fibrous root systems.

Inulin- Type of fructan found primarily in certain species of *Compositae* that consists mainly of β (2→ 1) fructosyl-fructose linkages .

Legume- Members of the *Fabaceae* plant family. Legumes are dicots (produce two seed leaves), produce seed in pods, have "netted" leaf venation, and usually have taproots.

Levan- Type of fructan found primarily in temperate forages (C_3) that consists mainly of β (2→ 6) fructosyl-fructose linkages. Levans can also be of bacterial origin.

Non-Fiber Carbohydrate (NFC)- Nonstructural carbohydrate fraction estimated by forage laboratories as NFC = 100- (CP % + (NDF % -NDICP %) + Fat % + Ash %)

Nonstructural carbohydrates (NSC)-Sugars, starches and fructans that accumulate in plant cells and are then readily mobilized for metabolism or translocation to other plant parts.

Phlein- A levan type of fructan found in temperate forages (see levan).

Starch- Polymer of glucose, composed of D-glucopyranose units with α (1→ 4) glycosidic links, readily hydrolyzed by digestive enzymes. Starches are not water soluble. Amylase and amylopectin are the two polysaccharide types of starch. Starches are reserve carbohydrates in forages, and the *primary* reserve carbohydrate in legumes.

Sugar- Mono- and di-saccharides, such as glucose and fructose, and sucrose respectively. Sugars are water soluble and hydrolysable.

Warm Season Plants- Warm season species make the majority of their growth during the warmest months of the year. They are typically planted in the spring or early summer.

Water-Soluble Carbohydrate (WSC)-The carbohydrate fraction in plants that is soluble in water including mono- and disaccharides and fructans.

CHAPTER I

Introduction

Laminitis is a systemic disease in horses and ponies that ultimately affects the laminar tissue binding the pedal bone to the inner hoof wall. In the later stages of laminitis, the pedal bone becomes detached and rotates downward to the sole of the hoof causing great suffering and pain to the affected animal. There are many causes of laminitis; however, the digestive and metabolic origins are associated with equine nutrition and are the most common. A recent survey of equine practitioners ranked laminitis first in need of research (AAEP, 2003). Much of today's research is focused on treating the disease within the hoof, when structural damage is irreversible. Other research is focusing on nutritional countermeasures for the avoidance of equine laminitis through the management of risk factors in pastures, feeds and the animals themselves. Ultimately, horse owners and those working directly with them need applicable, yet accurate information to maintain the health and performance of their horses.

Laminitis has been linked with intakes of nonstructural carbohydrates (NSC) in feeds and forages. Nonstructural carbohydrates have been implicated with acute digestive disorders due to their rapid fermentation, and chronic metabolic disorders associated with high glycemic and insulinemic responses that they may cause (Kronfeld & Harris, 2003; Hoffman et al., 2003). Intakes of inulin (a type of fructan) (Pollitt et al., 2003), and starch (Potter et al., 1992), have been shown to directly elicit laminitis. Intakes of simple sugars and starch can lead to IR, which has also been linked to laminitis (Treiber et al., 2006b). Nonstructural carbohydrates are risk factors for laminitis, but making recommendations for avoiding NSC in forages can be difficult because there are a lack of data on actual

ranges and the various factors which influence their accumulation in various forages. Avoiding high intakes of starch from cereal grain meals is straightforward- simply reduce the amount of feed given in a single meal (Potter et al., 1992). In contrast, grazing management practices to avoid excessive intakes of forage NSC in pastures requires further research on grazing behavior and intake, and variables that influence NSC content and profiles.

Factors that influence the NSC content in forages are of interest to equine researchers because of the need to predict when grazing animals are at a heightened risk and develop management strategies to reduce the incidence of laminitis. Studies have shown that not only do variables inherent to the plant affect NSC status, but that environmental conditions influence circadian and seasonal patterns in NSC content. There is also knowledge gap in our understanding of the fate of the various NSC fractions (sugars, starches, and fructans) within the equine digestive system. Thus, the overall objectives of our study were to identify risk factors for laminitis in pastures and horses:

OBJECTIVES

1. Evaluate circadian and seasonal variation in pasture forage nonstructural carbohydrates (NSC), including starches, simple sugars, and fructans, and how they are affected by the environment.
2. Evaluate circadian and seasonal variation in carbohydrate metabolism in grazing horses and possible relationships with forage NSC.
3. Evaluate digestive and metabolic variables in horses grazing spring pasture.
4. Identify basal proxies for insulin sensitivity and pancreatic β-cell responsiveness in grazing horses compared those confined to stalls and fed hay.

CHAPTER II

REVIEW OF LITERATURE

LAMINITIS IN HORSES

Laminitis is a disabling, common and costly disease of the horse and pony most often associated with digestive and metabolic disorders, which are linked to equine nutrition. Overall, annual incidence of laminitis in the U.S. is reported to be 2%, but this rises to about 5% in the spring and summer (USDA. Kane, 2000), and nearly half of all reported cases of laminitis in the US occurred in horses and ponies grazing "lush pasture" (Figure 1) (USDA, 2000):

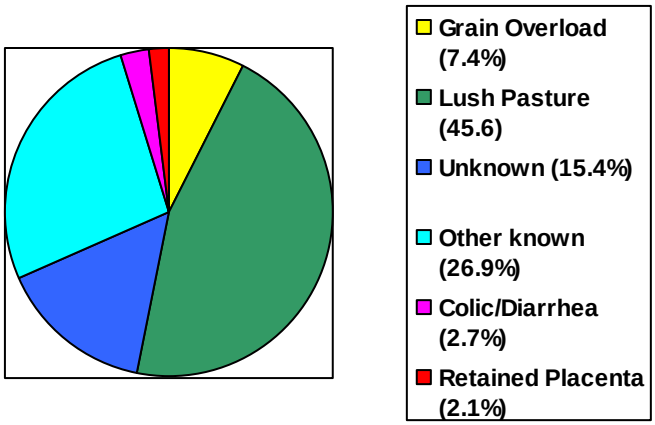

Figure 2.1. Perceived causes of annual incidence of laminitis in the U.S.

Equine laminitis is a systemic disease that may lead to inflammation of the lamina of the hoof, and downward rotation of the third phalanx (pedal or coffin bone) (Hood, 1999). Treatment of laminitis is difficult because researchers currently do not fully understand the pathogenesis of the disease and the trigger factors which elicit their effects within the hoof. Clinical signs of laminitis become obvious after metabolic, inflammatory, and degenerative cellular changes have occurred within the laminae

(Bailey et al., 2004). Research identifying risk factors for the avoidance of laminitis is critical because the disease has extensive implications for equine welfare. However the mechanisms, or trigger factors, which links digestive and metabolic disorders with the hoof remain unclear.

NONSTRUCTURAL CARBOHYDRATE PROFILES IN FORAGES

Accumulation of NSC in forages. Forage plants produce simple sugars through the biochemical process known as photosynthesis. During photosynthesis, plants take in carbon dioxide from the atmosphere, and use sunlight for energy to produce simple sugars, which they in turn use for growth and reproduction. When sugar production exceeds the energy requirement of the plant for growth and development, they are converted into storage forms of carbohydrates within the vegetative (non-reproductive) tissues. Cool-season (C3) pasture grasses accumulate fructan as their storage carbohydrate. Fructan is stored in the vacuoles of the leaves and transported to those of the stem, where they are stored until the plant needs them as an energy source. Fructan content can become quite high because they are stored in the vacuoles and there is hence no self-limiting mechanism. Starches are the primary storage carbohydrate of warm-season (C4) grasses and legumes and they do not produce fructans (Bailey et al., 2004; Chatterton, 1989). Starch production and storage takes place in the chloroplasts of the leaf, and this is a self-limiting process because once the chloroplasts become saturated, starch production stops. Therefore, in C3 grasses the stems contain the highest concentration of fructan, whereas in C4 plants and legumes, the leaves are the primary sites of starch accumulation.

Storage carbohydrates fluctuate in forages as a result of changes in photosynthetic activity and the changing needs of the plants for growth and development throughout a growing season. Circadian variations in storage carbohydrates also occur as photosynthetic rates and carbohydrate utilization change throughout the day. Circadian utilization of the sugars produced from photosynthesis typically results in lowest NSC content in the early morning, peaks in the afternoon, and declining content overnight (Bowden, 1968; Holt, 1969; Longland, 1999). Similar circadian patterns of storage carbohydrate accumulation have been reported for legumes (Lechtenberg et al.,1971). Seasonal variations in the storage carbohydrate content of grasses and legumes are associated with varying energy demands at different stages of growth, with concentrations being highest in late spring, lowest in the summer and winter, and intermediate in autumn (Smith, 1973; Waite, 1953). Although these patterns of circadian and seasonal variation in storage carbohydrates are observed under ideal environmental conditions, they may be subject to change because of the cumulative effects of various environmental factors, including varying light intensity, temperature, soil nutrients, and water status.

Environmental factors that affect NSC accumulation. Studies have shown that environmental conditions can lead to significant fluctuations in the amounts of NSC that accumulate in forage plants. The water soluble carbohydrate (WSC) content, which is comprised of simple sugar and fructan, of a given plant species ranged from 95 to 560 g/kg DM with corresponding fructan amounts of 32 to 439 g/kg DM, depending on the temperature at which it was grown, with higher and lower values being associated with

cooler (5-10º C) and warmer (15-25º C) temperatures, respectively (Chatterton, 1989). Temperature dictates NSC accumulation in plants because photosynthesis and subsequent storage of sugars continues at temperatures below the limit for growth (Pollock, 1986). Light intensity, or solar radiation, also affects the NSC content of forages, thus shading *Phalaris aqautica* L. pastures for an average of 43 h resulted in NSC contents of 62 and 126 g/kg NSC DM for shaded vs. un-shaded pastures, respectively (Ciavarella, 2000). Furthermore, in a study on the effects of drought on different varieties of orchardgrass in the Mediterranean, fructan content increased as drought conditions progressed, reaching 350 to 400 g/kg DM in stem bases at the end of a 3 mo drought period (Volaire and Lelievre, 1997). Thus factors which reduce growth, but do not affect sugar production, results in the accumulation of elevated concentrations of NSC in plants. Conversely, factors which promote growth generally result in a reduction of NSC content. For example, increased growth in response to application of N fertilizer reduced the WSC content of forage (Belesky et al., 1991a).

*Circadian and seasonal variation in NSC***.** Both circadian and seasonal variation in pasture NSC content has been reported in a number of studies. Circadian variation in WSC content of vegetative tissues of *Lolium perenne* cultivars were observed to double during daylight hours with increases in WSC content from 160 to 240 g/kg DM occurring within a 3 h period.; the lowest and highest amounts occurred in the early morning and late afternoon/evening, respectively (personal communication with Longland, in preparation). However, under conditions of low light intensity and mild ambient temperatures, there was comparatively little circadian fluctuation in WSC content.

Seasonal variation in pasture NSC constituents is well documented (Smith, 1973). A study of mixed species pastures from 10 horse farms in Germany that took place from the months April to November, fructan content ranged from 18 to 57 g/kg DM, where the highest concentrations occurred in May, August had the lowest content, and intermediate concentrations occurred in October (Vervuert et al., 2005). Another study determined the WSC contents of various ryegrass monocultures throughout the growing season in three consecutive years at nine sites in five Northern European countries (Germany, Ireland, Norway, Sweden, and the UK). The sites were as far north as the Arctic circle, and as far south as latitude 52. Over the three year study, concentrations of WSC ranged from less than 100 g/kg DM to over 385 g/kg DM, the highest concentrations occurred where there were cooler temperatures. The WSC fractions (glucose, sucrose, fructose, and fructan) were measured in the swards at the UK site across two growing seasons. The fructan content ranged from 75 to 279 g/kg DM, accounting for 55 to 75 % of the WSC fraction. After fructan, sucrose was the next most abundant component of WSC, accounting for 16 to 22 % of the total WSC fraction, with amounts of fructose and glucose being 6 to 12%, and 3 to 10% of the WSC fraction, respectively (Longland et al., personal communication).

Carbohydrate analysis and nomenclature. There are several systems used for the classification of carbohydrates. Carbohydrates are typically referred to as mono-, di, or oligo- polysaccharides. Monosaccharides include sugars (glucose, sucrose, fructose, galactose, mannose, xylose and arabinose) and are constituents of poly- and oligosaccharides. Disaccharides are less common and include lactose (glucose and lactose), important to nursing foals, and maltose (glucose and glucose), which is

produced by starch digestion in the small intestine. Oligosaccharides are made up of short chains of monosaccharides (DP 3 to 10) and includes fructooligosaccharides, raffinose, and stachyose. Fructooligosaccharides and inulin are types of fructans. Fructan is a collective name for all oligo- and poly-fructosyl sucrose (Cairns, 2003). There are three general classes of fructans: Inulin, bacterial levans and levans found in grasses (phleins or graminan). The three groups vary in degree of polymerization (DP) and branching. Inulins are linear β(2→ 1) linked furanoses linked at their end to a glucose residue. The DP of inulin can be as high as 70 residues. They are found in Jerusalem artichokes, chicory and garlic (Suzuki, 1993). Inulins have been used to experimentally induce laminitis in equines (Bailey et al., 2003; Bailey et al., 2002; Milinovich et al., 2006). Phleins (termed levan if they are of bacterial origin) are large linear molecules with β(2→ 6) linkages that can consist of up to 100,000 fructose units (Bonnett, 1994). The third types are those commonly found in C3 grasses- the graminans. These fructans are of the mixed type and contain both β(2→ 1) and β(2→ 6) linkage bonds between the fructose units (Cairns, 1997)**.** Other polysaccharides include starches (amylose and amylopectin), cellulose, hemicellulose, and pectins.

Some methods have classified carbohydrates according to the role they play in plants, while others have classified them according to how they are digested by animals. Carbohydrate analyses also vary, and some carbohydrate fractions are easier to measure than others. The most common method for carbohydrate analysis in feeds was developed by Van Soest in the late 1960's where neutral detergent solubles or neutral detergent fiber (NDF) were measured. The NDF included cellulose, hemicellulose and lignin. In this system, the amount of nonstructural carbohydrates (NSC) in feed was calculated by

difference (subtracting NDF, protein, ash, and ether extract from the total DM). Nonfiber carbohydrate (NFC) has also been used, but it is also calculated by difference and includes all carbohydrates not found in the NDF component. Forage laboratories in the U.S. were established initially in association with universities to support the Dairy Herd Improvement Association (DHIA), and their proximate analysis of carbohydrates was designed to be appropriate to the digestive physiology and metabolism of bovids as opposed to equids. Ideally, the NSC fraction should include mono- and disaccharides, oligosaccharides, polymeric fructan, and starch. More definitive techniques for measuring NSC and its constituents are being developed and utilized by commercial, government, and university laboratories. These techniques include water and ethanol extraction, enzymatic methods or chromatographic techniques. However at this time, the enzymatic and chromatographic techniques have not been routinely adopted by commercial laboratories, and such detailed information is unlikely to be available to the majority of the equine community. Currently, laboratories are performing ethanol extractions where ethanol soluble carbohydrate (ESC) is being reported, and in relation to forage analysis, this fraction consists mainly of sugar (glucose, sucrose, fructose, and some oligosaccharides). Water extractions are being performed and reported as water soluble carbohydrate (WSC), and formerly ‘sugar’- this analysis includes both sugars and fructans (Hall, 1999). Starches are measured by enzymatic techniques, which are typically similar between laboratories. The total NSC should then include both the WSC and starch fractions. However, most laboratories calculate NSC as the sum of ESC and starch, and fructans are not being included in the measurement. Techniques for the measurement of fructans, such as chromatographic techniques are being developed

through corporate and university research, but they are unavailable to horse owners. Therefore, in the analysis of forage for equines, it is imperative that the user fully understands which fractions are being reported by their laboratory, to enable development of suitable feeding regimes for equines predisposed to laminitis.

DIGESTIVE AND METABOLIC PROFILES ASSOCIATED WITH LAMINITIS

Proposed mechanisms for induction of laminitis. There is meager evidence of association and stronger evidence of causation between laminitis and rapid intakes of NSC, whereby excessive intakes of NSC have been implicated in acute digestive disturbances associated with their rapid fermentation, and chronic metabolic disorders associated with high glycemic and insulinemic responses (Kronfeld and Harris, 2003). Thus, laminitis in equines can be caused experimentally by the administration of high amounts of starch which exceed the digestive capacity of the small intestine, the undigested material flowing into the hindgut (Garner et al., 1977). Furthermore, fructans are thought not to be digested by mammalian enzymes (Roberts, 1975), but although some may be partially susceptible to acid hydrolysis or fermentation (de Fombelle et al., 2004) in the foregut, it is probable that much passes into the hindgut relatively unchanged, and administration of high amounts of fructan to horses routinely resulted in laminitis (Pollitt, 2003). The appearance of large amounts of starch or fructan in the hindgut is believed to result in the proliferation of lactic acid producing amylolytic and saccharolytic bacteria. This may result in reduced hindgut pH, which, in addition to hindgut acidosis, may lead to a cascade of events culminating in compromised blood flow (and thereby reduced nutrient supply) to the foot resulting in laminitis. Laminitis is also

associated with insulin resistance in equines, whereby the uptake of circulating glucose by tissue cells normally potentiated via insulin is reduced, leading to impoverished glucose supply to cells (or its metabolism within them), including those of the foot. Insulin resistance is often seen in very fat horses and ponies, and may be exacerbated by high intakes of sugars and or starch (Hoffman, 2003; Marlow, 1983). Clearly pastures contain each of the carbohydrate types that have been implicated in the elicitation of laminitis.

Intake of NSC and grazing behavior. One of the challenges in determining the effects of NSC on digestion and metabolism in grazing horses is that there are limited data on pasture intake. Estimates of pasture intake are critical to relate NSC in forages to the physiologic responses in grazing horses. Daily intake of pasture forage in horses has been shown to range from 1.5 to 5.2 % BW (Marlow, 1983; McMenniman, 2000). Although the highest of these reported intakes is exceptional, Argo et al (2002) reported similar intakes of a pelleted feed by ponies. A northern European study has demonstrated that potential intakes of fructan for a 500 kg horse can range from 3.7 and 7.3 kg/d, these amounts being similar to and nearly double the amount of fructan known to elicit laminitis when single doses were administered orally (Pollitt, 2003). The maximum amount of starch recommended to be fed to horses in a single meal is ranges from 2 to 4 g/kg BW in order to prevent hindgut disorders related to rapid fermentation (Potter, 1992). The higher amount of starch is very similar to the 3.75 kg fructan administered by Pollit et al. (2003) to experimentally induce laminitis. Amounts of simple sugars ingested by a 500 kg horse can range from 0.19 to 1.3 kg/d and 2.7 kg/d if the highest level of intake were to be achieved. Some of the forages in the N. European study contained

simple sugar concentrations up to 100 g/kg DM, which might contribute to the occurrence of insulin resistance in susceptible animals. Horses are selective grazers, and are known to prefer feeds with higher sugar content. For this reason, horse feed manufacturers add flavors and sweeteners to many of their products to increase palatability. It is likely that horses also prefer forages that are "sweeter'" and have higher NSC content, which can contribute to elevated intakes during certain times of the year, such as the spring, when forage NSC content is known to be at its highest.

Insulin resistance and laminitis. Insulin resistance (IR) is the inability of a normal concentration of insulin to produce a normal response from target tissue (Kahn, 1978). Insulin resistance may develop with chronic adaptation to meals high in sugar or starch, resulting from the effects of repeated large fluctuations in glycemia and insulinemia after these meals. In horses, IR is an identified as thrifty metabolism that spares glucose and conserves energy, a necessary trait under conditions of sparse nutritional resources. This would be an important feature for feral horses or ponies who have limited availability of forages during the summer and winter in many geographic locations.

The association between IR and laminitis was first studied in the 1980's through oral glucose and inter-venous insulin tolerance tests. Laminitic ponies were reported to be intolerant to glucose and significantly less sensitive to insulin than non-laminitic controls (Coffman and Colles, 1983). More recently, a specific quantitative method for assessing insulin resistance (the minimal model) has demonstrated that ponies genetically predisposed to laminitis have a reduced ability for insulin to induce hypoglycemia compared to normal ponies (Treiber et al., 2006a). Glucose intolerance was also observed in fat ponies with a history of laminitis after oral glucose loading (1g/kg BW). In ponies

with a previous history of laminitis, peak glucose concentrations were higher than normal ponies, and concentrations never returned to baseline. When signs of laminitis emerged in these studies, the insulin response became exaggerated leading to failure of the pancreatic β cells. Results from studies with IR and ponies indicate a changing role of IR in laminitis where 1) a compensated predisposing factor in healthy but genetically predisposed ponies, 2) a pathogenic component as transient exaggerated compensation during the onset of laminitis, and finally 3) uncompensated IR later in the course of the disease (Treiber et al., 2006a).

Recently, statistically standardized proxies to test for insulin sensitivity and insulin response have been derived from basal glucose and insulin concentrations in horses (Treiber et al., 2005b). The proxies are specific indicators of insulin resistance with a predictive power of 78% (Kronfeld et al., 2006). The reciprocal of the square root of insulin (RISQI) identifies insulin sensitivity. The modified insulin to glucose ratio (MIRG) identifies the pancreatic β-cell response. RISQI and MIRG were developed to estimate specific quantitative parameters, insulin sensitivity (SI) and insulin response (AIRg) of the minimal model. These proxies require a single resting basal blood sample, usually collected in the morning hours. The equations to calculate the RISQI [1] and MIRG [2] from basal plasma insulin (mIU/L) and glucose (mg/dL) are as follows (Treiber et al., 2006b):

$$[1]\ \text{RISQI} = 1 / \sqrt{\text{insulin}} = \text{insulin}^{-0.5}$$

$$[2]\ \text{MIRG} = [800 - 0.30\ (\text{insulin} - 50)^2] / (\text{glucose} - 30)$$

The proxies were used to identify insulin sensitivity and responsiveness in 160 Welsh and Dartmoor ponies (Hess, 2005; Treiber et al., 2006a). Ponies predisposed to

laminitis had lower insulin sensitivity (RISQI) and higher insulin response (MIRG), indicating a compensatory exaggeration of pancreatic β-cell insulin excretion (Treiber et al., 2005b). Cut off points were determined for RISQI and MIRG that differentiated ponies predisposed to laminitis from ponies without any predisposition with an accuracy of 70%. The criteria for insulin resistance was RISQI < 0.32, and the criteria for compensatory pancreatic β-cell response was MIRG > 5.6 (Treiber et al., 2005b).

This was the first study to apply specific proxies to determine insulin resistance in the equine species. Although this study indicated an association between insulin resistance (as determined by proxies) and laminitis, it may not be directly applicable to other breeds of horses or horse under natural grazing situations.

AVOIDING PASTURE LAMINITIS

Although a direct relationship between NSC intake from pasture forages and the onset of laminitis in horses is difficult to assess, research has identified possible mechanisms linking digestion and metabolism of the various NSC fractions with the disease. It is therefore recommended it to avoid pasture laminitis by reducing risk factors in the animals themselves and in the pastures on which they graze. This can be achieved through a combination of both pasture and horse management practices. The NSC content of forages needs to be reduced and grazing forages when they are known to have elevated NSC content such as during the spring when environmental conditions favor NSC accumulation should be avoided. Legumes and C4 grass species, or C3 species or varieties which tend to accumulate low contents of NSC are optimal for equines at risk of developing laminitis. Mowing or grazing pastures to maintain short, leafy stands (Watts

and Chatterton, 2004), together with maintenance of appropriate soil moisture and fertility, encourages growth and utilization of NSC as opposed to storage. Grazing should be restricted to the night and early morning hours for avoiding the highest NSC contents occurring during the day, and grazing shaded pastures, or under overcast conditions should help to reduce NSC intakes. Conditions which favor high NSC content typically occur in the spring and autumn where cool season plants are predominant, and extra caution should be taken at these times of the year. Furthermore, pastures which have 'gone to seed', should be avoided, as should recently harvested stubble fields, where high fructan content accumulates in stem bases. Grazing muzzles can also be used to avoid excessive forage, and thus NSC intakes, by horses at pasture. The use of grazing muzzles reduces the amount of forage that can be consumed and restricts intakes to the tops of leaves, where the concentrations of NSC tend to be lowest. Animals on restricted grazing regimes require an alternative source of forage, and this is most commonly given in the form of hay or a hay replacement pellet. However, preserved forages such as hay are also capable of having high concentrations of NSC, and therefore all forages fed to susceptible animals should be analyzed to determine NSC content. It is also likely that horses and ponies on these restricted diets will require mineral and vitamin supplementation, so feed programs should be developed accordingly.

Although only a few individuals within a group of horses or ponies may develop laminitis on a given pasture, the majority of the individuals do not. A predisposition to the disease likely exists in some animals whereby the threshold for developing laminitis is reduced. It is therefore possible that the amount of NSC or its constituents required to elicit the onset of laminitis in susceptible animals may be somewhat lower than that used

to induce the disease experimentally in healthy animals. However, horses also do not consume large doses of NSC at once, they typically graze for 12 to 17 h per day, (Crowell-Davies, 1985; Gallagher and McMeniman, 1989) so small amounts are trickle-fed through the digestive system throughout the grazing period. Another scenario is that continued ingestion of forages with elevated NSC content results in a chronic proliferation of lactate producing bacteria and lowering in hindgut pH, and also may lead to IR. Circadian and seasonal patterns in forage NSC content of pastures clearly influence metabolism and digestion in grazing horses. The link between pasture NSC content and laminitis likely involves a number of risk factors in pasture forages and the animals themselves.

CHAPTER THREE
MANUSCRIPT 1

Circadian and seasonal variation of pasture nonstructural carbohydrates

ABSTRACT: Nonstructural carbohydrates (NSC) and its constituents have been implicated with disorders in horses, including laminitis and insulin resistance. The NSC content of a 5-ha grass/legume horse pasture was evaluated in a series of 36 h studies in April, May, August, October 2005, and January 2006. The pasture was visually split into four quadrants and forage biomass yield and botanical composition of the pasture were evaluated. Environmental variables were measured corresponding with the collection of pasture forage samples hourly for 36 h. Macroclimatic variables measured were ambient temperature, humidity, solar radiation. Microclimatic variables measured were canopy temperature, soil temperature, and photosynthetically active radiation (PAR). Two approaches were used for the determination of carbohydrate profiles in forage samples. For the first (LAB1), a 400 g composite sample of the 4 quadrants (n = 32) was oven dried and submitted to a regional forage laboratory (Dairy One, Ithaca, NY) to determine starch and sugar. Sugar was the water soluble carbohydrate fraction extracted prior to analysis for starch, and included fructans. The NSC was the sum of starch and sugar. For the second (LAB2), a 300 g forage composite sample from each quadrant (n = 128) was frozen in liquid nitrogen, freeze dried, and analyzed for specific NSC fractions using hydrolytic enzymes, with the addition of HCL for the determination of fructans including graminans, the type of fructans in cool season grasses. Tall fescue was the dominant species in each trial. Mean biomass yield was 2,612 kg/ha DM, and there were no differences between trials. Both the LAB1 and LAB2 analyses revealed circadian and

seasonal patterns in forage NSC and its constituents. The highest NSC content was found in April, and was comprised mostly of simple sugars. Overall, simple sugars accounted for 57% of NSC, fructans accounted for 29% of NSC, and starch accounted for 14% of NSC. (LAB2). The circadian fluctuations of NSC and sugar (LAB1) were described by empirically fitted third degree polynomials in April ($R^2 = 0.64$, $P < 0.001$; $R^2 = 0.63$, $P < 0.001$, respectively), May ($R^2 = 0.71$, $P < 0.001$; $R^2 = 0.61$, $P < 0.001$, respectively), and August ($R^2 = 0.41$, $P < 0.01$; $R^2 = 0.44$, $P < 0.001$, respectively). In April, NSC (LAB1) was lowest between 0400 and 0500 (17.6 ± 0.3%), and highest between 1600 and 1700 (22.2 ± 0.3%). Forage NSC content (LAB1) was associated with environmental variables in all months with strongest correlations in April; ambient temperature ($r = 0.72$, $P < 0.001$), solar radiation ($r = 0.62$, $P < 0.001$), and humidity ($r = -0.84$, $P < 0.001$). Overall, LAB1 and LAB2 were in agreement for estimation of forage NSC and starch. A notable observation was the low fructan and relatively high simple sugar content. Simple sugars rather than, or in addition to, fructans may be important in the pathogenesis of the metabolic and digestive disorders (e.g. laminitis) that occur in grazing horses.

Keywords: Pasture, NSC, environment

Introduction

The spring and early summer months coincide with increased content of nonstructural carbohydrates (NSC), including sugars, starches, and fructans in temperate pastures. High pasture NSC content is associated with laminitis in horses. Laminitis is a systemic disease that affects the hoof, which is associated with metabolic and digestive

disruptions caused by high intake of NSC. Overall, annual incidence of laminitis in the U.S. is reported to be 2%, but this rises to about 5% in the spring and summer (Kane, 2000), and nearly half of all reported cases of laminitis in the US occur in animals at pasture (USDA, 2000). Therefore, factors that influence the NSC content in forages are of interest to equine researchers because of the need to predict when grazing animals are at a heightened risk for certain diseases such as laminitis.

Large doses of starch and fructan have been shown to elicit laminitis experimentally (Garner et al., 1975; Pollitt, 2003). However, these studies administered large boluses which were not representative of the carbohydrate profile a horse would consume while grazing pasture forages. Sugars (glucose, sucrose and fructose) may elicit laminitis similarly to starch because they are substrates for rapid fermentation (Clarke et al., 1990), in addition to their direct glycemic-insulinemic effects (Hoffman et al., 2003). Persistent intakes of high sugar in pastures may lead to insulin resistance, which is a predisposing condition for laminitis (Treiber et al., 2006b).

Studies have shown that not only do variables inherent to the plant affect carbohydrate profiles, but that environmental conditions influence the concentration of NSC in pasture forages (Chatterton, 1989). Variables such as temperature (Dias-Tagliacozzo, 1999; Thorsteinsson, 2002; Vagujfalvi, 1999), solar radiation (Ciavarella, 2000), water status (Volaire, 1997), and nutrient status (Belesky, 1991b) have all been show to influence NSC content and its constituents. Because environmental conditions change over the seasons, and throughout the day, NSC content has also been shown to fluctuate seasonally (Burns, 2000; Dubbs et al., 2003) and diurnally (Holt, 1969; Lechtenberg, 1971; Longland, 1999).

Factors that influence the NSC profiles in forages are of interest to equine researchers because of the need to predict when grazing animals are at a heightened risk. The objective of this study was to evaluate the circadian and seasonal variation in NSC content and to identify effects of environmental factors on NSC. The study also aimed to evaluate seasonal changes in botanical composition and forage biomass yield.

Materials and Methods

Five 36 h studies in April, May, August, October 2005, and January 2006, investigated the forage NSC content, forage botanical composition and biomass yield, and environmental conditions of a 5-ha horse pasture at the Middleburg Agricultural Research and Extension Center in northern Virginia.

Pasture establishment and management. A 10-ha field was sprayed with Roundup™ in June and late August 2003, and a broadleaf herbicide was applied in July 2003. The field was seeded in late September 2003 with following seeding rates: 11 kg/acre Max Q™ tall fescue, 3.5 kg/acre Kentucky bluegrass and 1 kg/acre Patriot white clover. Soil was tested for fertility and in March 2004, 18 kg/acre nitrogen fertilizer was applied. The clover was not well established by March 2004, so clover was planted again at a seeding rate of 1 kg/acre. The pasture was established by September 2004 and was grazed by a group of no more than 10 horses until March 2005. The pasture was cut regularly with a batwing bush hog to maintain a sward height of no more than 24 cm from the time of establishment through the duration of the study (at least 10 d prior to each 36 h study). The pasture was not limed or fertilized for the duration of the study.

Forage sample collection. The 10- ha pasture was sectioned off by electric tape fencing to maintain a grazing area of approximately 5-ha which was used for the studies. The 5-ha grazing area was visually divided into four equally sized quadrants marked by orange rubber cones and plastic t-posts. Forage samples were collected hourly from 0600 to 2200 the first day, then overnight at 2400, 0200, 0400, and hourly sample collection resumed gain on the second day from 0600 to 1800. The purpose of staggering the sampling overnight was to eliminate labor, while still allowing for sample collection.

Approximately 400 g (wet weight) of clipped forage was collected into cloth bags. Samples were collected by walking in a "W" pattern in each quadrant and clipped (no more than 2.5 cm from the base) every 5 meters. Samples were immediately taken to the laboratory where an approximately 100 g sub sample was taken from the each of the four hourly samples and composited into an oven dried paper bag. The approximately 400 g sample was then weighed and dried at 70º C in a drying oven to determine DM. The remaining four 300 g samples in cloth bags were individually preserved in liquid nitrogen and stored at –80º C until analysis.

Forage sample analysis. The oven dried forage samples were submitted to a commercial laboratory for proximate analysis (Dairy One, Ithaca NY) (LAB 1) (Table 1). The NSC content was calculated as the sum of sugar and starch. Sugar was determined as water soluble carbohydrates, so it included both sugars and fructans, which were extracted prior to starch extraction (Hall, 1999). Starch was determined using a glucoamylase enzyme and measuring dextrose in an automated biochemical analyzer (YSI 2700 SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA, Application Note Number 319).

The frozen samples were shipped to the USDA-ARS Laboratory in Logan, Utah (LAB 2) via freezer truck where they were analyzed for specific NSC fractions using hydrolytic enzymes (Megazyme International Ireland Ltd., Wicklow, Ireland), with the addition of HCL for the determination of fructans including graminans, the type of fructans in cool season grasses. Samples were freeze dried and ground prior to analysis. It should be noted that all white clover was removed from samples from the April and May studies due to error. Because this is a new technique not commercially available, following is a detailed description of the laboratory methods.

Carbohydrate analysis (LAB2). Approximately 40 mg of ground tissue or control powder was weighed into 16 x 125 mm glass, screw-top tubes. Two tubes were left empty as "enzyme blanks," two tubes were used for sucrose-cellulose controls (Megazyme International Ireland Ltd., Wicklow, Ireland) two tubes were used for fructan (inulin-cellulose controls) (Megazyme International Ireland Ltd., Wicklow, Ireland) and one tube was left blank for a borohydride control. Four ml of deionized water was added to each tube and tubes were shaken gently to suspend tissue. Tubes were then placed in a water bath at 95 ºC for 20 min. Samples were then centrifuged for 15 min at 1240 x g in a Sorvall GLC-2 centrifuge with a swinging bucket rotor. Supernatants were decanted into 16 x 125 mm polystyrene screw-cap tubes. Samples were extracted once more in an additional 8 ml of water, and each supernatant was combined with the first extract of that sample. Volume was then brought up to a line molded into the tube (14.46 ml). Tubes were capped and stored at 4 ºC overnight. On the second day, samples were warmed at 70 ºC for 20 minutes and tube contents mixed thoroughly before analysis.

Water soluble carbohydrate (WSC) was measured according to AOAC Method

999.03 for fructan measurement (Megazyme's Fructan Assay Procedure, AOAC Method 999.03, AACC Method 32.32) adapted to include determination of smaller sugars (mainly glucose, fructose and sucrose) as well as fructan. Furthermore, fructan was hydrolyzed with HCl, as the fructanase in the Megazyme kit did not completely hydrolyze 2-6-linked fructan (graminan). The method was also adapted to allow samples to be read in a 96-well plate reader. A total of 80 wells were used for the samples and controls; the remaining 16 wells were saved for standards. For determination of small sugars, 50 µl of water extract of the tissue or control powder (Megazyme International Ireland Ltd., Wicklow, Ireland) was combined with 50 µl of sucrase/amylase/ pullulanase/maltase (made up as per kit instructions) and incubated at 40 ºC for 30 min. A 25 µl aliquot was then pipetted into a deep well reaction plate (1 ml well capacity) and set aside for determination of simple sugars with PAHBAH reagent (Sigma, St. Louis, MO). Simple sugars in the amylase reaction were converted to sugar alcohols by the addition of 40 µl of 10 mg/ml sodium borohydride, incubated at 40 ºC for 30 min. Excess borohydride was liberated by the addition of 100 µl 0.2 N acetic acid. The resultant foaming was contained within the deep-well plate. Foam was broken by centrifuging the plate at 5000 x g for 1 min in a Quiagen 4-15 ºC centrifuge. Following centrifugation, 100 µl of the reaction solution (Solution "S" in the Megazyme procedure) was pipetted into a clean deep-well plate to which had already been added 50 µl of 1.5 N HCl, producing a final HCl concentration of 0.5 N. Mixture was incubated at 70 ºC for 60 min. After cooling, 25 µL was removed for simple sugar determination with PAHBAH reagent.

PAHBAH determination of simple sugars produced by sucrase reaction: 25 µL

of digest was pipetted into a deep-well plate. Standards for the enzyme digest were 25 µL of an equimolar mixture of glucose and fructose at combined concentrations of 0, 50, 100, 150, 200, 250, 300 and 350:M. After pipetting both extract and standards into the plate, 300 µL of PAHBAH reagent (made up as per Megazyme instructions) was added to the plate and the mixture was incubated for 6 min at 95 ºC and cooled for 10 min by placing plate in water at room temperature; 200 µl was then transferred to a flat-bottomed, optically clear microplate which was read in a SpectraMax Plus (Molecular Devices Corp., Sunnyvale, CA) plate reader at 410 nm.

Simple sugars produced by HCl digestion of fructan were measured in the same way, except the standard was 25 µL of fructose at the same concentrations. It was found that the presence of HCl in the PAHBAH reaction caused an increase in color production, so before the PAHBAH reagent was added, 10 µL of 1.25 N HCl was added to the standards and 10 µL of deionized water was added to the digest samples. Reaction was run and measured as above. Values obtained from measurements on the sucrose and fructan controls were used as correction factors if needed (e.g. if the fructan control measured 95% of the known value, sample values were multiplied by 1.05). Solutions were made up as prescribed in Megazyme's Fructan Assay Procedure (AOAC Method 999.03, AACC Method 32.32) and Megazyme's Total Starch Assay Procedure (AOAC Method 996.11, AACC Method 76.13).

For the measurement of starch, approximately 40 mg of ground tissue or control powder was weighed into 16 x 125 mm glass screw-cap tubes. Thermostable α-amylase was diluted from stock solution at the ratio of 0.1 ml amylase to 2.0 ml 0.05 M MOPS, pH 7.0 with 5 mM calcium chloride. A slight excess of enzyme solution was made up for

80 samples, sufficient enzyme solution was made up for 82 samples (8.2 ml amylase plus 164 ml buffer). The amyloglucosidase solution was made up in the same proportions, with the enzyme being diluted in 0.2 M sodium acetate buffer, pH 4.5. Solutions were made up according to Megazyme's Total Starch Assay Procedure (AOAC Method 996.11, AACC Method 76.13). To begin digestion, 300 µl of 80% (v/v) ethanol was added to each tube. Using a bottle-top dispenser, a 25 µL aliquot of the samples and standards was pipetted into a clean deep-well plate, followed by 250 µl of the GOPOD reagent. The mixture was incubated in a 50 ºC water bath for 20 min, then cooled and 200 µl was pipetted into an optically clear 96-well plate and read in the plate reader at 510 nm.

Pasture composition and yield. Forage botanical composition and biomass yield of the 5-ha pasture was determined in all trials except for May 2005. Botanical composition was assessed by the double DAFOR Scale Abaye (1997), which was adapted from the method of Brodie (1985). The scale (D = dominant, A = abundant, F = frequent, O = occasional, R = rare) was used to measure the relative abundance of forage species within a given area of pasture, where separate classifications were given for forage and weed species. A rank of abundant was given to species that covered one half to three quarters of the area. A ranking of frequent was assigned to species that covered less than half of the area, but were well scattered throughout the site. Occasional species were those that were found a few times, and rare were those that occurred only one or two times in a given area. The double DAFOR Scale was used to asses the species composition within ten 0.25 m^2 quadrates randomly placed in each of the four quadrants.

Forage mass was measured by clipping the forage within the quadrates (n = 40) and contents were placed in pre-weighed bags and oven dried to calculate DM yield.

Environmental conditions. Macro- and micro environmental conditions were measured and recorded hourly during each of the five 36 h studies. The macro-environmental conditions, temperature (ºC), humidity (%), and solar radiation (watts/m^{-2}) were measured and recorded hourly by a weather station (Texas Weather Instruments, Dallas, Texas). Forage canopy temperature (ºC) was measured from each of the four quadrants using an infrared thermometer (Mikron Infrared, Inc**.**, Oakland, NJ). Soil surface temperature (ºC) was measured using Watch Dog 100-Temp 2K data loggers installed just below the Ao horizon at approximately 12 cm (Spectrum Technologies, Inc. Plainfield, Il). Photosynthetically active radiation (PAR) (µmol m^{-2} s^{-1}) was measured using an AccuPAR Model LP-80 point sensor above and below the canopy (Decagon Inc., Pullman, WA).

Statistical analysis. All data are summarized as means ± SE. A one-way ANOVA with Bonferroni's post test was performed to compare results from LAB1 and LAB2, and to compare biomass yield between the seasons. Polynomial regression analysis of pasture NSC, sugar, and starch (LAB1) were performed using the REG procedure of the SAS System (SAS Institute Inc, ver. 9.13, Cary, NC). This analysis fit a repeated measures ANCOVA model with month, polynomials across sampling hours, and their interactions. Data from months was sliced and analyzed separately by fitting polynomial through sixth-order testing for significance of Type I hypotheses. Polynomials were then reduced to their highest order term and significant at alpha = 0.05). Cubic curves were fit to the pasture carbohydrate (LAB1) and environmental

variables to visualize patterns in circadian variation. Partial correlation coefficients were calculated to estimate the strength of linear association between pasture variables (LAB1) and environmental variables while partialing out the linear effect of sampling hour. The calculations were performed using the CORR procedure of the SAS System (ver. 9.13 SAS Institute Inc, Cary, NC 27513). Since data from LAB2 were received at a much later date than LAB1, statistical analyses included only nonlinear regression to evaluate circadian patterns in NSC and its constituents, and a one-way ANOVA with Bonferroni's post test to determine differences in the fractions across the months (GraphPad Prism ver. 4.00, San Diego, CA).

Results.

Botanical composition and yield. Grass species were dominant throughout all quadrants (Table 1) and tall fescue was the dominant species (Figure 1) during all of the studies. Kentucky bluegrass occurred frequently in some instances, but was usually ranked occasionally. White clover was rare and was found mostly in quadrant two. Weeds observed were horse nettle, plantain, crabgrass and dandelion, but their occurrence was rare and found mostly in quadrant 2. Mean forage biomass yield was 2,612 kg/ha DM, and there were no differences between trials (Figure 2).

Nonstructural carbohydrate content. The nutrient composition of the pasture for each of the five 36 h trials is shown in Table 2 (LAB1). Specific mean carbohydrate fractions reported by LAB 1 and LAB 2 are summarized in Table 3. Ranges of NSC and its constituents were similar between LAB1 and LAB2 (Table 4 and 5, respectively). Both LAB1 and LAB2 analyses revealed circadian and seasonal patterns in forage NSC

and its constituents, where seasonal patterns are shown in Table 3. Polynomial fits for each month separately (LAB1) revealed circadian fluctuation for NSC and sugar in April, May and August (Figure 3 and Figure 4, respectively). In October and January the polynomial fits were first order and linear, consequently there was a lack of significant circadian variation. There was no circadian variation in starch in any of the months. LAB2 showed similar patterns in circadian variation for NSC and sugar, but the fructan and starch did not show circadian fluctuation (Figure 5). Fructan was however higher in April than in the other months (Figure 6).

Environmental effects. Macroclimate and microclimate data are summarized in Table 6 and Table 7, respectively. Overall, the greatest ranges in macroclimate and microclimate variables occurred in April (eg. ambient temperature from night to day ranged from 2.8 to 16.7 ºC, respectively). August was extremely hot (ambient temperature 26.4 ± 0.2 ºC, and a maximum of 31.6 ºC). Conditions were overcast and rainy during much of the October and January trials and consequently there is little circadian fluctuation in environmental variables.

Correlation coefficients for environmental data and pasture variables (LAB1) are reported in Table 8 through Table 12 for April through January, respectively. Plots of corresponding pair-wise comparisons of these correlations are shown in Appendix Figures 1 through 21, and are grouped by environmental variable. Overall, there were significant correlations between environmental variables and LAB1 pasture NSC, sugar and starch, although results were inconsistent between the months. In April and May, there were correlations between the environmental variables and NSC and sugar, but few with starch. During the trials in August, October and January, there were correlations

between all of the environmental variables and starch, with exception for PAR below the canopy in October. During all of the months, there was a positive correlation between ambient temperature and solar radiation and forage NSC content. There was a negative correlation between relative humidity and forage NSC content during all of the months. Infrared temperature of the canopy, like ambient temperature, had a positive correlation with forage NSC content. The PAR data was inconsistent, and there was no PAR data for the January trial.

Discussion

Nonstructural carbohydrates are of interest to equine research because they are involved in both digestive and metabolic diseases in horses, such as insulin resistance (Treiber et al., 2005a) and laminitis (Pollitt, 2003). Concentrations of NSC and its constituents are influenced by environmental conditions; therefore this study identified some of the environmental effects on pasture NSC. This study illustrated circadian and seasonal variation in pasture NSC, and was evidence for how the environment plays a role in shaping these patterns.

Overall, the pasture carbohydrate profiles in this study were consistent with those previously reported for mixed grass/legume pastures in Northern Virginia, where NSC content ranged from about 4 to 23% DM, and in this study they ranged from 2.3 to 25.3% DM over the five 36 h trials. Nonstructural carbohydrate content in tall fescue sampled in October and December in North Carolina averaged 10.3 to 15.7 % DM over three sampling years, while the highest individual NSC was 21.6 % DM (Burns, 2000). During the 36 h trials in October and January, NSC contents were likely lower than those

previously reported in other studies because overcast skies with precipitation predominated most of the sampling period. Low light intensity and rain have been shown to result in lower NSC content (Ladyman, 2003; Longland, 1999). Temperatures were also very mild during the October trial, and there was little difference between the day and night temperatures, which is a major factor in accumulation of NSC in cool season plants (Chatterton, 1989). April also had the greatest difference between night and day time temperatures. In previous studies, WSC content of a given plant species ranged from 95 to 560 g/kg DM with corresponding ranges in fructan of 32 to 439 g/kg DM depending upon the temperature at which it was grown, with higher and lower values being associated with cooler (5-10º C) and warmer (15-25º C) temperatures, respectively (Chatterton, 1989). Although the mean temperatures were similar between April, May and October, minimum and maximum temperatures varied.

Circadian and seasonal variation in NSC in this study were consistent with other studies (Burns and Chamblee2000; Chatterton, 1989; Ciavarella, 2000; Longland, 1999). Hoffman et al. (2003) reported peaks in NSC at April and November. This study found forage NSC content to be highest in April, but when a secondary peak was expected in late October, environmental conditions resulted in low NSC content. Circadian patterns were observed in April, May and August for NSC and sugar, with the most extreme fluctuation occurring in April. In all months, pasture forage NSC and sugar were lowest in the early morning hours (between 0400 and 0600) and highest in the late afternoon (between 1600 and 1800).These results are consistent with reports that NSC tends to increase throughout the day from 0600 to 1800 (Lechtenberg, 1971).

Circadian and seasonal variations of forage NSC content in cool season pastures are a result of various environmental influences on the plants, and the plants themselves (ie. stage of maturity and species). The environmental influences are related to the regulation of photosynthesis and respiration. Photosynthesis is the process by which plants synthesize sugars (which are stored as starches, and fructans in cool season grasses, such as those in this study). In plant leaves, the uptake of water and CO_2 necessary for photosynthesis occurs through stomata, and is regulated by the opening and closing of their guard cells. The activity of these pores is coupled with environmental factors such as light intensity, water status and humidity. Under conditions of high light intensity, guard cells swell, the stomata open, and CO_2 diffuses into the leaf cells and is assimilated in photosynthesis (Roelfsema and Hedrich, 2005). In the context of this study, environmental conditions in April favored photosynthesis, therefore sugar production was high. Under conditions of low light intensity, low humidity and low water availability, the apertures may be reduced, thus a reduction in photosynthesis may occur, providing one explanation as to why forage NSC content was lowest in the October and January 36 trials. Although humidity was high in October, light intensity was low and resulted in decreased photosynthesis, and hence decreased sugar and NSC content

Plant maturity and species also affect forage NSC and its components in pastures. Typically, immature plants are higher in NSC and lower in fiber, while mature plants are lower in NSC and higher in fiber. In a recent study, sugar (glucose, sucrose and fructose) concentrations in vegetative tissues of oat forage were higher in younger plants (15 % dry weight) than in mature plants (1-2 % dry weight) (Chatterton et al., 2006). In the April and May 36 h studies plants were young and vegetative (tiller to

flower) and were generally higher in NSC than the other months. In the study by Chatterton et al. (2006), starches increased with maturity (3-4% to 10-15%), and fructans did not appear to be affected by stage of maturity. Starch content never approached the amount reported by Chatterton et al. (2006), but the pasture was mown to maintain the sward height at 14 to 20 cm, and seed head production (which accounts for high starch content) was minimal. Based on the carbohydrate analyses performed by LAB1, fructan content was unknown, LAB2 results indicated that only one-third of the 'sugar' fraction was fructan and actual sugar probably accounted for the other two-thirds. The pasture was predominantly tall fescue though, which may not have accumulated the high concentrations of fructan such as those reported in other species including perennial ryegrass and oat forage (> 20% DM) (Chatterton et al., 2006; Longland, 1999).

Conclusions

The amount of NSC, sugar, and starch varied throughout the day and over the season during these five 36 h trials, which has implications on the management of horses prone to laminitis and insulin resistance. The environmental observations and correlations between pasture NSC, sugar, and starch, provide insight into how the environment affects NSC profiles in forages, and when grazing should be avoided. These are necessary data for advancing future research on the avoidance of disorders associated with elevated pasture forage NSC and its constituents. A notable observation was the low fructan and relatively high simple sugar content. Simple sugars rather than, or in addition to, fructans may be important in the pathogenesis of the metabolic and digestive disorders (e.g. laminitis) that occur in grazing horses. However, the laboratory techniques utilized by

LAB2 in these studies need further validation. Further work is also needed to determine what “safe” amounts of NSC are for managing horses prone to disorders exacerbated by high concentrations of NSC, such as laminitis and insulin resistance. Most feed companies now offer “low starch” and “safe” feeds for these types of at-risk horses, thus determining cut off points for NSC content of feeds and forages alike would benefit the feeding management of equines. While these studies provided great insight to the NSC content of forages throughout the year and over a circadian period, they were short windows of time for only one year, thus more data are needed to determine factors that influence NSC content of forages and how grazing horses may be affected.

Table 3.1. Pasture composition for each of the four quadrants using 0.25 m^2 quadrates (n = 10) in April, August, October 2005 and January 2006 presented as total ground cover (%), grass species (%), legume species (%), and weed species (%).

Quadrant	Component	April	August	October	January
1	Ground cover	76.5	92.0	94.0	58.5
	Grass	100	99.5	99.5	100
	Legume	0	5.0	5.0	0
	Weed	0	0	0	0
2	Ground cover	82.5	87.5	91.5	45.5
	Grass	89.5	92.0	97.0	100
	Legume	14.4	26.7	7.0	0
	Weeds	11.9	20.0	5.0	0
3	Ground cover	84.5	92.0	99.0	40.5
	Grass	100	98.5	99.0	100
	Legume	0	10.0	0	0
	Weed	0	5.0	0	0
4	Ground cover	85.5	88.0	100	36.3
	Grass	100	99.5	100	100
	Legume	0	5.0	0	0
	Weeds	0	0	0	0

Table 3.2. Nutrient composition on a DM basis of the pasture forage (n = 33) and hay forage (n = 2) (LAB1) during the five 36 h trials[1].

Item	April	May	August	October	January	Hay
DE, Mcal/kg	2.8 ± 0.01^{a}	2.2 ± 0.01^{b}	2.1 ± 0.01^{c}	2.1 ± 0.01^{c}	2.1 ± 0.01^{c}	1.9 ± 0.02^{d}
CP, %	21.3 ±1.4^{a}	14.0 ± 0.2^{b}	12.7 ± 0.2^{c}	12.9 ± 0.2^{c}	14.2 ± 0.3^{b}	11.3 ± 0.5^{d}
ADF, %	25.4 ± 0.24^{a}	35.3 ± 0.2^{b}	36.9 ± 0.5^{c}	37.9 ± 0.2^{c}	39.1 ± 0.3^{d}	41.7 ± 0.4^{e}
NDF, %	46.4 ± 0.33^{a}	64.2 ± 0.3^{b}	64.0 ± 0.7^{c}	66.2 ± 0.4^{c}	66.5 ± 0.5^{d}	62.1 ± 1.3^{e}
EE, %	2.8 ± 0.04^{a}	2.8 ± 0.04^{b}	4.3 ± 0.1^{c}	3.8 ± 0.06^{a}	2.2 ± 0.09^{d}	1.6 ± 0.3^{e}
NSC, %	20.3 ± 0.41^{a}	11.7 ± 0.4^{b}	9.2 ± 0.5^{c}	6.9 ± 0.2^{d}	7.1 ± 0.2^{d}	8.9 ± 0.1^{c}
Sugar, %	18.9 ± 0.40^{a}	10.2 ± 0.4^{b}	7.6 ± 0.5^{c}	5.7 ± 0.2^{d}	6.1 ± 0.2^{d}	6.1 ± 0.2^{d}
Starch, %	1.4 ± 0.04^{a}	1.5 ± 0.06^{a}	1.5 ± 0.06^{a}	1.1 ± 0.05^{b}	1.0 ± 0.03^{b}	2.8 ± 0.1^{c}
Ash, %	8.8 ± 0.09	5.9 ± 0.04	8.3 ± 0.08	7.0 ± 0.1	6.4 ± 0.1	7.6 ± 0.4
Ca, %	0.48 ± 0.004^{a}	0.27 ± 0.003^{b}	0.44 ± 0.006^{c}	0.53 ± 0.01^{d}	0.38 ± 0.004^{e}	0.9 ± 0.07^{f}
P, %	0.40 ± 0.001^{a}	0.33 ± 0.003^{b}	0.40 ± 0.005^{a}	0.32 ± 0.004^{b}	0.21 ± 0.004^{c}	0.23 ± 0.01^{d}
Mg, %	0.18 ± 0.0001^{a}	0.13 ± 0.003^{b}	0.26 ± 0.003^{c}	0.24 ± 0.004^{c}	0.15 ± 0.002^{d}	0.20 ± 0.01^{e}
K, %	3.1 ± 0.03^{a}	2.1 ± 0.02^{b}	2.4 ± 0.3^{c}	2.0 ± 0.04^{b}	0.95 ± 0.03^{d}	3.0 ± 0.08^{a}
Na, %	0.005 ± 0.0002^{a}	0.006 ± 0.0002^{a}	0.005 ± 0.0006^{a}	0.006 ± 0.0002^{a}	0.002 ± 0.0002^{b}	0.10 ± 0.04^{c}
Fe, %	175.6 ± 6.6^{a,b}	83.5 ± 5.9^{a}	161.7 ±14.4^{a}	186.6 ± 11.1^{a,b}	494.8 ± 50.6^{c}	129.0 ± 15.0^{d}
Cu, %	6.9 ± 0.13^{a}	5.2 ± 0.09^{b}	6.3 ± 0.11^{c}	5.4 ± 0.09^{b}	5.0 ± 0.11^{b}	6.5 ± 0.5^{c}
Zn, %	22.9 ± 0.30^{a}	20.2 ± 0.3^{b}	15.1 ± 0.2^{c}	15.6 ± 0.3^{c}	14.0 ± 0.3^{d}	17.5 ± 0.5^{e}

[1]Analyses (AOAC, 1990) performed by Dairy One, Ithaca, NY

^{a,b,c,d,e,f} Columns with different letter superscripts are different ($P < 0.05$)

Table 3.3. Pasture and nonstructural carbohydrate (NSC) profiles during the five 36 studies from LAB1 (n = 33) and LAB2 (n = 142). Data are summarized as mean ± SE.

		Month				
Profile	Lab	April	May	August	October	January
NSC, % DM	LAB1	20.3 ± 0.4^{a}	$11.7 \pm 0.4^{b,*}$	9.2 ± 0.5^{c}	6.9 ± 0.2^{d}	$7.1 \pm 0.2^{d,*}$
	LAB2	19.6 ± 0.3^{a}	13.9 ± 0.3^{b}	9.0 ± 0.3^{c}	7.6 ± 0.2^{d}	8.6 ± 0.2^{c}
Sugar[1], % DM	LAB1	$18.9 \pm 0.4^{a,*}$	$10.2 \pm 0.4^{b,*}$	$7.6 \pm 0.5^{c,*}$	$5.7 \pm 0.2^{d,*}$	$6.1 \pm 0.2^{d,*}$
	LAB2	11.4 ± 0.2^{a}	7.6 ± 0.2^{b}	5.9 ± 0.3^{c}	4.2 ± 0.1^{d}	4.5 ± 0.1^{d}
Starch, % DM	LAB1	1.4 ± 0.04^{a}	1.5 ± 0.06^{a}	1.5 ± 0.06^{a}	1.1 ± 0.05^{b}	1.0 ± 0.03^{b}
	LAB2	2.7 ± 0.1^{a}	2.3 ± 0.1^{b}	1.2 ± 0.03^{c}	0.7 ± 0.04^{d}	1.2 ± 0.03^{c}
Fructan, % DM	LAB1	-	-	-	-	-
	LAB2	5.5 ± 0.1^{a}	3.9 ± 0.1^{b}	1.9 ± 0.05^{d}	2.6 ± 0.06^{c}	2.8 ± 0.07^{c}

[1]LAB1 Sugar = water soluble carbohydrate (WSC) = sugar + fructan

[a,b,c,d] Means with different letter superscripts differ within a row ($P < 0.05$).

[*] Means with different symbol superscripts differ between profiles ($P < 0.05$).

Table 3.4. Pasture and nonstructural carbohydrate (NSC) profiles during the five 36 studies (LAB1). Data are summarized as mean ± SE, minimum (Min), and maximum (Max) (n= 33) [1].

	NSC[2]			Sugar[3]			Starch		
Month	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max.
April	20.3 ± 0.4[a]	15.8	25.3	18.9 ± 0.40[a]	14.6	23.7	1.4 ± 0.04[a]	0.9	2.1
May	11.7 ± 0.4[b]	6.5	16.0	10.2 ± 0.4[b]	5.4	14.0	1.5 ± 0.06[a]	0.7	2.1
August	9.2 ± 0.5[c]	2.3	14.5	7.6 ± 0.5[c]	1.2	12.7	1.5 ± 0.06[a]	0.8	2.2
October	6.9 ± 0.2[d]	3.9	10.1	5.7 ± 0.2[d]	3.6	8.2	1.1 ± 0.05[b]	0.2	1.6
January	7.1 ± 0.2[d]	4.8	9.6	6.1 ± 0.2[d]	3.7	8.5	1.0 ± 0.03[b]	0.7	1.4

[1]Analysis (AOAC, 1990) performed by Dairy One, Ithaca, NY

[2]NSC = WSC + starch

[3]Sugar is water soluble carbohydrates (WSC) and includes fructans.

[a,b,c,d,e] Means within the same column with different letter subscripts differ ($P < 0.05$).

Table 3.5. Pasture and nonstructural carbohydrate (NSC) profiles during the five 36 studies (LAB2). Data are summarized as mean ± SE, minimum (Min), and maximum (Max) (n = 142) [1].

	NSC[2]			Sugar[3]			Fructan			Starch		
Month	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max.
April	19.6 ± 0.3[a]	16.3	23.2	11.4 ± 0.2[a]	8.5	13.4	5.5 ± 0.1[a]	4.5	6.5	2.7 ± 0.1[a]	1.9	3.5
May	13.85 ± 0.3[b]	9.9	16.6	7.6 ± 0.2[b]	5.4	9.2	3.9 ± 0.1[b]	2.8	5.2	2.3 ± 0.1[b]	1.7	3.1
August	9.0 ± 0.3[c]	6.1	12.1	5.9 ± 0.3[c]	3.6	8.3	1.9 ± 0.05[d]	1.2	2.7	1.2 ± 0.03[c]	0.7	1.5
October	7.6 ± 0.2[d]	5.7	10.2	4.2 ± 0.1[d]	3.6	6.0	2.6 ± 0.06[c]	2.0	3.2	0.7 ± 0.04[d]	0.4	1.4
January	8.6 ± 0.2[c]	6.6	10.9	4.5 ± 0.1[d]	3.5	6.0	2.8 ± 0.07[c]	2.0	4.1	1.2 ± 0.03[c]	0.9	1.5

[1]Analysis performed by USDA-ARS (Logan, UT) using enzymatic procedures adapted from Megazyme Inc. (Wicklow, IRE)

[2]NSC = Sugar + fructan + starch

[3]Sugar is the sum of glucose, sucrose and fructose

[a,b,c,d,e] Means within the same column with different letter subscripts differ ($P < 0.05$)

Table 3.6. Summary of macroclimate variables over the five 36 h studies. Data are summarized as mean ± SE, minimum (Min), and maximum (Max) (n = 258).

	Temperature (ºC)			Humidity (%)			Solar radiation (watts/m^{-2})		
Month	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max
April	10.3 ± 0.3	2.8	16.7	46.7 ± 0.8	25	68	430.5 ± 21.6	0	920
May	12.9 ± 2.5	8.3	16.7	61.8 ± 0.5	49	77	377.7 ± 20.5	0	1100
August	26.4 ± 0.2	18.9	31.6	78.7 ± 1.1	53	100	410.0 ± 21.1	0	870
October	9.7 ± 0.1	6.6	13.8	85.7 ± 0.6	67	100	92.1 ± 8.9	0	520
January	5.1 ± 0.1	2.8	8.9	80.5 ± 0.8	63	100	116.7 ± 10.2	0	480

Table 3.7. Summary of microclimate variables over the five 36 h trials Data are summarized as mean ± SE, minimum (Min), and maximum (Max) (n = 258).

	Soil Temperature (ºC)			IR Temp (%)			PAR above (µmol m^{-2} s^{-1})			PAR below (µmol m^{-2} s^{-1})		
Month	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max	Mean ± SE	Min	Max
April	11.5 ± 0.1	7.9	14.3	10.8 ± 0.4	0.2	19.5	1293.0 ± 54.9	8.9	2308.0	157.36 ± 9.3	0	411.3
May	17.01 ± 0.1	15.1	18.6	14.7 ± 0.3	3.8	22.0	846.4 ± 42.1	0	2074.0	74.5 ± 5.1	0	273.4
August	24.3 ± 0.1	22.6	26.2	27.1 ± 0.3	17.5	32.9	639.8 ± 41.7	0	1963.0	106.5 ± 6.1	0	281.3
October	14.3 ± 0. 02	13.5	15.2	11.9 ± 0.02	8.3	18.6	145.4 ± 7.0	2.0	281.3	70.5 ± 7.5	0	329.8
January	4.7 ± 0.02	3.7	5.4	8.6 ± 0.2	-1.0	14.3	na	na	na	na	na	na

Table 3.8. Linear correlation between nonstructural carbohydrate (NSC), sugar and starch (LAB1), and environmental variables, with sampling hour partialed out, during April 2005. Correlation were significant if ($P < 0.001$).

	Pasture Variable[a]		
Environmental Variable	NSC[b]	Sugar[c]	Starch
Air temperature (ºC)	r = 0.72	r = 0.74	r = 0.08
	$P < 0.001$	$P < 0.0001$	$P = 0.27$
Ambient relative humidity (%)	r = - 0.84	r = -0.85	r = - 0.15
	$P < 0.001$	$P < 0.0001$	$P = 0.04$
Radiation (watts/m^{-2})	r = 0.62	r = 0.61	r = 0.19
	$P < 0.001$	$P < 0.0001$	$P = 0.007$
Soil temperature (ºC)	r = 0.73	r = 0.74	r = 0.13
	$P < 0.001$	$P < 0.0001$	P = 0.08
IRtemp (ºC)	r = 0.66	r = 0.66	r = 0.16
	$P < 0.001$	$P < 0.0001$	P = 0.03
PAR above(µmol m^{-2} s^{-1})	r = 0.31	r = 0.30	r = 0.20
	$P < 0.001$	$P < 0.0001$	P = 0.006
PAR below(µmol m^{-2} s^{-1})	r = 0.09	r = 0.08	r = 0.18
	P = 0.21	P = 0.26	P = 0.01

[a]Analysis (AOAC, 1990) performed by Dairy One, Ithaca, NY

[b]NSC = sugar + starch

[c]Sugar is measured as water soluble carbohydrates and includes fructans

Table 3.9. Linear correlation between nonstructural carbohydrate (NSC), sugar and starch (LAB1), and environmental variables, with sampling hour partialed out, during May 2005. Correlations were significant if $P < 0.001$.

	Pasture Variable[a]		
Environmental Variable	NSC[b]	Sugar[c]	Starch
Air temperature (ºC)	r = 0.40	r = 0.42	r = 0.10
	$P < 0.0001$	$P < 0.0001$	$P = 0.12$
Ambient relative humidity (%)	r = - 0.59	r = -0.59	r = -0.35
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Radiation (watts/m^{-2})	r = 0.42	r = 0.45	r = 0.09
	$P < 0.0001$	$P < 0.0001$	$P = 0.20$
Soil temperature (ºC)	r = 0.40	r = 0.39	r = 0.25
	$P < 0.0001$	$P < 0.0001$	P = 0.0002
IRtemp (ºC)	r = 0.59	r = 0.61	r = 0.20
	$P < 0.0001$	$P < 0.0001$	P = 0.002
PAR above (µmol m^{-2} s^{-1})	r = 0.53	r = 0.55	r = 0.25
	$P < 0.0001$	$P < 0.0001$	P = 0.0001
PAR below (µmol m^{-2} s^{-1})	r = 0.41	r = 0.42	r = 0.21
	$P < 0.0001$	$P < 0.0001$	P = 0.0016

[a]Analysis (AOAC, 1990) performed by Dairy One, Ithaca, NY

[b]NSC = sugar + starch.

[c]Sugar is measured as water soluble carbohydrates and includes fructans

Table 3.10. Linear correlation between nonstructural carbohydrate (NSC), sugar, and starch (LAB1), and environmental variables, with sampling hour partialed out, during August 2005. Correlations were significant if $P < 0.001$.

	Pasture Variable[a]		
Environmental Variable	NSC[b]	Sugar[c]	Starch
Air temperature (ºC)	r = 0.38	r = 0.28	r = 0.61
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Ambient relative humidity (%)	r = -0.32	r = -0.21	r = - 0.60
	$P = 0.0002$	$P = 0.0002$	$P < 0.001$
Radiation (watts/m^{-2})	r = 0.47	r = 0.35	r = 0.69
	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
Soil temperature (ºC)	r = 0.25	r = 0.16	r = 0.47
	$P = 0.004$	$P = 0.08$	P < 0.0001
IRtemp (ºC)	r = 0.36	r = 0.27	r = 0.54
	$P < 0.001$	$P = 0.002$	P < 0.0001
PAR above (µmol m^{-2} s^{-1})	r = 0.15	r = 0.03	r = 0.48
	$P = 0.08$	$P = 0.71$	P < 0.0001
PAR below (µmol m^{-2} s^{-1})	r = 0.24	r = 0.13	r = 0.60
	P = 0.005	$P = 0.13$	P < 0.0001

[a]Analysis (AOAC, 1990) performed by Dairy One, Ithaca, NY

[b]NSC = sugar + starch

[c]Sugar is measured as water soluble carbohydrates and includes fructans.

Table 3.11. Linear correlation between nonstructural carbohydrate (NSC), sugar and starch, and environmental variables, with sampling hour partialed out, during October 2005. Correlations were significant if $P < 0.001$.

	Pasture Variable[a]		
Environmental Variable	NSC[b]	Sugar[c]	Starch
Air temperature (ºC)	r = 0.72	r = 0.69	r = 47
	$P < 0.0001$	$P < 0.0001$	$P < 0.001$
Ambient relative humidity (%)	r = - 0.75	r = - 0.70	r = -0.58
	$P < 0.0001$	$P < 0.0001$	P < 0.001
Radiation (watts/m^{-2})	r = 0.58	r = 0.53	r = -0.50
	$P < 0.0001$	$P < 0.0001$	$P < 0.001$
Soil temperature (ºC)	r = 0.59	r = 0.57	r = 0.41
	$P < 0.001$	$P < 0.001$	$P < 0.001$
IRtemp (ºC)	r = 0.49	r = 0.47	r = 0.35
	$P < 0.001$	$P < 0.001$	$P < 0.001$
PAR above (µmol m^{-2} s^{-1})	r = 0.69	r = 0.62	r = 0.65
	$P < 0.001$	$P < 0.001$	P < 0.001
PAR below (µmol m^{-2} s^{-1})	r = 0.23	r = 0.25	r = 0.06
	P = 0.005	P = 0.003	P = 0.49

[a]Analysis (AOAC, 1990) performed by Dairy One, Ithaca, NY

[b]NSC = 100 –water – CP – fat – ash - NDF.

[c]Sugar is measured as water soluble carbohydrates, including fructans

Table 3.12. Linear correlation between nonstructural carbohydrate (NSC), sugar and starch (LAB1), and environmental variables, with sampling hour partialed out, during January 2006. Correlations were significant if $P < 0.001$.

	Pasture Variable[a]		
Environmental Variable	NSC[b]	Sugar[c]	Starch
Air temperature (ºC)	r = 0.29	r = 0.30	r = 47
	$P < 0.0001$	$P < 0.0001$	$P < 0.001$
Ambient relative humidity (%)	r = - 0.34	r = - 0.35	r = -0.58
	$P < 0.0001$	$P < 0.0001$	P < 0.001
Radiation (watts/m^{-2})	r = 0.34	r = 0.38	r = -0.50
	$P < 0.0001$	$P < 0.0001$	$P < 0.001$
Soil temperature (ºC)	r = 0.05	r = 0.05	r = 0.41
	$P = 0.40$	$P = 0.43$	$P < 0.001$
IRtemp (ºC)	r = 0.25	r = 0.27	r = 0.35
	$P < 0.001$	$P < 0.001$	$P < 0.001$

[a]Analysis (AOAC, 1990) performed by Dairy One, Ithaca, NY

[b]NSC = 100 –water – CP – fat – ash - NDF.

[c]Sugar is measured as water soluble carbohydrates, including fructans

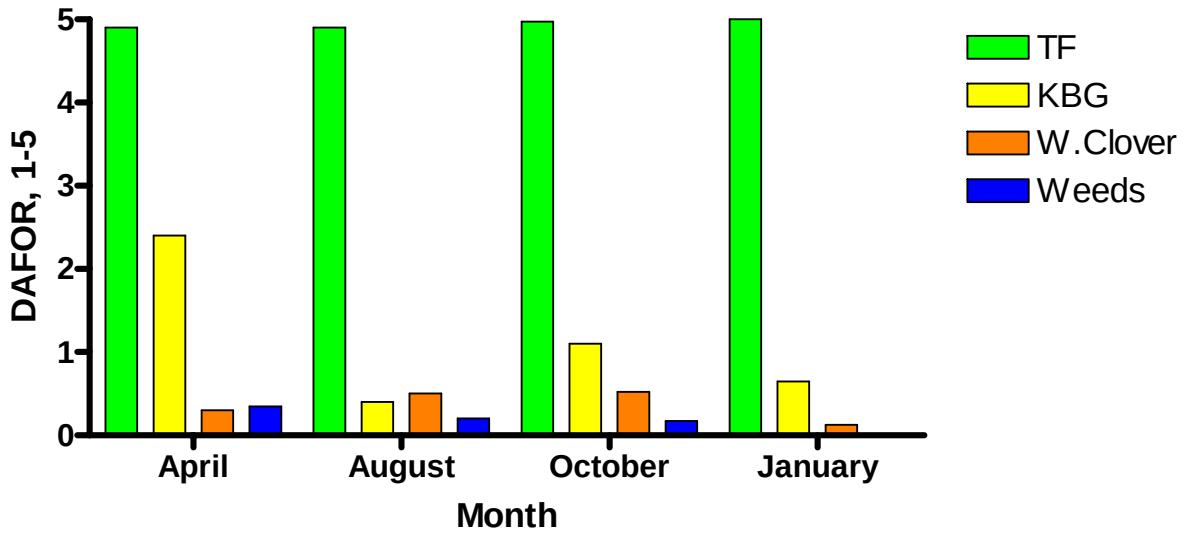

Figure 3.1. Visual evaluation of botanical composition using the Double DAFOR Scale for tall fescue (TF), Kentucky bluegrass (KBG), white clover (W. Clover) and miscellaneous weeds (Weeds) during 36 h studies in April, August, October 2005, and January 2006.

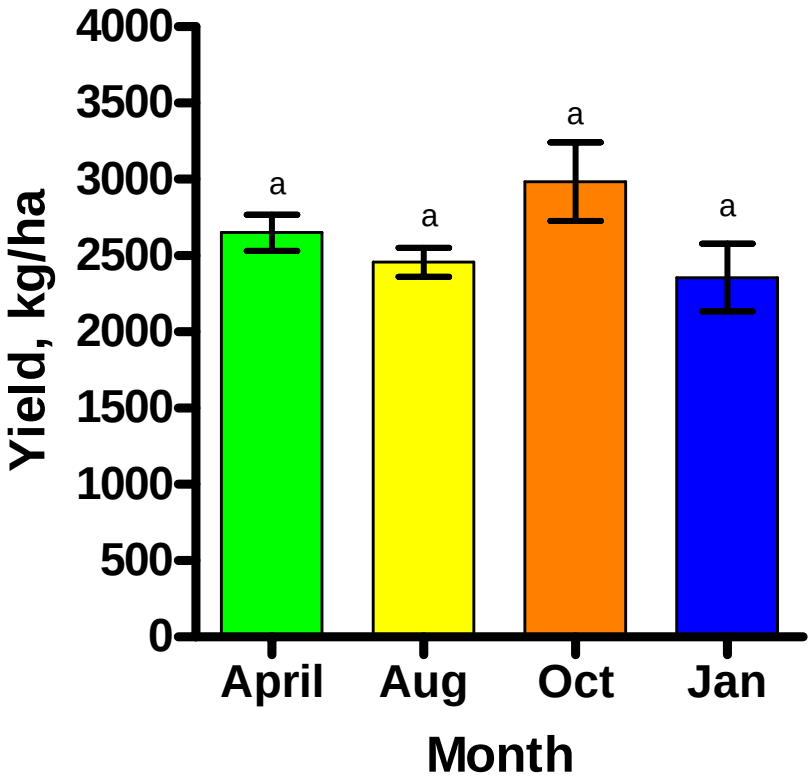

Figure 3.2. Biomass yield (kg/ha) of all forages in the 5-ha pasture in April, August, October 2005, and January 2006.

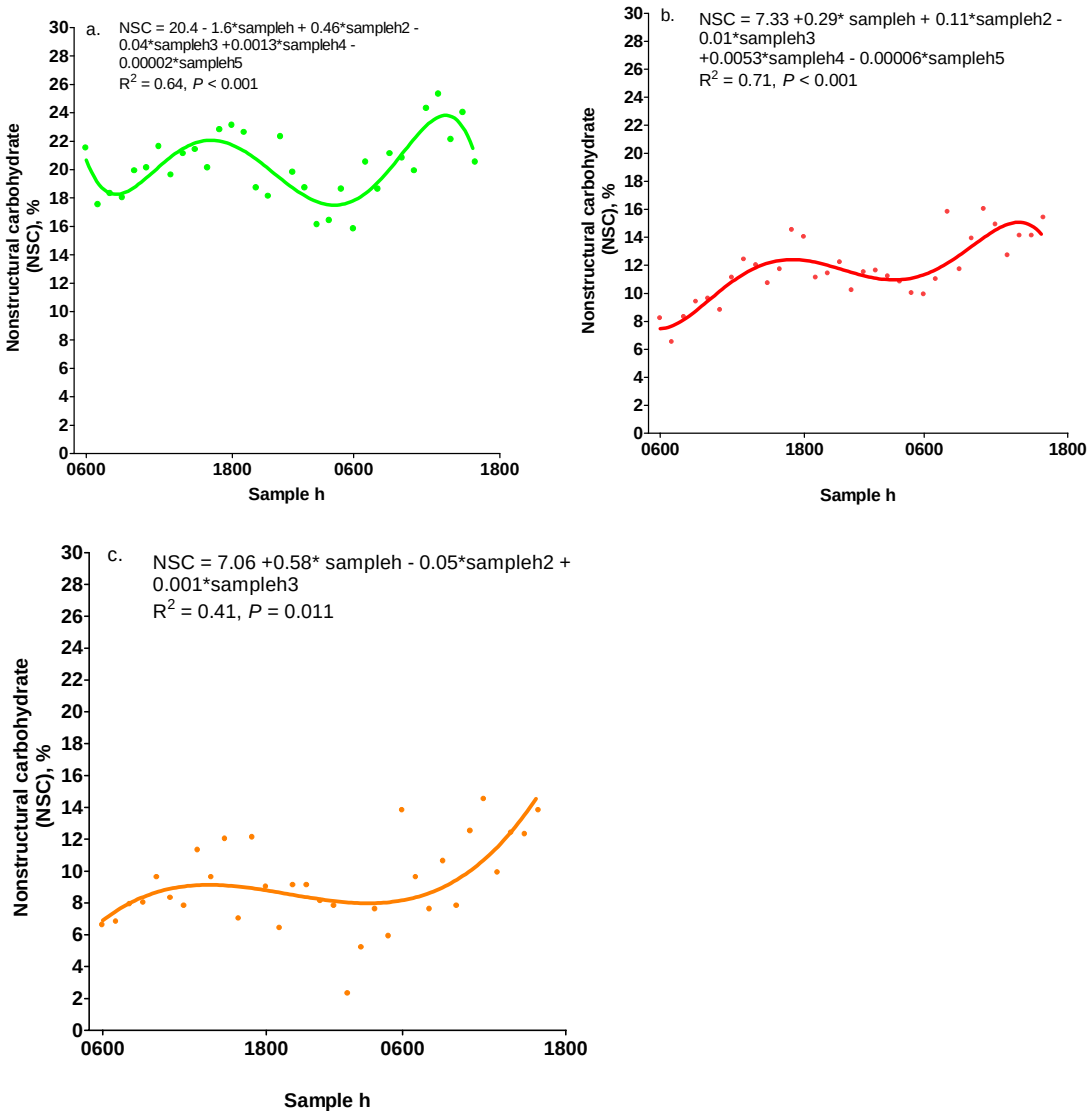

Figure 3.3. Polynomial regression of pasture forage nonstructural carbohydrate (NSC, % DM) (LAB1) (a.) April fifth order, quintic), (b.) May fifth order, quintic) and (c.)August (third order, cubic).

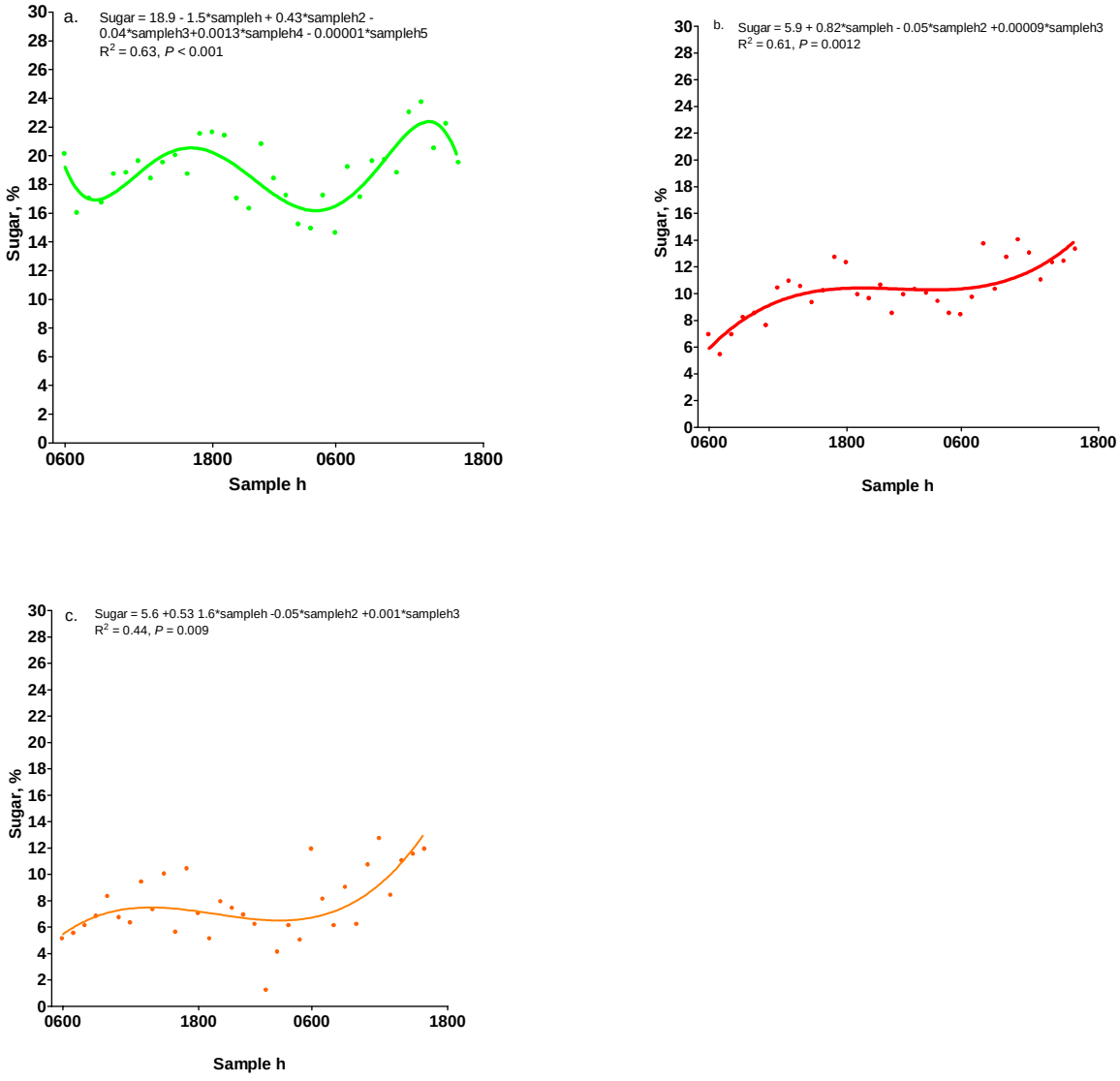

Figure 3.4. Polynomial regression of pasture forage sugar (% DM) (LAB1) in (a.) April (fifth order, quintic), (b.) May (fifth order, quintic), and (c.) August (third order, cubic).

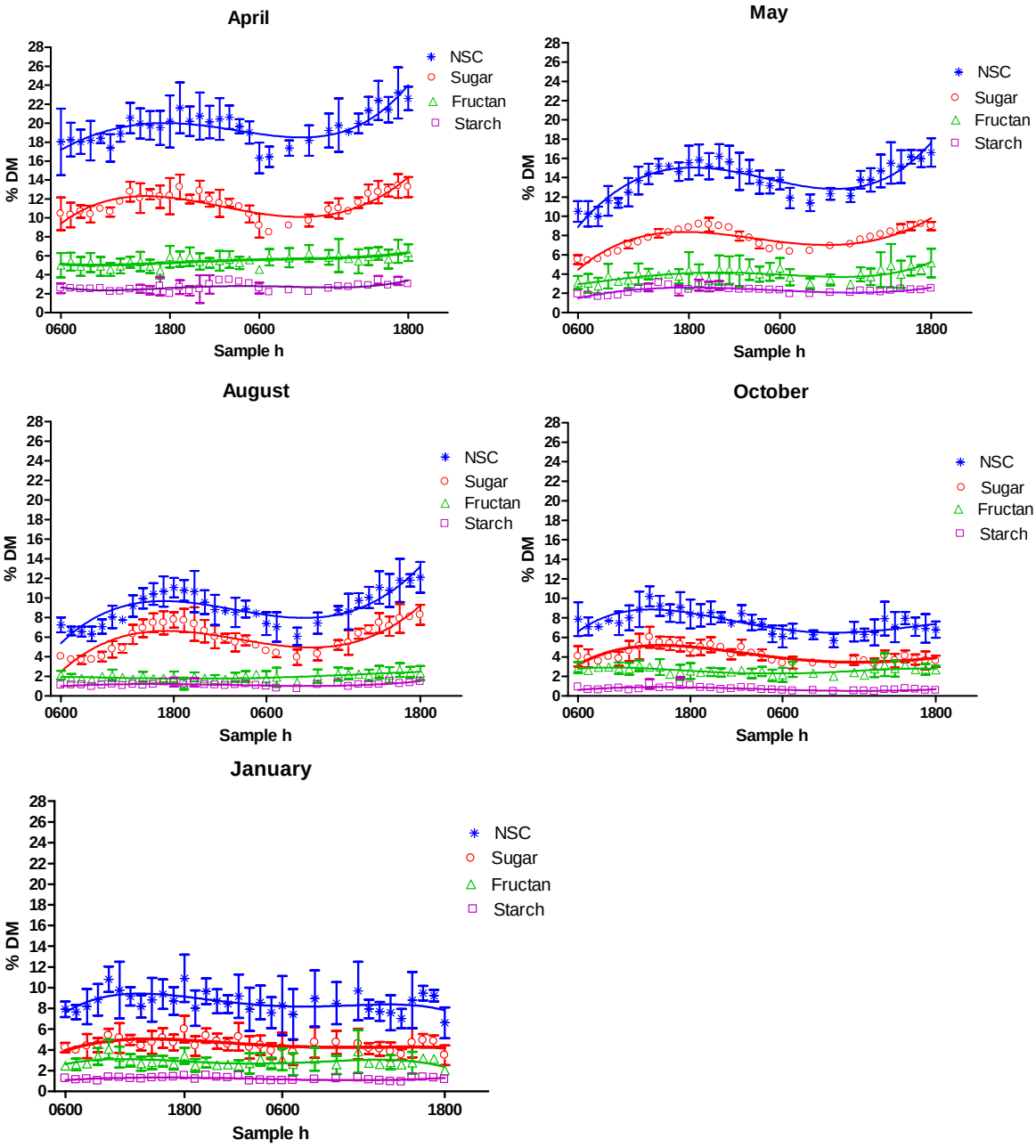

Figure 3.5. Pasture forage nonstructural carbohydrates (NSC, % DM), sugar (% DM), fructan (% DM), and starch (% DM) (LAB2) during 36 h studies in April, May, August, October and January.

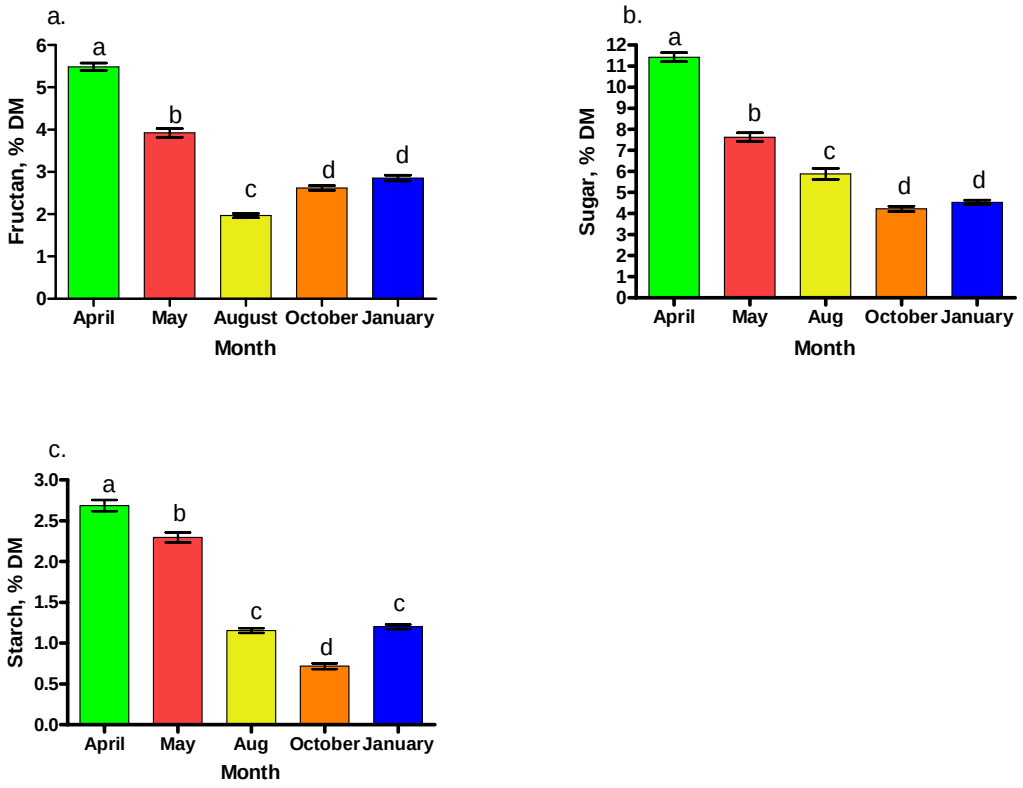

Figure 3.6. Nonstructural carbohydrate (NSC) profiles (a. fructan, b. sugar, and c. starch, % DM) (LAB2) of the pasture over the five 36 h studies. Different letters indicate significant differences between months ($P < 0.05$).

CHAPTER 4
MANUSCRIPT 2

Circadian and seasonal fluctuations of glucose and insulin concentrations in grazing horses

ABSTRACT: The nonstructural carbohydrate (NSC) content of pasture forages undergoes circadian and seasonal fluctuation which may influence carbohydrate metabolism and risk for laminitis in grazing horses. The objective of this study was to examine the circadian and seasonal fluctuation of glucose and insulin concentrations in horses grazing pasture or fed hay, and identify possible relationships with forage NSC content. Plasma glucose and insulin concentrations were measured hourly in Thoroughbred mares for 36 h in April, May, August, and October 2005, and January 2006. Fourteen mares were randomly assigned to grazing (housed on a 5-ha predominantly tall fescue pasture; n = 10) or control (stabled within the pasture and fed timothy/alfalfa hay; n = 4) groups. The mares were 11 ± 5 yr old, weighed 596.0 ± 14.5 kg, and body condition scores ranged from 4.5 to 7.5 (on a scale of 1 to 9). Hourly pasture samples were submitted to a commercial laboratory (Dairy One, Ithaca, NY) for measurement of starch and sugar, where "sugar" was water soluble carbohydrates and included fructans. The NSC was the sum of starch and sugar. The effects of diet treatment and sampling month on mean glucose and insulin concentrations (averaged over each 36 h period) were evaluated by two-way repeated measures ANOVA with Dunn's post test. For grazing horses, Pearson correlations were used to describe the association between glucose and insulin with forage NSC, sugar and starch. There was a significant NSC x mo interaction effect ($P < 0.0001$). Plasma insulin was highest in grazing horses in April ($P < 0.001$) followed by May ($P < 0.001$). Plasma insulin was

higher in grazing compared to control horses at all sample points in April, and a circadian pattern was evident ($P = 0.012$). Plasma insulin was higher in grazing horses than control horses in April ($P < 0.001$) and May ($P < 0.001$). In grazing horses, plasma glucose was higher in April than all months except for May, and plasma glucose was higher in grazing horses compared to control in April. In grazing horses, plasma insulin was significantly correlated with NSC and sugar in April (r = 0.69 and r = 0.67, respectively); May (r = 0.46 and r = 0.47, respectively); and January (r = 0.44 and r = 0.46, respectively). Higher plasma glucose and insulin concentrations in April and May, and the pronounced circadian pattern in April, corresponded to the patterns in pasture forage NSC content and may reflect increased intake and digestion of hydrolysable carbohydrates. These alterations in glucose and insulin dynamics during spring may increase risk of laminitis via exacerbation of insulin resistance.

Keywords: Horses, insulin, glucose, pasture, carbohydrates

Introduction

Laminitis is an inflammatory disease which manifests itself in the hoof (Bailey et al., 2004). Pasture associated laminitis accounts for nearly half of the reported cases of clinical laminitis in the U.S. (USDA, 2000). Intervention of laminitis needs to aim at countermeasures for avoiding laminitis since clinical treatment after its onset is unlikely to prevent disability. Avoiding high intakes of nonstructural carbohydrates (NSC), including sugars, starches and fructans, is a countermeasure that can be taken to avoid laminitis.

There is evidence of association and causation between laminitis and rapid and long term elevated intakes of NSC. Nonstructural carbohydrates have been implicated in acute digestive disturbances associated with their rapid fermentation, and chronic metabolic disorders associated with high glycemic and insulinemic responses (Kronfeld & Harris, 2003). Laminitis may involve both digestive and metabolic disorders (Harris et al., 2006; Hoffman et al., 2003; Pollitt, 1999). Research has shown that intakes of fructan (Pollit et al., 2003), and starch in meals of cereal grains (Potter et al., 1992), can cause laminitis. Fructans and starches are associated with acute digestive disorders because their rapid fermented in the hindgut, disrupts the microflora with a proliferation in lactic acid producing bacteria and decreased hindgut pH. Alteration of the hindgut microflora is thought to result in the release of trigger factors for laminitis (including endotoxins, exotoxins, and vasoactive amines) (Bailey et al., 2004). However, the links between these trigger factors from the hindgut and the pathophysiology in the hoof during laminitis have yet to be identified. In addition, these studies employ unusually high bolus doses of their respective carbohydrate, greater than those grazing on pasture would ever encounter, and often diarrhea and endotoxemia result, which are not characteristic of naturally occurring laminitis. Intakes of sugars from forage may also be associated with laminitis. High intakes of sugar are associated with metabolic disorders such as insulin resistance, and they are associated with digestive disorders since they are highly fermentable within the hindgut if intake exceeds the digestive capacity of the small intestine. Several studies have identified insulin resistance as a predisposing condition for laminitis (Coffman and Colles, 1983; Jeffcott et al., 1986; Treiber et al., 2006a; Treiber et al., 2006b). Insulin resistance causes a disruption in insulin signaling, which

can lead to scenarios associated with laminar separation, such as; altered glucose transport into cells, vasoconstriction and endothelial damage, and an elevated inflammatory response (DeFronzo and Ferrannini, 1991; Fonseca et al., 2004). Therefore recommendations have been made to avoid fructan, sugar and starch as risk factors, to reduce the incidence of laminitis. This is a difficult recommendation to act on due to the omnipresence of NSC in forages, and a lack of data on actual ranges to avoid in real world feeding scenarios.

Avoiding excessive starch from cereal grain meals involves a relatively straightforward reduction in the amount fed (Potter et al., 1992). In contrast, grazing management practices to avoid excessive intakes of NSC requires further research to better understand forage intake, and variables influencing NSC concentration. Factors that influence the NSC profiles in forages are of interest to equine researchers because of the need to predict when grazing animals are at a heightened risk. Studies have shown that not only do variables inherent to the plant affect NSC status, but that environmental conditions affect the accumulation of NSC(Chatterton et al., 2006; Holt, 1969).

The objective of this study was to examine the circadian and seasonal fluctuation of glucose and insulin concentrations in grazing horses and those fed hay, and identify possible relationships with forage NSC content. In identifying how carbohydrate profiles in forages affect the metabolic profiles of grazing horses improved management recommendations for horses predisposed to laminitis can be made.

Materials and Methods

Five 36 h studies investigated the influence of pasture carbohydrate profiles on metabolic profiles in horses. A group of 10 grazing mares and four stalled horses were housed on a 5-ha pasture at the Middleburg Agricultural Research and Extension Center in northern Virginia. The pasture consisted of 75% Max Q tall fescue, 20% Kentucky bluegrass, and 5% white clover. Corresponding blood samples from horses and pasture samples were collected in April, May, August, September, October 2005, and January 2006. Sampling procedures for horses and pastures were the same over the five studies.

Horses. Ten mares were housed on a 5-ha pasture and four horses were housed in temporary 4 X 4 m stalls in a run-in shed within the same pasture, and offered timothy/alfalfa hay only. All horses had ad libitum access to white salt and fresh water. The horses were aged 11 ± 5 yr old (range 6 to 16 yr). Mean weight of the horses over the five months was 596.0 ± 14.5 kg, and mean body condition score was 6.2 ± 0.2 (range 4.5 to 7.5 on a scale of 1 to 9) (Henneke et al., 1983). The horses were acclimated to the pasture for 7 d before sampling began and control horses were placed in the stalls 36 h before each study to acclimate. Hourly blood samples were collected from all 14 horses during the five 36 h trials.

Jugular catheters were inserted at 0500 and collection of blood samples began at 0930. Hourly samples were collected until 0930 the second day, and then continued at two h intervals until 2130 the second day. Blood was collected into two 7 ml heparinized vaccutainer tubes (Fisher Scientific, St. Louis, MO), placed on ice, and taken to the lab where plasma was separated by centrifugation at 3000 x g for 10 min. Plasma was stored at -20˚C until analysis. Plasma was analyzed for glucose (mg/dL) and insulin (μIU/mL).

Glucose and was measured by colorimetric assay (UV visible chemistry). Insulin was measured by a validated chemiluminescent immunoassay (Appendix I).

Pasture. The 10- ha pasture was sectioned off by electric tape fencing to maintain a grazing area of approximately 5-ha which was used for the studies. The 5-ha grazing area was visually divided into four equally sized quadrants marked by orange rubber cones and plastic t-posts. Forage samples were collected hourly from 0600 to 2200 the first day, then overnight at 2400, 0200, 0400, and hourly sample collection resumed gain on the second day from 0600 to 1800. The purpose of staggering the sampling overnight was to eliminate labor, while still allowing for sample collection. Approximately 400 g (wet weight) of clipped forage was collected into cloth bags. Samples were collected by walking in a "W" pattern in each quadrant and clipped (no more than 2.5 cm from the base) every 5 meters. Samples were immediately taken to the laboratory where an approximately 100 g sub sample was taken from the each of the four hourly samples and composited into an oven dried paper bag. The approximately 400 g sample was then weighed and dried at 70º C in a drying oven to determine DM. The remaining four 300 g samples in cloth bags were individually preserved in liquid nitrogen and stored at –80º C until analysis. The oven dried samples were submitted to a regional forage laboratory (Dairy One, Ithaca, NY) (LAB1) to determine starch and sugar. Sugar was water soluble carbohydrates (WSC) extracted prior to analysis for starch, and included fructans. The NSC was the sum of starch and sugar. For the second (LAB2), four 300 g forage samples (one from each quadrant) were frozen in liquid nitrogen, freeze dried, and analyzed for specific NSC fractions using hydrolytic enzymes, with the

addition of HCL for the determination of fructans including graminans, the type of fructans in cool season grasses (Chapter 3, manuscript 1).

Hay. Core samples of the timothy/alfalfa hay offered to the stalled horses were collected, composited, and dried at 70º C to determine DM. Sugar, starch and NSC were determined by the same analyses as the pasture samples (LAB1 only).

Statistical analysis. All data are summarized as means ± SE. A repeated measures ANOVA was used to compare the grazing and stall kept horses. Pearson correlations were used to describe the linear relationships between horse variables (glucose and insulin) and pasture variables (NSC, sugar and starch) for grazing horses. For glucose and insulin in horses (to account for sample hour and repeated measurements within individual horses), a repeated measures mixed effects ANCOVA was used to test for effects of the categorical variable month and the continuous variables NSC and starch, as well as the interactions of month with each of the continuous variables. Sugar was left out of the model because it was too similar to NSC to be included. Covariation among repeated measurements was modeled using a spatial power law covariance structure. The MIXED procedure of the SAS System (ver. 9.13 SAS Institute Inc., Cary, NC) was used to perform the calculations. Model adequacy was assessed using standardized residual plots. Predicted values were plotted across predictor variables (month, NSC, and starch) to visualize the nature of the relationships.

Results

Forage carbohydrate profile. Mean forage carbohydrate profiles are shown in Table 1. During the 36 h trial in April, pasture NSC and sugar content were at least

double that of the other four monthly 36 h trials. In April, NSC ranged from 15.8 to 25.3% DM, May ranged from 6.5 to 16.0 % DM, August ranged from 2.3 to 14.5 % DM, October ranged from 3.9 to 10.1% DM, and January Ranged from 4.8 to 9.6% DM. Pasture starch content was less than 2% DM for all months, and differences between months were negligible. Further enzymatic analysis performed by the USDA-ARS laboratory revealed that approximately two-thirds of the sugar fraction was comprised of simple sugars (glucose, sucrose and fructose) and fructan accounted for the remaining one-third. Hay NSC was 10 % DM, where sugar comprised 8% and starch 2% (lab1). The amount of simple sugars and fructan that comprised the sugar fraction in the hay is unknown.

Grazing versus hay. Mean insulin and glucose concentrations for grazing horses and horses offered hay are shown in Table 2. Circadian patterns in insulin for grazing and hay fed horses during each 36 h trial are shown in Figure 1a-e. In April, insulin was higher in grazing horses than horses fed hay at all sampling hours. Differences between dietary groups were less apparent during the trials held in May, August, October and January. There were no differences in glucose s between grazing and hay fed horses during any of the five 36 h trials.

Effects of pasture. The correlation coefficients used to describe linear relationships between insulin and glucose in grazing horses and pasture variables are shown in Table 3. There was a linear relationship between both pasture NSC and sugar with insulin in grazing horses in April ($r = 0.69$ and $r = 0.67$, respectively), May ($r = 0.46$ and $r = 0.47$), and January ($r = 0.44$ and $r = 0.46$). There were no relationships between pasture carbohydrates and glucose, however in April, when both glucose and pasture

NSC were at their highest, there was a tendency for a linear relationship (r = 0.40, P = 0.05).

When the effects of NSC on insulin in grazing horses were evaluated within the context of the full model, there was a significant NSC x mo interaction effect (P < 0.0001). In April NSC content was significantly higher (P < 0.0001) than in other months, and insulin concentrations were also very high (Table 1 and Table 2). However, within the month of April there was only a small and insignificant (P = 0.15) linear relationship between insulin and NSC (Figure 2a). The situation was similar in May, only to a lesser degree. There were significantly higher insulin concentrations and NSC content in May compared to August, October, and January, but, the linear relationship between NSC and insulin was not significant in May (P = 0.078). In the other months insulin concentrations and NSC content were both lower, and although there were significant linear relationships between NSC and insulin within each of those months, they were inconsistent across months. Insulin decreased with increasing NSC content in August (P = 0.0019), but insulin increased with increasing NSC in October and January (P = 0.0277 and P = 0.0025) (Figure 2a). The linear relationship between starch and insulin was also inconsistent across months. Within the months of April, May, August, and January, linear relationships between starch and insulin were small and insignificant. In October, insulin decreased with increasing starch content (P = 0.0004) (Figure 2b).

When the effects of NSC on glucose in grazing horses were evaluated within the context of the full model, there was a significant NSC x mo interaction effect (P < 0.0001). In April NSC content was significantly higher (P < 0.0001) than in other months, and glucose concentrations were higher, although not significantly so (Table 1

and Table 2). Within the month of April there was only a small and insignificant (P = 0.38) linear relationship between glucose and NSC (Figure 3a). Although glucose concentrations in May were not significantly different than the other months, within the month of May, glucose s increased as NSC increased (P = 0.02). In the other months where glucose did not differ, and NSC content was lower, there were no significant linear relationships between NSC and glucose. There were also no linear relationships between starch and glucose within any of the months during the 36 h trials (Figure 3b).

Discussion

The results illustrated seasonal and circadian variation in pasture carbohydrate profiles, and these patterns appeared to influence circulating blood glucose and insulin in the grazing horses. Intakes of NSC can cause acute digestive disturbances (associated with their rapid fermentation), and metabolic disorders (associated with elevated glycemic and insulinemic responses), thus increasing the risk of laminitis (Kronfeld, 2003; Treiber et al., 2006a).

Overall, the pasture carbohydrate profiles in this study were similar to those previously reported for Middleburg, VA (Hoffman et al., 2001), where NSC content ranged from about 4 to 23% DM for 107 pasture samples, and in this study they ranged from 2.3 to 25.3% DM over all of the months. Several studies have also shown similar patterns in circadian and seasonal variation in NSC (Burns and Chamblee2000; Chatterton, 1989; Longland, 1999). It is difficult to directly compare patterns in seasonal and circadian variation of NSC because the environmental conditions (and hence geographic location) dictate rate of synthesis and utilization of carbohydrates. Hoffman et al. (2003) analyzed pasture samples that were collected from Northern Virginia, and

reported peaks in NSC at April and November. This study found NSC content to be highest in April, but in late October, when a secondary peak was expected, content was quite low. The low content in the October 36 h trial was a reflection of the environmental conditions. The temperatures were warmer than usual (ranging from 8 to 11º C), and overcast and rainy conditions persisted throughout most of the trial. The low content of NSC in August and January trial was consistent with previous studies reporting nadirs in the summer and winter months (Burns and Chamblee, 2000; Hoffman et al., 2001). The circadian variation was also dictated by the environmental conditions, where April showed the greatest fluctuation in pasture NSC which was lowest between 0400 and 0500 (17.6 ± 0.3%), and highest between 1600 and 1700 (22.2 ± 0.3%). These results are consistent with reports that NSC tends to increase throughout the day from 0600 to 1800 (Lechtenberg, 1971).

These seasonal and circadian fluctuations influenced the metabolic profiles of the grazing horses, which was characterized by their insulinemic responses, and to some degree their glycemic response. The most apparent insulinemic response in grazing horses occurred during the April 36 h trial. Individual insulin concentrations in grazing horses in April ranged from 10.99 to 241.02 μIU/mL, and the overall mean was 54.5 ± 9.9 μIU/mL. In some instances, these insulin s exceed those reported for horses fed grain concentrates (Fowden et al.1984; Hoffman et al., 2003). Fowden et al. (1984) reported mean insulin concentrations of 24 ± 1.5 before feeding, and 62.4 ± 10.1 μIU/mL four to six hours after feeding a grain meal. The highest insulin concentrations in Fowden's study were from a group of pregnant mares (<270 d) where the mean was 61.6 ± 6.6 before feeding, and 130.5 ± 15.0 μIU/mL after the grain meal was fed. Lower

concentrations were reported by Hoffman et al. (2003) for pregnant mares adapted to a feed high in sugar and starch (40.3 ± 6.4 mIU/L) or high in fat and fiber (25.9 ± 6.34 mIU/L).

The results from this study are more comparable to a study where plasma insulin and glucose values were evaluated in a group of 160 ponies to quantitatively determine a pre-laminitic metabolic profile (Treiber et al., 2006b). The study revealed that insulin concentrations in ponies with clinical cases of laminitis were 92 ± 2 μIU/mL. Insulin was lower in the group that had previously had episodes of laminitis (32 ± 6 μIU/mL), and lower yet in ponies that had never exhibited laminitis (15 ± 2 μIU/mL). Similar to this study no differences were found between groups for glucose concentrations. It seems unusual that this study would find insulin concentrations similar to laminitic ponies since it evaluated healthy Thoroughbreds, a breed not typically prone to laminitis or obesity, as is the case with ponies. This fact emphasized the impact of pasture carbohydrate content on the metabolic status of grazing horses.

Conclusions

This study was unique in that it was the first to reveal nutritional correlations between pasture carbohydrate composition and metabolic profiles in grazing horses. The circadian and seasonal patterns observed in forage carbohydrates, and insulin in horses, is evidence for improving the management of equines at risk for metabolic and digestive disorders. For horses that are a prone to insulin resistance and associated laminitis, grazing pastures should be avoided at certain times of the day and at certain times of the year, as this study showed a direct relationship between pasture NSC and insulin in

grazing horses. This study demonstrated that NSC content of the pasture, and subsequent insulin concentrations in the horses, were highest in April, particularly in the afternoon hours. It is important to emphasize that this study consisted of a series of 36 h trials that provided only a short window of examination within each month. It is essential to consider all of the variables that affect NSC content in forages and metabolic profiles in horses when managing at risk horses. Overall, avoiding NSC concentrations that pose a risk must be balanced with the clear benefits of raising and maintaining horses on pasture. Future studies should aim at defining cut-off points for safe amounts of NSC and its constituents for horses prone to laminitis.

Table 4.1. Pasture and nonstructural carbohydrate (NSC) profiles during the five 36 studies from LAB1 (n = 33) and LAB2 (n = 132). Data are summarized as mean ± SE.

		Month				
Profile	Lab	April	May	August	October	January
NSC, % DM	LAB1	20.3 ± 0.4[a]	11.7 ± 0.4[b,*]	9.2 ± 0.5[c]	6.9 ± 0.2[d]	7.1 ± 0.2[d,*]
	LAB2	19.6 ± 0.3[a]	13.9 ± 0.3[b]	9.0 ± 0.3[c]	7.6 ± 0.2[d]	8.6 ± 0.2[c]
Sugar[1], % DM	LAB1	18.9 ± 0.4[a,*]	10.2 ± 0.4[b,*]	7.6 ± 0.5[c,*]	5.7 ± 0.2[d,*]	6.1 ± 0.2[d,*]
	LAB2	11.4 ± 0.2[a]	7.6 ± 0.2[b]	5.9 ± 0.3[c]	4.2 ± 0.1[d]	4.5 ± 0.1[d]
Starch, % DM	LAB1	1.4 ± 0.04[a]	1.5 ± 0.06[a]	1.5 ± 0.06[a]	1.1 ± 0.05[b]	1.0 ± 0.03[b]
	LAB2	2.7 ± 0.1[a]	2.3 ± 0.1[b]	1.2 ± 0.03[c]	0.7 ± 0.04[d]	1.2 ± 0.03[c]
Fructan, % DM	LAB1	-	-	-	-	-
	LAB2	5.5 ± 0.1[a]	3.9 ± 0.1[b]	1.9 ± 0.05[d]	2.6 ± 0.06[c]	2.8 ± 0.07[c]

[1]LAB1 Sugar = water soluble carbohydrate (WSC) = sugar + fructan

[a,b,c,d] Means with different letter superscripts differ within a row ($P < 0.05$).

Table 4.2. Mean plasma insulin and glucose concentrations in grazing and control horses across monthly 36 h studies (means ± SD).

	Insulin (μIU/mL)		Glucose (mg/dL)	
Month	Grazing	Control	Grazing	Control
April	54.6 ± 9.9[a],*	11.7 ± 3.4[a]	116.5 ± 3.4[a],*	105.7 ± 3.2[a]
May	20.8 ± 3.4[b,*]	14.7 ± 3.6[a]	110.9 ± 2.1[a,b]	109.2 ± 4.2[a]
August	10.9 ± 1.7[c]	8.6 ± 2.6[a]	98.1 ± 3.8[b,c]	94.5 ± 3.1[a]
October	13.2 ± 1.4[c]	9.9 ± 2.6[a]	93.2 ± 2.3[c]	94.5 ± 2.7[a]
January	11.5 ± 1.4[c]	9.3 ± 2.9[a]	96.4 ± 1.6[b,c]	100.5 ± 4.9[a]

[*] Means differ between grazing and control for respective variable ($P < 0.05$).

[a,b,c,d] Means with different letter subscripts differ within the same column ($P < 0.05$).

Table 4.3. Pearson correlation coefficients[a] for horse variables (insulin and glucose) and pasture carbohydrate variables (nonstructural carbohydrate [NSC][b], sugar[c] and starch) for grazing horses. Correlations were significant if $P < 0.05$.

Horse variable		Carbohydrate variable		
	Month	NSC	Sugar	Starch
Insulin	April	r = 0.69	r = 0.67	r = 0.31
		$P < 0.001$	$P < 0.001$	$P = 0.14$
	May	r = 0.46	r = 0.0.47	r = 0.21
		$P < 0.05$	$P < 0.05$	$P = 0.31$
	August	r = 0.22	r = 0.24	r = 0.05
		$P = 0.28$	$P = 0.25$	$P = 0.81$
	October	r = 0.27	r = 0.32	r = 0.05
		$P = 0.19$	$P = 0.12$	$P = 0.82$
	January	r = 0.44	r = 0.46	r = 0.03
		$P < 0.05$	$P < 0.05$	$P = 0.89$
Glucose	April	r = 0.40	r = 0.39	r = 0.10
		$P = 0.05$	$P = 0.05$	$P = 0.63$
	May	r = 0.25	r = 0.27	r = 0.02
		$P = 0.23$	$P = 0.19$	$P = 0.93$
	August	r = 0.10	r = 0.09	r = 0.10
		$P = 0.63$	$P = 0.65$	$P = 0.64$
	October	r = 0.30	r = 0.32	r = 0.06
		$P = 0.15$	$P = 0.12$	$P = 0.79$
	January	r = 0.09	r = 0.05	r = 0.33
		$P = 0.64$	$P = 0.82$	$P = 0.12$

[a] probability P > |r| under H0: Rho = 0), n = 25

[b]Nonstructural carbohydrate (NSC) = sugar + starch

[c]Sugar = water soluble carbohydrate

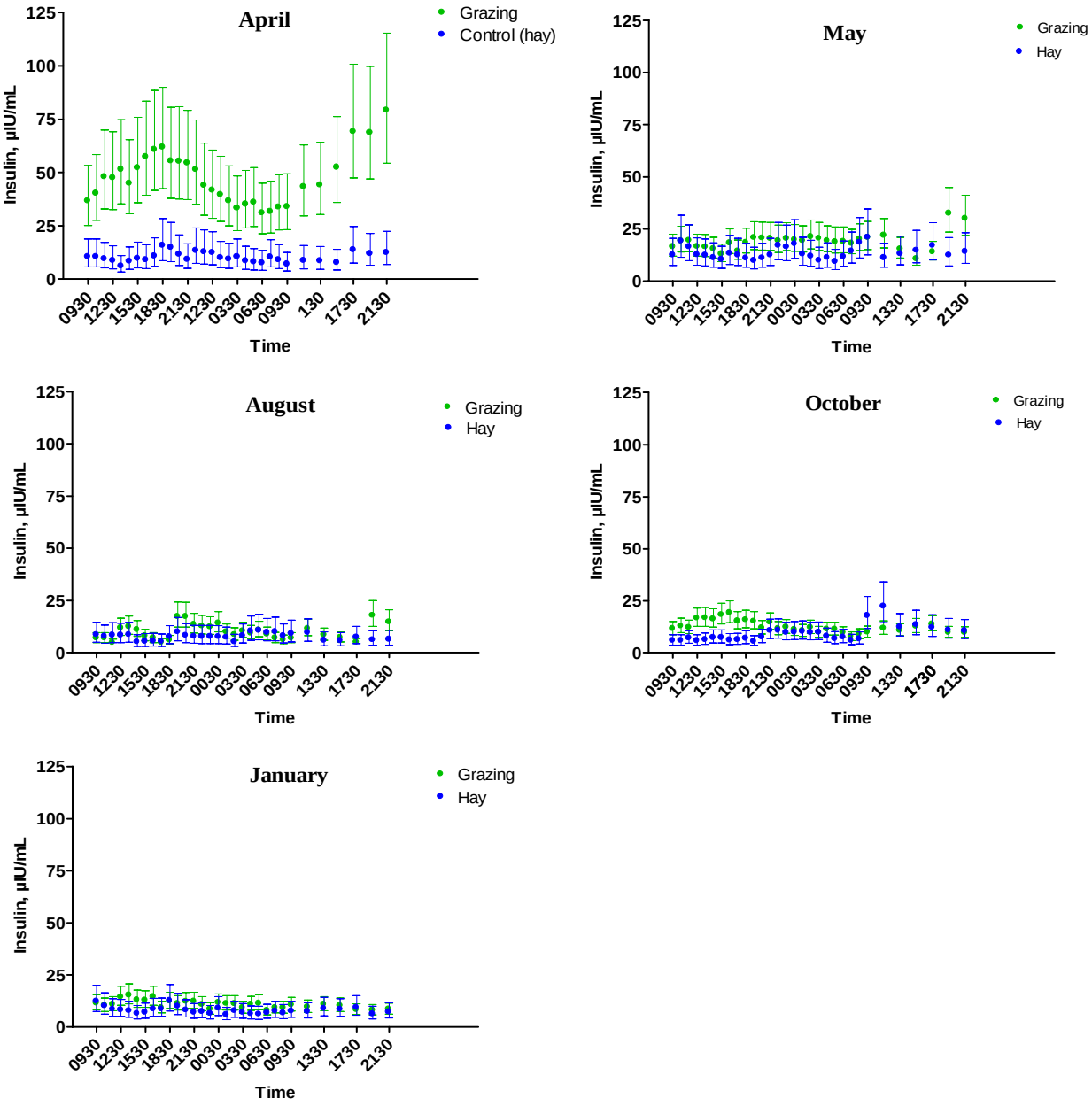

Figure 4.1. Plots of geometric means of insulin in grazing horses (green) and control horses fed hay (blue) during the five 36 h studies.

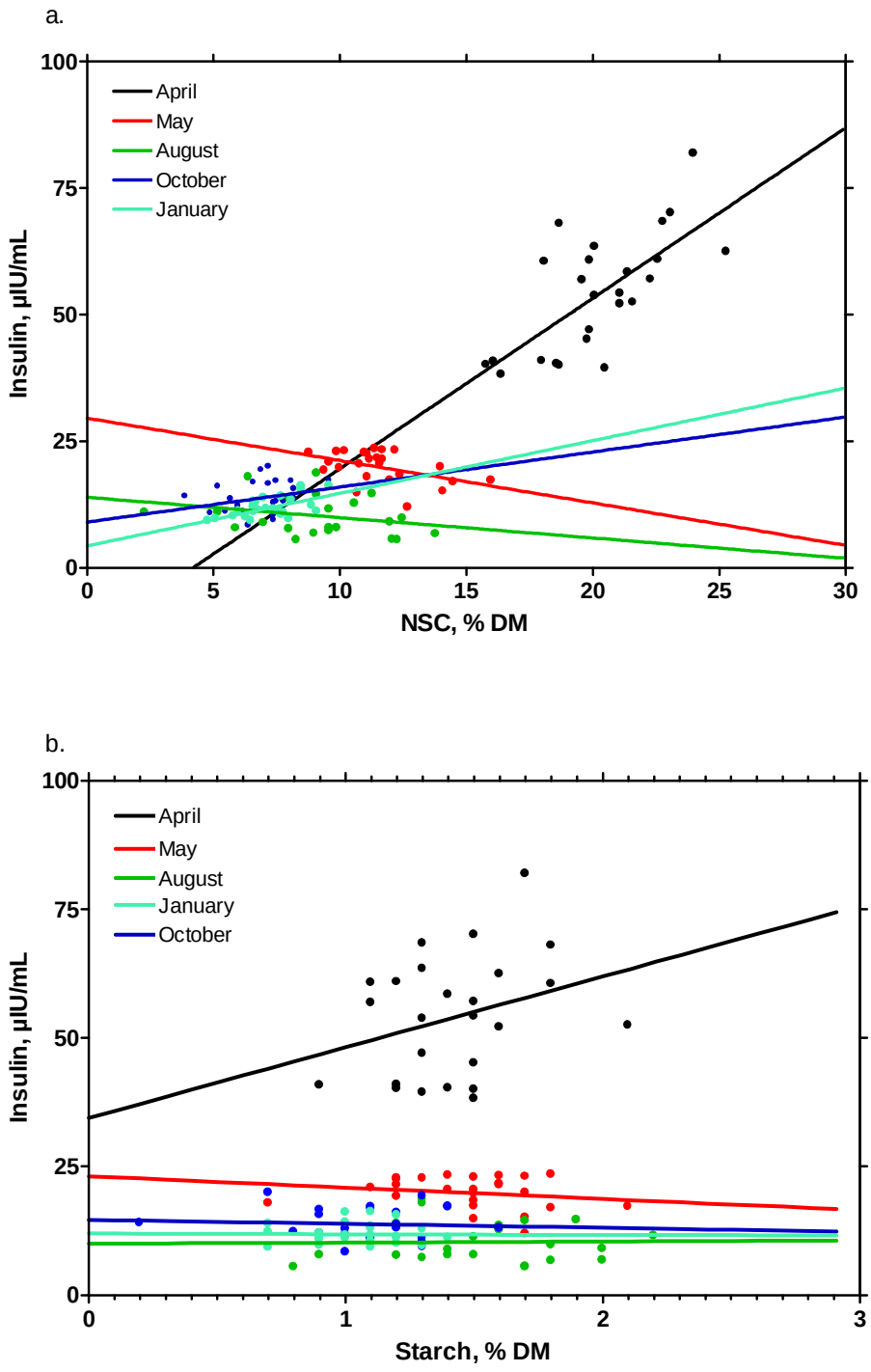

Figure 4.2. Mean plasma insulin (µIU/mL) means in grazing horses and (a.) NSC (% DM) and (b.) Starch (% DM) (LAB1) during the five 36 h studies.

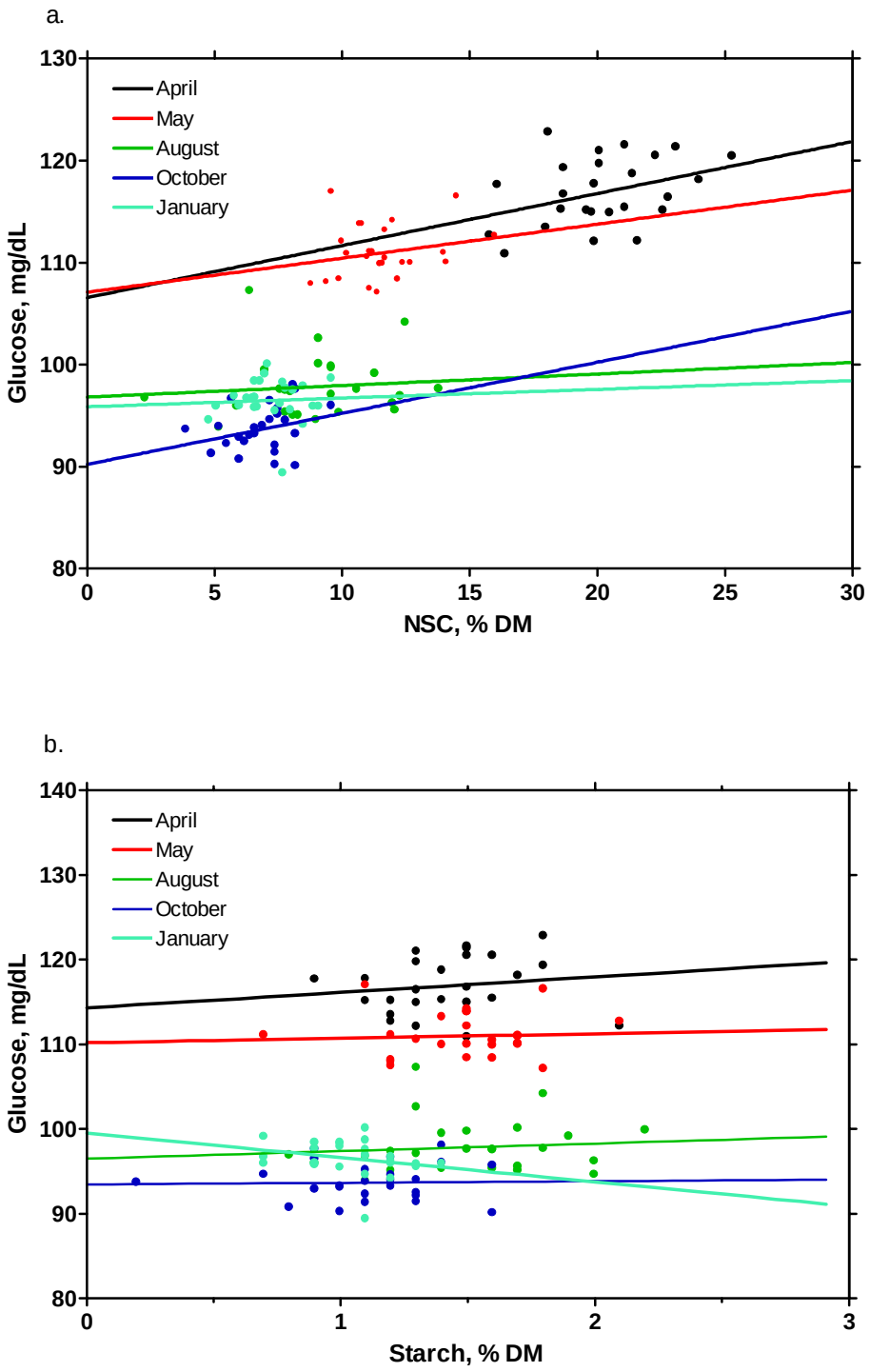

Figure 4.3. Plots of mean glucose (mg/dL) in grazing horses and (a.) NSC (%DM), (b.) Starch (%DM) (LAB1) during the five 36 h studies.

CHAPTER FIVE
MANUSCRIPT 3

Metabolic and digestive variables in horses grazing spring pasture

ABSTRACT: Laminitis often occurs in the spring and may be associated with dietary nonstructural carbohydrates (NSC). Sugars, starches and fructans comprise NSC and can fluctuate rapidly under typical spring environmental conditions. In April 2005, a 36 h study took place in northern Virginia where 14 mares were randomly assigned to grazing (housed on a 5-ha pasture predominantly tall fescue; n = 10) or control (stabled within the pasture and fed timothy/alfalfa hay; n = 4) groups. The mares were 11 ± 5 yr old, weighed 596.0 ± 14.5 kg, and body condition scores ranged from 4.5 to 7.5 (on a scale of 1 to 9). Plasma glucose, insulin and L-lactate concentrations, and fecal pH, L-lactate, D-lactate, and volatile fatty acids (VFA) were measured hourly. Hay samples and hourly pasture samples were analyzed for starch and sugar, where "sugar" is water soluble carbohydrates (and includes fructans), and NSC was the sum of starch and sugar. The NSC content of the hay was 8.9 ± 0.05 % DM, and pasture NSC content ranged from 15.8 to 25.3 % DM. Grazing horses had higher overall insulin and glucose than control horses ($P < 0.05$). A circadian pattern in insulin in grazing horses correlated to forage NSC content ($r = 0.601$, $P = 0.008$). Individual mean insulin response was proportional to the increase in insulin per increase in unit of NSC ($r^2 = 0.033$, $P < 0.001$). Sinusoidal circadian patterns in NSC ($r^2 = 0.51$, $P < 0.001$) and insulin in grazing horses ($r^2 = 0.12$, $P < 0.001$) had similar frequency ($P = 0.36$), with changes in insulin delayed by 30 min. Plasma L-lactate was higher in grazing horses (0.64 mmol/L) than control horses (0.40 mmol/L) ($P < 0.001$). Fecal pH was lower in grazing horses (pH 6.9) than control horses

(pH 7.2) (P = 0.008). Fecal VFAs, including acetic acid, butyric acid, and D- and L-lactate were higher in grazing horses compared to control horses ($P < 0.05$). The alterations in metabolic and digestive variables observed in grazing horses may reflect increased intake and digestion of hydrolysable and rapidly fermentable carbohydrates that were present in spring forages

Keywords: Horses, grazing, forage, spring

Introduction

Overall, annual incidence of laminitis in the U.S. is reported to be 2%, but this rises to about 5% in the spring and summer (Kane, 2000), and nearly half of all reported cases of laminitis in the U.S. occurred in animals at pasture (USDA, 2000). Seasonal variation in forage NSC content has been well documented with higher concentrations occurring in the spring months (Longland, 1999). Daily patterns in NSC are also seen under conditions where cool nights followed by bright sunny days favor photosynthesis as opposed to growth (Chatterton et al., 2006; Holt, 1969).

Nonstructural carbohydrates (NSC) in pasture may affect insulin dynamics in grazing horses, providing a possible link between NSC and laminitis (Treiber et al., 2006b; Watts, 2004). The objective of this study was to evaluate forage NSC content in a spring pasture and its effect on metabolism and digestion in grazing horses.

Materials and Methods

A 42 h field study began at 0600 April 14, 2005 and ended at 2130 April 15, 2005. The study consisted of hourly forage sampling from a 5-ha pasture corresponding with blood and fecal sample collection in horses grazing the pasture and horses kept in stalls and fed hay.

Horses. Fourteen horses were placed on a 5-ha pasture on April 7, 2005. Four of the horses were taken off the pasture at 0700 April 13 and placed in temporary stalls (in a run in shed within the pasture) and fed timothy/alfalfa hay and were used as a control group. The remaining 10 mares were continuously housed on the pasture throughout the study. All horses had ad libitum access to water and white salt.

Jugular catheters were inserted at 0500 and collection of blood samples began at 0930 April 14, 2005. Hourly samples were collected until 0930 April 15, and then continued at two h intervals until 2130 April 15. Blood was collected into two 7 ml heparinized vaccutainer tubes (Fisher Scientific, St. Louis, MO), placed on ice, and taken to the lab where plasma was separated by centrifugation at 3000 g for 10 min. Plasma was stored at -20˚C until analysis. Plasma was analyzed for glucose, insulin and L-lactate. Glucose was measured by colorimetric assay (UV visible chemistry). Insulin was measured by a chemiluminescent immunoassay (Appendix I). Plasma L-lactate was measured using a UV method enzymatic test and spectrophotometry (Boehringer Mannheim/ R-Biopharm Cat. No. 10 139 084 035, Darmstadt, Germany).

Freshly voided feces were obtained (100 g) every four h. Fecal samples were diluted in a 1:10 ratio with deionized water and mixed in a commercial blender. Fecal pH was measured using a digital pH meter (Corning model 340, Corning, NY) which was

calibrated using a chemical standard (SB107-500 Fisher Scientific, Fairlawn, NJ). The mixture was then filtered through cheese cloth to remove large particulate matter and an aliquot was used for quantification of short chain volatile fatty acids (VFAs), D-lactate acid, and L-lactate. For the measurement of VFAs, five mL of fecal fluid was pipetted into a culture tube containing 1 mL of 25% metaphosphoric acid and 5 mL of the internal standard 4-methyl valeric acid 5µM/mL. Contents were mixed and frozen until analyzed using gas chromatography (GC) with a helium carrier (Agilent 6890). Upon thawing, samples were centrifuged for 10 min at 3000 g to remove large particulate matter. A small aliquot was placed in a micro centrifuge tube and centrifuged for 10 min at 3,000 g at 4 ºC. The clear supernatant was placed in a syringe to filter through a 0.45 µm nylon syringe filter. The filtrate was collected into a GC vial, and the vial was crimped. A 1 µl sample was injected by the GC autoinjector. Concentrations were based on peak areas determined by the standards, where known quantities of the individual VFAs were injected. D- and L-lactate were measured using an enzymatic test kit and specrtophotometry (Boehringer Mannheim/ R-Biopharm Cat. No. 11 112 821 035, Darmstadt, Germany).

Forages. The 5-ha field was divided visually into four quadrants by plastic fence posts. Pasture forage samples (approximately 400 g) were collected hourly from 0600 April 14 to 1800 April 15, 2005 from each of the four quadrants and placed in cloth bags made of cotton. A 100 g grab sample was removed from each of the cloth bags and placed in a paper bag where they were hand mixed, weighed, and dried in an oven at 70º C to determine DM. The remaining 300 g forage samples in the cloth bags were preserved in liquid nitrogen and stored at -80º C and were analyzed for specific

carbohydrate fractions using enzymatic techniques, where sugar (glucose, sucrose and fructose), fructan, and starch were measured separately according to Chatterton et al. 2006 with slights modifications (ie. addition of HCL for measurement of fructans) (LAB 2). The oven dried forage samples were analyzed (Dairy One, Ithaca, NY) for starch and sugar, where "sugar" is water soluble carbohydrates (and includes fructans), and NSC was the sum of starch and sugar (Hall et al., 1999) (LAB 1). Starch was determined using a glucoamylase enzyme and measuring dextrose in an automated biochemical analyzer (YSI 2700 SELECT Biochemistry Analyzer, YSI Incorporated, Yellow Springs, Ohio, USA, Application Note Number 319).

Botanical composition was assessed by the double DAFOR Scale Abaye (1997), which was adapted from the method of Brodie (1985). The scale (D = dominant, A = abundant, F = frequent, O = occasional, R = rare) was used to measure the relative abundance of forage species within a given area of pasture, where separate classifications were given for forage and weed species. A rank of abundant was given to species that covered one half to three quarters of the area. A ranking of frequent was assigned to species that covered less than half of the area, but were well scattered throughout the site. Occasional species were those that were found a few times, and rare were those that occurred only one or two times in a given area. The double DAFOR Scale was used to asses the species composition within ten 0.25 m^2 quadrates randomly placed in each of the four quadrants. Forage mass was measured by clipping the forage within the quadrates (n = 40) and contents were placed in pre-weighed bags and oven dried to calculate DM yield.

Hay. Core samples of the timothy/alfalfa hay that was offered to the horses in stalls were collected (n = 2), and composite samples were placed in paper bags and dried at 70º C to determine DM. The samples were submitted to a commercial laboratory (Dairy One, Ithaca, NY) and the same analytical procedures were performed as for the pasture forage samples.

Environment. Environmental conditions were measured and recorded hourly during the 42 h trial. Ambient temperature (ºC), relative humidity (%), and solar radiation (watts/m^{-2}) were measured and recorded hourly by a weather station (Texas Weather Instruments, Dallas, Texas) located in close proximity to the 5-ha pasture.

Statistical analysis. Glucose and insulin in grazing and control horses were compared by repeated measures ANOVA using Bonferroni's post tests (SAS ver 9.1, Gary, NC). Mean plasma D- and L-lactate, fecal pH and fecal VFA's in grazing and control horses were compared using students t-test (GraphPad Prism version 4.00, GraphPad Software, San Diego CA). Correlations between pasture NSC and plasma insulin in grazing horses were determined by linear regression, and circadian patterns in NSC and insulin were established by nonlinear regression fitting sin waves (GraphPad Prism version 4.00, GraphPad Software, San Diego CA)

Results

The environmental conditions were typical for the spring in northern Virginia with an overnight low of 2.8º C and a daytime high of 16.7º C April 14, 2005 and a mean temperature of 9.8º C. The mean relative humidity was 47%, and was lowest in the mid-afternoon (around 32%) and highest in the early morning hours (around 68%). Maximum

solar radiation occurred in the mid-afternoon (1300-1400) at 920 watts/m^2 and the mean radiation throughout the day light hours was 597 watts/m^2. The dominant forage species was tall fescue, Kentucky bluegrass was frequent, and white clover was rare.

According to LAB 1, mean forage NSC content was 20.3 ± 0.4, sugar content was 18.8 ± 0.4, and starch content was 1.4 ± 0.04. There was a clear circadian variation in pasture NSC where it was lowest between 0400 and 0500 (17.6 ± 0.3%), and highest between 1600 and 1700 (22.2 ± 0.3%). Hay NSC was 8.9 ± 0.05% according to LAB1. Further carbohydrate analyses by LAB 2 revealed that the majority of the NSC was comprised of simple sugars (glucose, sucrose, and fructose) (Figure 1).

Insulin concentrations were significantly higher in grazing horses (54.6 ± 9.9 μIU/mL)) than in control horses (11.7 ± 3.4 μIU/mL) ($P = 0.012$). Insulin concentrations varied between horses, however, overall insulin concentrations in grazing horses correlated to NSC s in the pasture ($r^2 = 0.601$, $P = 0.008$). Thus, plasma insulin concentrations in the grazing horses also showed a pattern of circadian variation, while horses fed hay showed little fluctuation. Sinusoidal circadian patterns in NSC ($r^2 = 0.507$, $P < 0.001$) and insulin in grazing horses ($r^2 = 0.121$, $P < 0.001$) had similar frequency ($P = 0.36$), with changes in insulin delayed by 30 min (Figure 2). The percent change in insulin was 2.5 times that of NSC. Individual mean insulin response was proportional to the increase in insulin per increase in unit of NSC ($r^2 = 0.033$, $P < 0.001$) (Figure 3). Plasma glucose concentrations were higher in grazing horses (116.5 ± 3.4 mg/dL) than horses fed hay (105.7 ± 3.2 mg/dL), but a circadian pattern in variation was not evident (Figure 4).

Fecal pH was lower in grazing horses (pH 6.9) than control horses (pH 7.2) (P = 0.008). Plasma L-lactate was higher in grazing horses (0.64 mmol/L) than control horses (0.40 mmol/L) ($P < 0.001$) (Figure 5a). Fecal D- and L-lactate were higher in grazing horses (5.0 ± 0.3 and 3.9 ± 0.3 mmol/L, respectively) than control horses (3.6 ± 0.5 and 2.4 mol/L, respectively) (Figure 5b and 5c). Differences in short chain VFAs between grazing and control horses were varied. Mean fecal acetic acid concentration was higher in grazing horses (42.2 ± 1.4) than in control horses (32.2 ± 3.3) (P = 0.001) (Figure 6a). Fecal butyric acid was also higher in grazing horses (4.0 ± 0.3) compared to control horses (2.9 ± 0.3) (Figure 6c). Although the concentrations of isobutyric and isovaleric acid were relatively low and undetectable in some samples, they were still higher in grazing horses than control horses ($P < 0.05$) (Figure 6d and 6e). There were no differences between grazing and control groups in fecal concentration of propionic and valeric acid (Figure 6b and 6f).

Discussion

Changes in carbohydrate metabolism and digestion in grazing horses during spring may increase risk of laminitis via exacerbation of insulin resistance and rapid fermentation in the hindgut. Previous studies have shown an association between insulin resistance and laminitis (Coffman and Colles, 1983; Treiber et al., 2006a) . Laminitic ponies were reported to be intolerant to glucose and significantly less sensitive to insulin than non-laminitic controls during a series of IV glucose tolerance tests (Coffman and Colles, 1983). More recently, a specific quantitative method for assessing insulin resistance (the minimal model) has demonstrated that ponies genetically predisposed to

laminitis have a reduced ability for insulin to induce hypoglycemia compared to normal ponies (Treiber et al., 2006a). Glucose intolerance was also observed in fat ponies with a history of laminitis after oral glucose loading (1g/kg BW). In ponies with a previous history of laminitis, peak glucose concentrations were higher than normal ponies, and concentrations never returned to baseline. When signs of laminitis emerged in these studies, the insulin response became exaggerated leading to failure of the pancreatic β cells. Results from studies with IR and normal ponies indicate a changing role of IR in laminitis: 1) a compensated predisposing factor in healthy but genetically predisposed ponies, 2) a pathogenic component as transient exaggerated compensation during the onset of laminitis, and finally 3) uncompensated IR later in the course of the disease.

This study utilized Thoroughbred mares that were not known to be predisposed to laminitis; therefore none of the horses developed laminitis during the 42 h study. However, some of the horses had extremely high insulin concentrations (up to 250 mg/dL), indicating possible IR. During the study, there were four horses with average insulin concentrations over 50 μIU/mL, rivalling insulin concentrations in lactating mares fed a concentrate high in sugar and starch (Williams et al., 2001). Interestingly, it appeared the higher the insulin concentrations in the horses, the more sensitive they were to the changes in pasture forage NSC content. The sugar content of the forages was likely to be the cause of the elevated insulemic response in these grazing horses.

The circadian patterns in NSC were attributable to sugar which comprised approximately 93% of the total NSC according to LAB1, and 58% according to LAB2. The difference between the two laboratories in their measurement of sugar is that LAB1 included all water soluble carbohydrates, including fructans, thus overestimating this

fraction. LAB2 estimated fructan comprised 28% of the total NSC and starch 14%. Environmental factors, geographic location and plant species may all affect forage NSC content, so it should be kept in mind that this was one study over a very short period of time, and NSC status here is not indicative of what it may be elsewhere.

In addition to the sugar content of the forages being a possible cause of the insulinemic responses in horses, the forage sugars may also play a role in the digestive responses in the grazing horses. Other studies have identified that that the capacity of the small intestine to digest starch is limited, and that that fructan is poorly digested in the small intestine. In this study, the digestive profiles of the grazing horses compared to the control horses indicated effects on hindgut fermentation in line with what is observed with the onset of laminitis. It has been well documented that rapid fermentation of NSC leads to an increase in lactic acid producing microorganisms in the hindgut and a drop in cecal and colonic pH (Clarke et al., 1990; Crawford, 2005). In a laminitis induction study, fecal pH of horses dosed with 7.5 to 12.5 g/kg BW of oligofructose (fructan) decreased rapidly to below 5.0 eight h after the dose was administered, while control horses remained at just above 7.0 throughout a 48 h study. Plasma L-lactate and D-lactate concentrations were also higher than control horses (van Eps and Pollitt, 2006). In this study, fecal concentrations of D-lactate (which indicates rapid fermentation) was higher in grazing horses compared to control horses, as well as L-lactate, acetic acid, and other short chain VFAs. Plasma D-lactate was difficult to detect, yet plasma L-lactate was higher in grazing horses compared to controls, also indicating effects on hindgut fermentation that may be implicated with laminitis.

Conclusions

The alterations in metabolic and digestive variables observed in grazing horses may reflect increased intake and digestion of hydrolysable and rapidly fermentable carbohydrates that were present in spring forages. Horses at risk of developing laminitis should have limited access to spring pastures and grazing should be avoided at certain times of the day when forage NSC content is high. This study showed that NSC content of pasture forage was highest in the mid to late afternoon on a bright sunny day that followed a cool night and humidity was low. Changes in carbohydrate metabolism and digestion in grazing horses during spring may increase risk of laminitis via exacerbation of insulin resistance and rapid fermentation in the hindgut. While research has implicated fructans and starch with laminitis, no research has specifically examined the association between sugars and laminitis. Since sugar has direct effects on carbohydrate metabolism, and is also a substrate for rapid fermentation, more studies are needed to evaluate sugar metabolism in both forages and in horses.

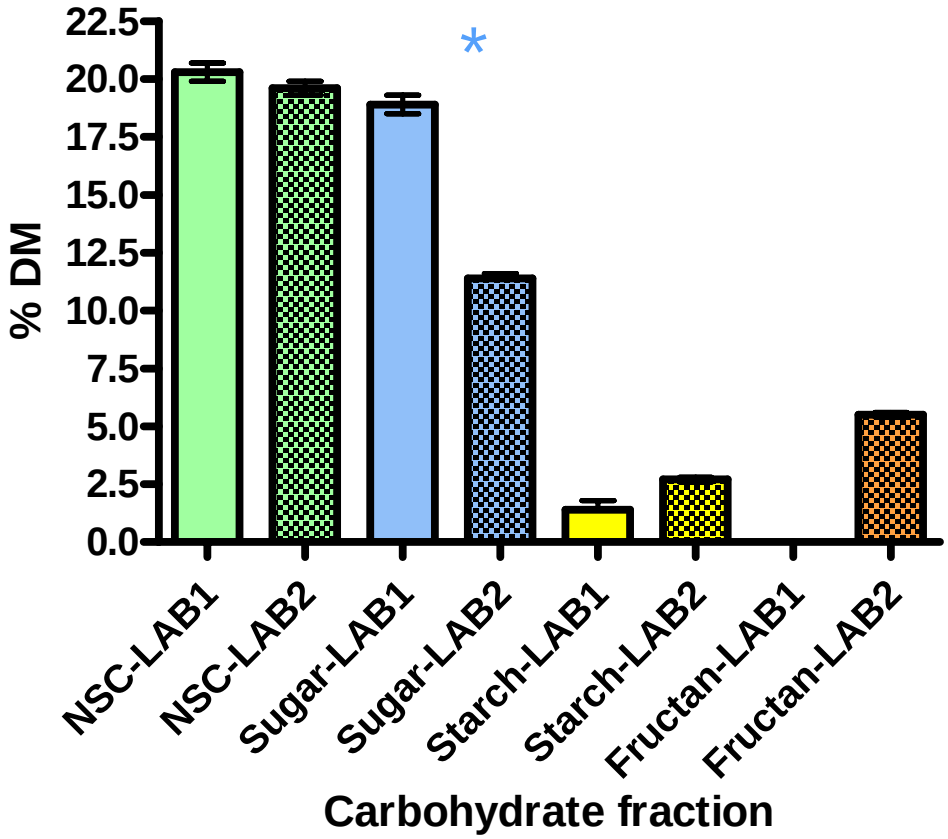

Figure 5.1. Mean NSC, sugar (LAB1 sugar includes fructans) starch, and fructan (% DM) as determined from Dairy One (LAB1) and enzymatic analysis of forage (LAB2) in April. Means were different if $P < 0.05$.

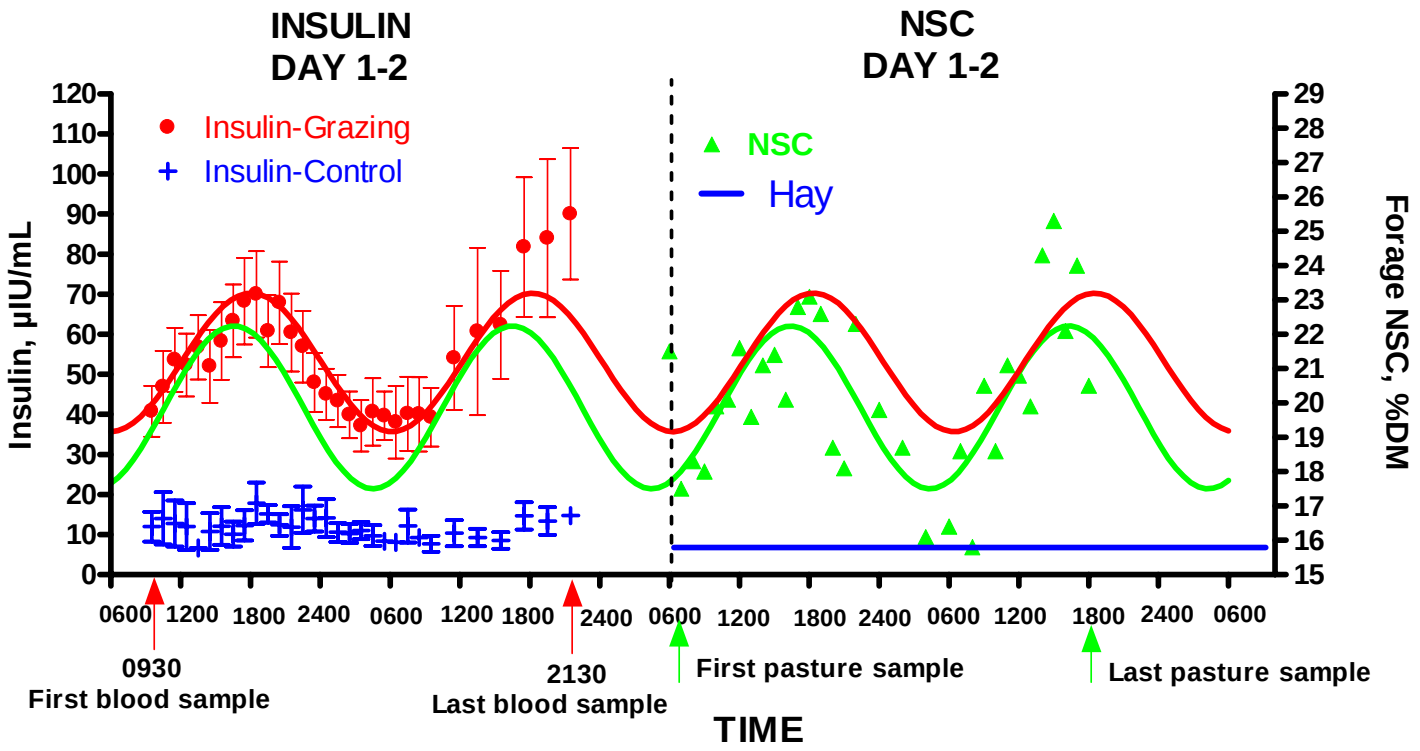

Figure 5.2. Sinusoidal circadian patterns in NSC (LAB1) and insulin in grazing horses.

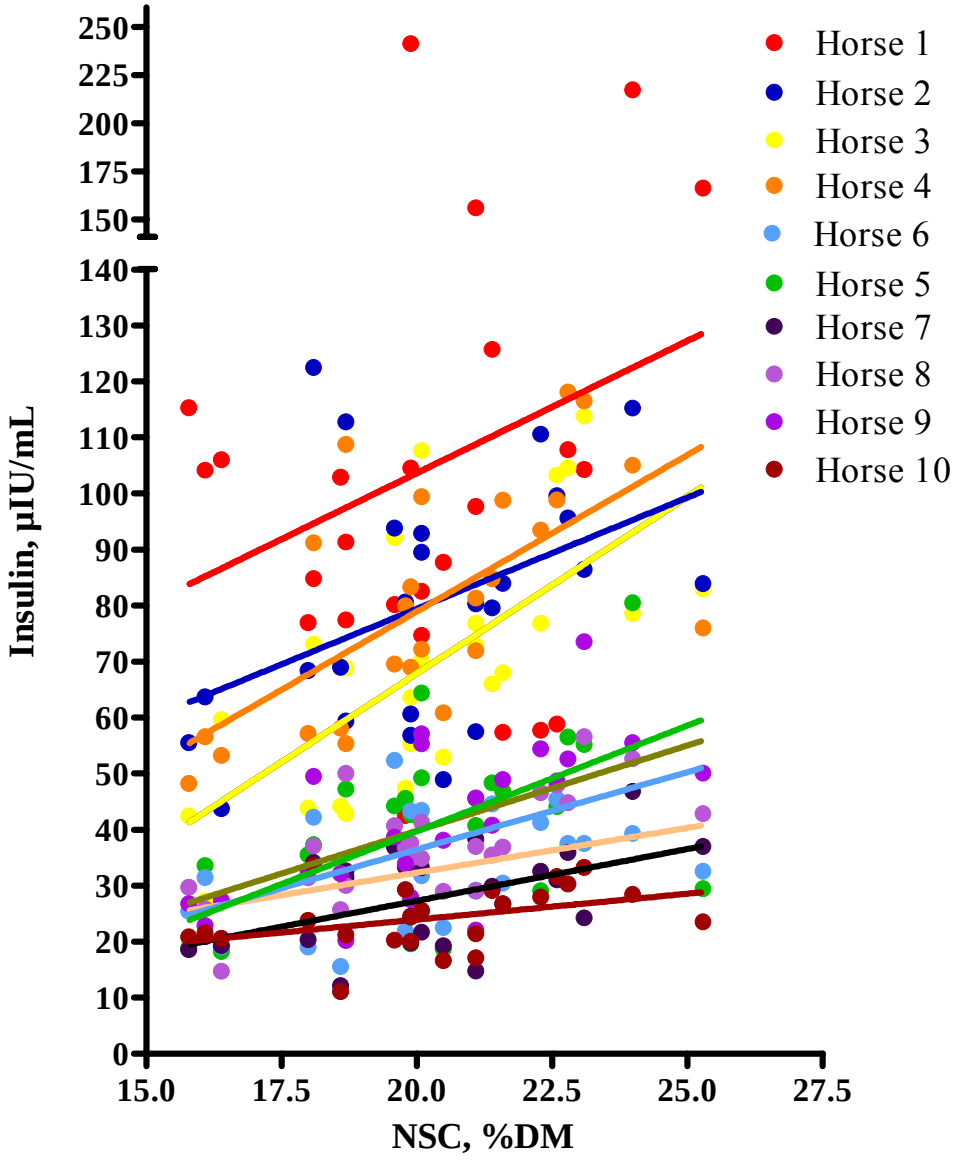

Figure 5.3. Insulin response (µIU/mL) to pasture NSC (LAB1) for individual horses grazing (n=10).

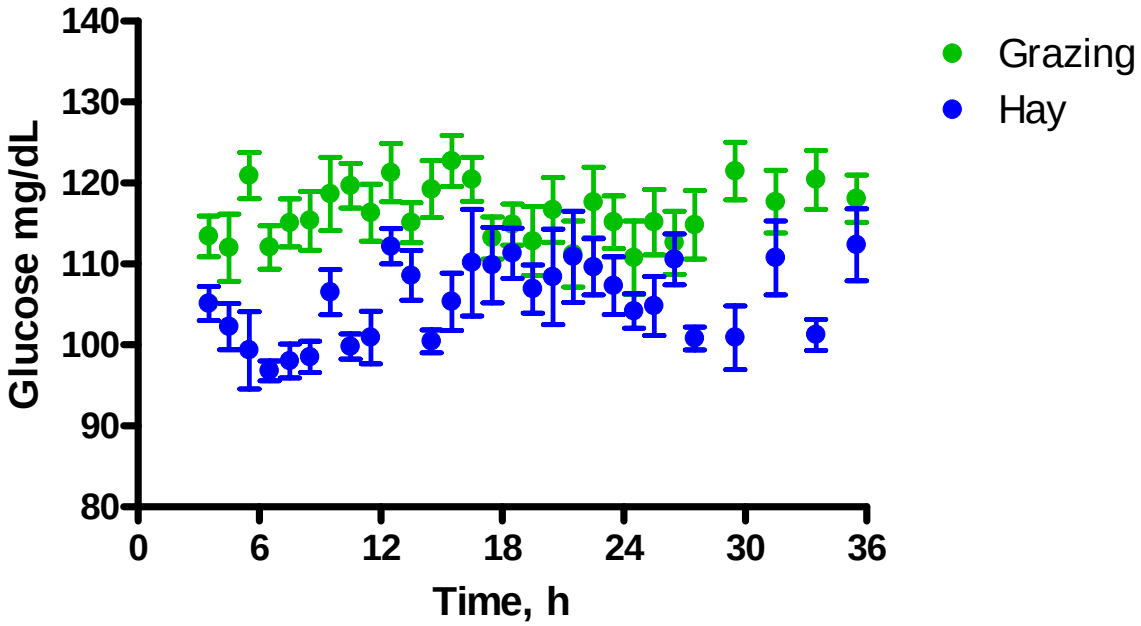

Figure 5.4. Glucose concentration (mg/dL) in grazing horses and horses fed hay over a 36 h sampling period in April 2005.

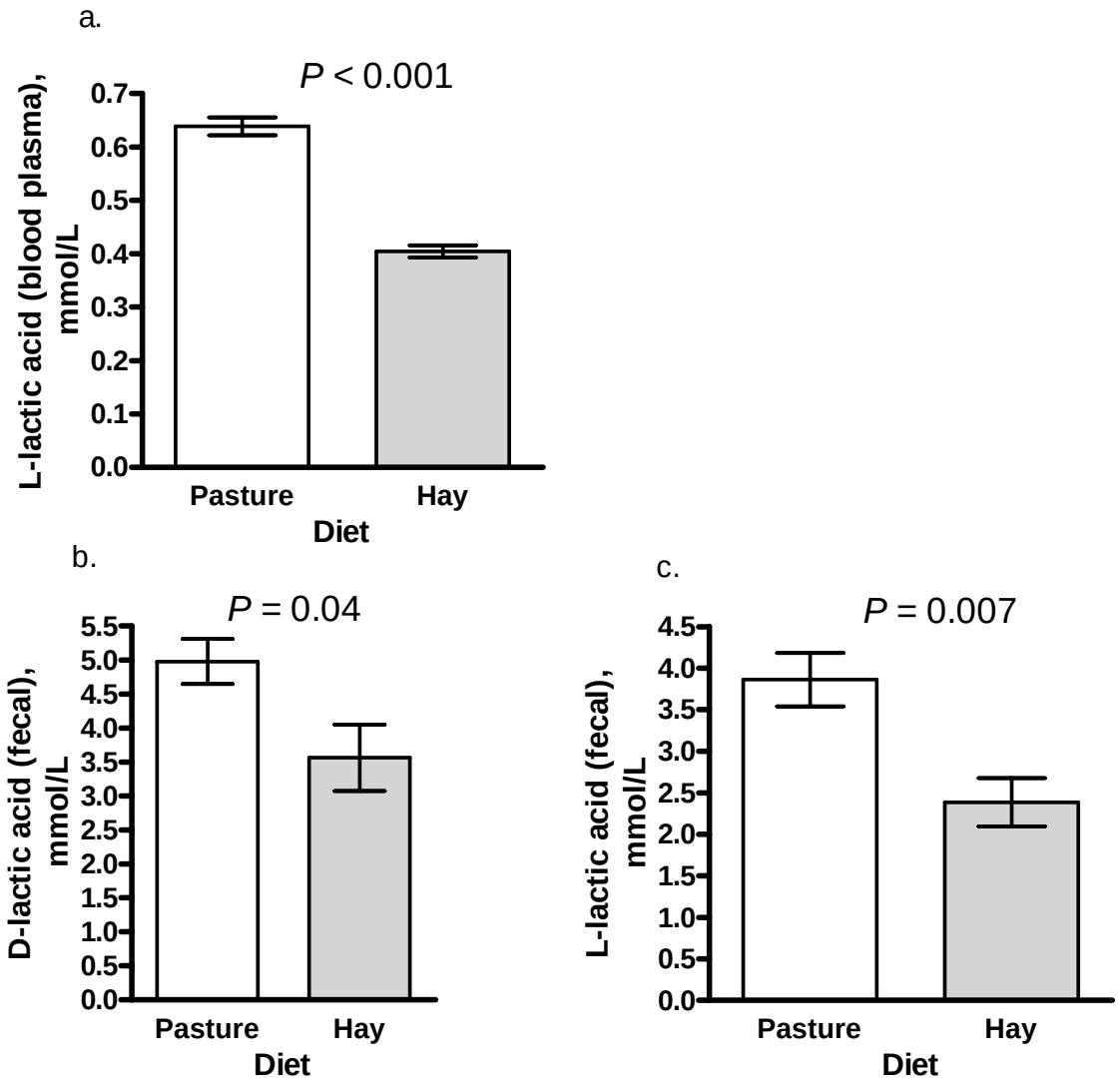

Figure 5.5. The plasma concentration of L-lactate (n = 31) (a) and fecal concentrations of D-lactate (b) and L-lactate (c) in grazing horses (n = 40) and control horses (n = 16).

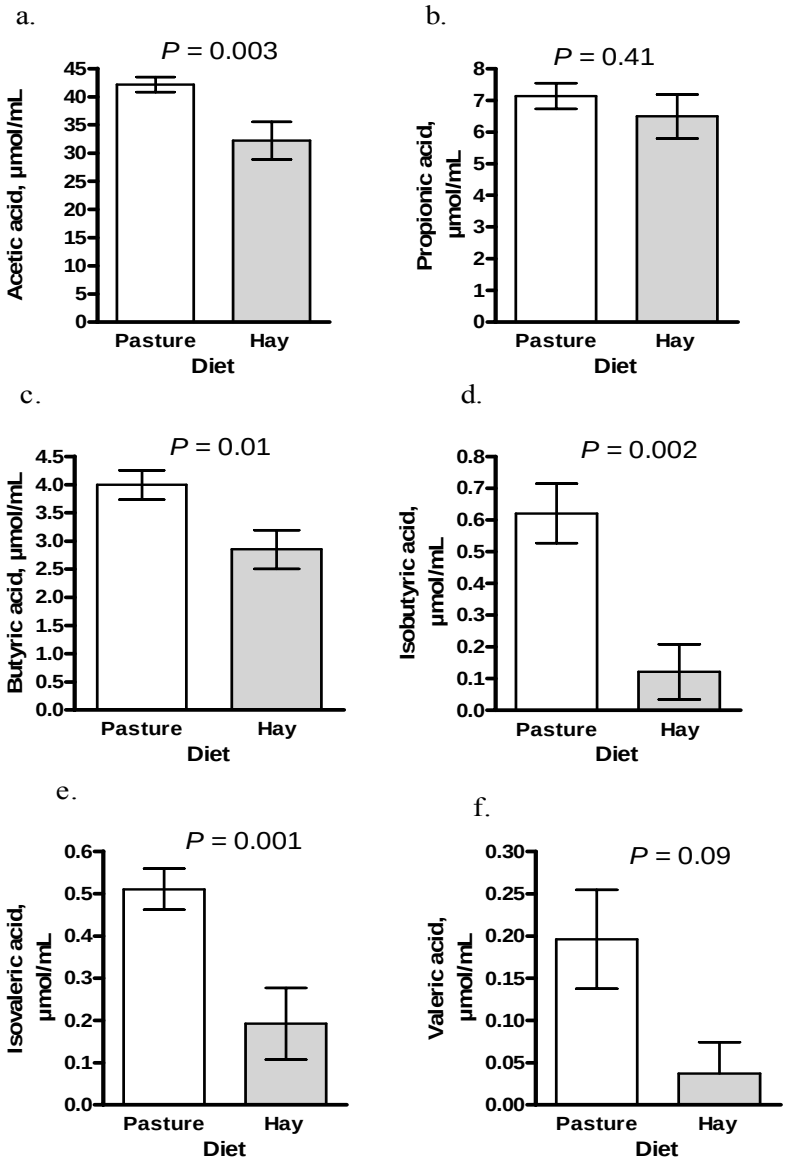

Figure 5.6. Mean fecal volatile fatty acid (VFA) concentrations in grazing horses and control horses; acetic acid (a), propionic acid (b), butyric acid (c), isobutyric acid (d), isovaleric acid (e), and valeric acid (f).

CHAPTER SIX
MANUSCRIPT 4

Seasonal variation in proxies for insulin resistance in Thoroughbred mares grazing pasture or fed hay

ABSTRACT: Basal proxies for insulin sensitivity (reciprocal of the square root of insulin [RISQI]) and pancreatic β-cell response (modified insulin-to-glucose ratio [MIRG]) were calculated in grazing horses (n=10) and horses restricted to hay diets (n=4) during a 36 h trial. Blood samples were collected in April, May, August, and October 2005, and January 2006 from venous catheters. Basal values for insulin and glucose were calculated from the mean of four hourly samples collected from 0530 to 0830 the second morning of the 36 h trials each mo. Seasonal variation in proxies was evident by marked differences in April from the other four mo. In April both RISQI and MIRG for grazing horses met the criteria for insulin resistance and compensatory pancreatic β-cell response, respectively. In April, RISQI was lower ($P < 0.001$) in grazing horses (0.18 ± 0.02 $[mU/L]^{-0.5}$) than horses offered hay (0.34 ± 0.03 $[mU/L]^{-0.5}$). The MIRG was higher ($P < 0.001$) in grazing horses in April (7.3 ± 0.5 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$) than horses offered hay (3.9 ± 0.8 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$). In May, August, October and January, there was no difference between grazing horses and horses offered hay for both RISQI and MIRG. In May, grazing horses met the criteria for insulin resistance based on RISQI (0.23 ± 0.02 $[mU/L]^{-0.5}$), as did the horses offered hay (0.28 ± 0.03 $[mU/L]^{-0.5}$). Seasonal variation in insulin sensitivity, independent of food composition, as occurred in our study may influence the onset of certain diseases such as laminitis. Horses or ponies predisposed to types of laminitis associated with insulin resistance may benefit from management which reduces access to pasture in the spring when environmental

conditions favor increased water soluble carbohydrate content may be elevated in certain forages.

Introduction

The most common causes of laminitis are linked to equine nutrition. Pasture composition (grass sugar and fructan, and legume starch) accounts for 54%, and another 8% of laminitis is associated with excessive grain intake (USDA, 2000). There is an increase in annual incidence of laminitis from 2% to about 5% in the spring and summer (Kane, 2000). The seasonal variation in the occurrence of laminitis is most likely linked to seasonal variation in pasture carbohydrate composition (Longland, 1999),

Laminitis has been associated with insulin resistance in horses and ponies (Treiber et al., 2006a). Insulin resistance is a thrifty pattern of metabolism that spares glucose and conserves energy, a beneficial trait for equine breeds that evolved in nutritional sparse environments. The association between insulin resistance and laminitis was first studied by glucose and insulin tolerance tests Laminitic ponies were reported to be intolerant to glucose and significantly less sensitive to insulin than non-laminitic controls (Coffman and Colles, 1983). More recently, a specific quantitative method for assessing insulin resistance (the minimal model) has demonstrated that ponies genetically predisposed to laminitis have a reduced ability for insulin to induce hypoglycemia compared to normal ponies (Treiber et al., 2006a). A pre-laminitic metabolic syndrome has been described statistically by cut off points for proxies for insulin sensitivity (RISQI) and pancreatic β-cell responsiveness (MIRG).

Previous studies evaluating metabolic predispositions and nutritional risk factors for pasture laminitis have involved fasted animals removed from pasture. To grasp the association between pasture composition and the metabolic responses in horses, research needs to focus on animals under natural grazing conditions. The objective of this study was to calculate RISQI and MIRG in grazing horses and those confined to stalls and offered hay only.

Materials and Methods

Five 36 h trials were performed to identify proxies for insulin resistance in grazing horses compared to those confined to stalls and fed hay only. The five trials took place in April, May, August, September, October 2005, and January 2006 at the Middleburg Agricultural Research and Extension Center (M.A.R.E.C.) in northern Virginia. A group of 10 grazing mares and four stalled horses were housed in a 5-ha grass/legume pasture. The pasture consisted of approximately 60% Max Q fescue, 35% Kentucky bluegrass, and 5% white clover. The stalls were located in a run-in shed within the confines of the pasture. Procedures for corresponding blood samples from horses and pasture samples were the same over the five months.

Horses. Ten Thoroughbred mares were housed on a 5-ha pasture and four Thoroughbred mares were housed in temporary stalls within the same pasture and offered timothy/alfalfa hay only (Table 1). Horses were a mean ± SD of 11 ± 5 yr old (range 6 to 16 yr). Mean weight of the horses over the five months was 596.0 ± 14.5 kg, and mean body condition score was 6.2 ± 0.2 (range 4.5 to 7.5 on a scale of 1 to 9) (Henneke et al., 1983) (Table 2). Horses had ad libitum access to water and white salt. Horses were

acclimated to the pasture for 7 d before each sampling period. Hourly blood samples were collected from all 14 horses during 36 h sampling periods during the months of April, May, August, and Ocotber 2005, and January 2006. Horses were sampled hourly for 36 h because this study paralleled others studies evaluating circadian patterns in variables in horses and pastures.

Jugular catheters were inserted at 0500 and collection of blood samples began at 0930. Hourly samples were collected until 0930 the second day, and then continued at two h intervals until 2130 the second day. Blood was collected into two 7 ml heparinized vaccutainer tubes (Fisher Scientific, St. Louis, MO), placed on ice, and taken to the lab where plasma was separated by centrifugation at 3000 g for 10 min. Plasma was stored at -20˚C until analysis. Plasma was analyzed for glucose and insulin. Glucose was measured by colorimetric assay (UV visible chemistry). Insulin was measured by a chemiluminescent immunoassay (Appendix I).

Proxies. Proxies for insulin sensitivity (RISQI) and pancreatic β-cell response (MIRG) were calculated from basal plasma concentrations of glucose (mg/dL) and insulin (mU/L) according to methods previously described by Treiber et al. (2005b), with minor modifications. The basal values of glucose and insulin were calculated by the mean from 0530 to 0830 on the second day of sampling during each month since there was continuous intake of pasture or hay and horses were never fasted. The peak vales of glucose and insulin were selected as the single highest value throughout the 36 h for each month.

Pasture. Composite pasture and hay samples were dried at 70º C to determine DM. Nutrient content was determined by proximate analysis (Dairy One, Ithaca NY) (Table 2).

Statistical analysis. A mixed effects, repeated measures analysis of variance was used to test for main effects of treatment and month as well as their interaction. The calculations were performed using the MIXED procedure of the SAS System (ver. 9.13, SAS Institute Inc., Cary, NC 27513). Significant interactions were further investigated using tests of simple main effects using the SLICE option. Model adequacy was assessed using plots of standardized residuals and quantile-quantile plots. Insulin and TG exhibited an apparent multiplicative error structure so the data was log-transformed to stabilize variances and back-transformed for presentation

Results

At all sampling times, all horses appeared to be in good health. Mean weights of the horses ranged from 572.7 ± 37.0 in April to 610.3 ± 37.8 in January, and BCS ranged overall from 5.7 ± 0.9 to 6.3 ± 0.6 (Table 1). Individual horses were similar in weight and BCS between dietary treatments (Table 2).

Basal and peak plasma insulin concentrations are shown in Table 3. Basal and peak plasma glucose concentrations are shown in Table 4. To the author's knowledge, this is the first study to examine seasonal variation in proxies for insulin resistance (ie. RISQI and MIRG) based on basal samples collected from horses with continuous access to pasture or hay.

There were differences in RISQI between months ($P < 0.001$) and a dietary treatment by month interaction was evident ($P < 0.001$) (Figure 1). In April, RISQI for grazing horses was lower than in May, August, October and January ($P< 0.001$). In May, RISQI for grazing horses was lower than August, October, and January values ($P < 0.001$). In August, October and January RISQI values for grazing horses did not differ.

In all months except for May, horses offered hay had RISQI values > 0.32 $[mU/L]^{-0.5}$. In horses offered hay, the April RISQI value was higher than May ($P = 0.009$), but lower than October ($P = 0.03$), and January ($P = 0.02$). The May RISQI value was lower than April, August, October and January ($P < 0.05$).). In August, RISQI was lower than October ($P = 0.004$) and January ($P = 0.004$). October and January RISQI values did not differ in horses offered hay.

In April, RISQI was lower ($P = 0.0004$) in grazing horses (0.18 ± 0.02 $[mU/L]^{-0.5}$) than horses offered hay (0.34 ± 0.03 $[mU/L]^{-0.5}$). In May, RISQI did not differ between grazing horses (0.23 ± 0.02 $[mU/L]^{-0.5}$) and those offered hay (0.28 ± 0.03 $[mU/L]^{-0.5}$). In August, RISQI did not differ between grazing horses (0.36 ± 0.02 $[mU/L]^{-0.5}$) and those offered hay (0.33 ± 0.03 $[mU/L]^{-0.5}$). In October, RISQI did not differ between grazing horses (0.33 ± 0.02 $[mU/L]^{-0.5}$) and those offered hay (0.40 ± 0.03 $[mU/L]^{-0.5}$). In January, RISQI did not differ between grazing horses (0.33 ± 0.02 $[mU/L]^{-0.5}$) and those offered hay (0.40 ± 0.03 $[mU/L]^{-0.5}$).

There was seasonal variation in the MIRG as it differed between months ($P = 0.01$), and a dietary treatment by month interaction was evident ($P = 0.03$) (Figure 2). MIRG did not differ between April and May in grazing horses. April MIRG for grazing horses was higher than August ($P < 0.001$), October ($P < 0.004$) and January ($P < 0.001$).

May MIRG was also higher than August ($P < 0.008$), October ($P < 0.02$), and January ($P < 0.005$) for horses on pasture. August, October and January MIRG were not different in grazing horses. There were also no differences between months in MIRG in horses offered hay in April.

In April, the MIRG was higher ($P = 0.0007$) in grazing horses (7.3 ± 0.5 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$) than horses offered hay (3.9 ± 0.8 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$). In May, MIRG did not differ between grazing horses (6.5 ± 0.5 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$) and horses offered hay (5.3 ± 0.8 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$). In August, MIRG did not differ between grazing horses (4.3 ± 0.5 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$) and horses offered hay (4.8 ± 0.8 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$). In October, MIRG did not differ between grazing horses (4.9 ± 0.5 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$) and horses offered hay (3.8 ± 0.8 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$). In January, MIRG did not differ between grazing horses (4.7 ± 0.5 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$) and horses offered hay (3.6 ± 0$mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$).

Horses grazing pasture in April and May met the criteria for insulin resistance ($RISQI < 0.32\ [mU/L]^{-0.5}$) previously described by Treiber et al. (2005). In May, the group of horses offered hay met the criteria of insulin resistance.

Horses grazing pasture in April and May also met the criteria for compensatory β-cell secretory response ($MIRG > 5.6\ mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$) previously described by Treiber et al. (2005). Horses offered the hay diet never had MIRG values greater than 5.6 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$.

Discussion

To the author's knowledge, the study reported here is the first to apply the proxies RIQI and MIRG to a group of grazing horses over the four seasons. To the author's knowledge this is also the first study to compare groups of horses on pasture with those in stalls and offered a hay diet only.

Proxies for RISQI and MIRG were first assessed for horses using the minimal model of glucose-insulin dynamics (Treiber et al., 2005b). In a subsequent study examining metabolic differences between ponies either with a history of laminitis, or with no history laminitis, Treiber et al (Treiber et al., 2006b), statistically derived cut off points for insulin resistance (RISQI < 0.32 $[mU/L]^{-0.5}$), and compensatory β-cell secretory response (MIRG > 5.6 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$). The proxies for RISQI and MIRG were among the criterion used to identify a pre-laminitic metabolic syndrome in apparently healthy ponies. The other criterion included obesity (BCS > 6.0 with fat deposition at the neck and tail head), and hypertriglyceridemia (triglyceride concentration > 57.0 mg/dL). Individual criteria had predictive powers of more than 70% for the development of laminitis. In this study the proxies associated with insulin resistance (RISQ and MIRG), were calculated in Thoroughbred mares with no history of laminitis. Further, the author's did not expect to identify a pre-laminitic metabolic syndrome in the Thoroughbreds since the breed is not typically prone to laminitis (USDA, 2000). In the contrary, the grazing horses in this study did meet the criteria for insulin resistance and pancreatic β-cell response in the spring, particularly in the month of April. Therefore, it may be potentially misleading to associate the cut-off points for RISQI and MIRG with a pre-laminitic metabolic syndrome in the horses in our study.

The horses potentially could have been responding to the relatively high of water soluble carbohydrate ('sugar') during the spring, particularly in April. Insulin resistance develops with chronic adaptation to meals high in sugar and starch and the resulting fluctuations in the glycemic and insulinemic response (Treiber et al., 2005a). Results from our study suggest that insulin resistance may also develop as a chronic adaptation to elevated water soluble carbohydrate content in the spring pasture. In the spring, environmental conditions favor accumulation of water soluble carbohydrates and starches, leading marked circadian patterns in pasture carbohydrate profiles (Longland and Byrd, 2006). Large amounts of water soluble carbohydrate ingested at once may mimic the overload models and rapid fermentation could lead to the production of trigger factors associated with insulin resistance and laminitis (Bailey et al., 2004).

Although it did appear the horses were responding to increased WSC content in their diet, it is possible that the cut off points for insulin sensitivity and β-cell response, which have previously been used to identify insulin resistance and a pre-laminitic metabolic syndrome, may be inappropriate to apply to other horse populations. Thoroughbreds are not typically prone to insulin resistance or laminitis, yet all of the ten grazing horses fit the criteria for insulin resistance and compensatory β-cell response in April and most in May. In May the horses fed hay only also met the criteria for insulin resistance based on RISQI, despite that they were fed the same hay during each of the five month trials. Healthy Thoroughbred mares grazing pasture in this study were similar to a group of ponies with previous signs of clinical laminitis, where RISQI was 0.25 ± 0.01 $[mU/L]^{-0.5}$ and 0.12 ± 0.01 $[mU/L]^{-0.5}$ in March and May 2004, respectively (Treiber et al., 2006b). In our study, basal values of insulin and glucose were determined by

averaging the early morning samples (0530 to 0830), to simulate a basal sample, even though the horses were never fasted. In the pony study the animals were removed from pasture at 0700 and a single blood sample was collected between 0800 to 1000. This seemingly small distinction in protocol could alter the outcome of the proxy calculations. Unless groups of animals are treated exactly the same, this study suggests it is difficult to compare proxies for insulin resistance between studies.

Conclusions

This study indicated that there are seasonal differences in insulin sensitivity and pancreatic β-cell response in grazing horses, which may be attributed to increased amounts of water soluble carbohydrate in springtime pastures. Avoiding factors that contribute to insulin resistance may decrease risks to certain diseases such as laminitis. More research is needed to assess RISQI and MIRG in horses under natural grazing conditions to implement appropriate cut off points used to predict a prelamintic metabolic syndrome.

Table 6.1. Weights (kg) and body condition scores (BCS) of horses (n= 14) over the five sampling trials. Data are summarized as mean ± SD.

	April	May	August	October	January
Weight	572.7 ± 37.0	595.9 ± 35.5	605.4 ± 33.6	596.0 ± 31.0	610.3 ± 37.8
BCS	5.7 ± 0.9	6.1 ± 0.7	6.4 ± 0.5	6.4 ± 0.5	6.3 ± 0.6

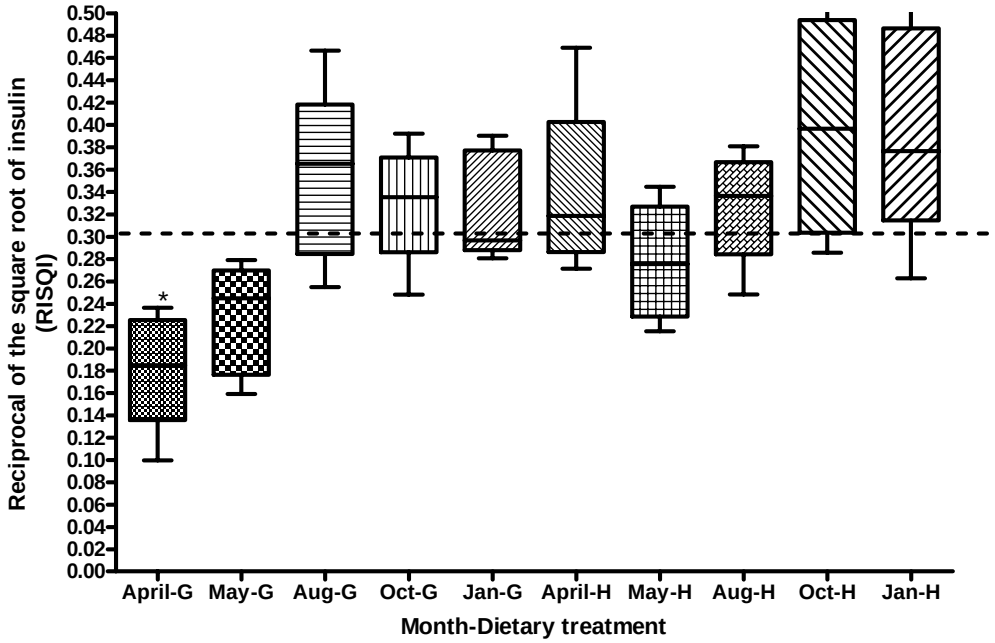

Figure 6.1. Calculated RISQI for grazing horses (G) and horses fed hay (H) in April, May, August, October and January. Dashed line represents cut off pint for criteria identifying insulin resistance (RISQI < 0.32 $[mU/L]^{-0.5}$).

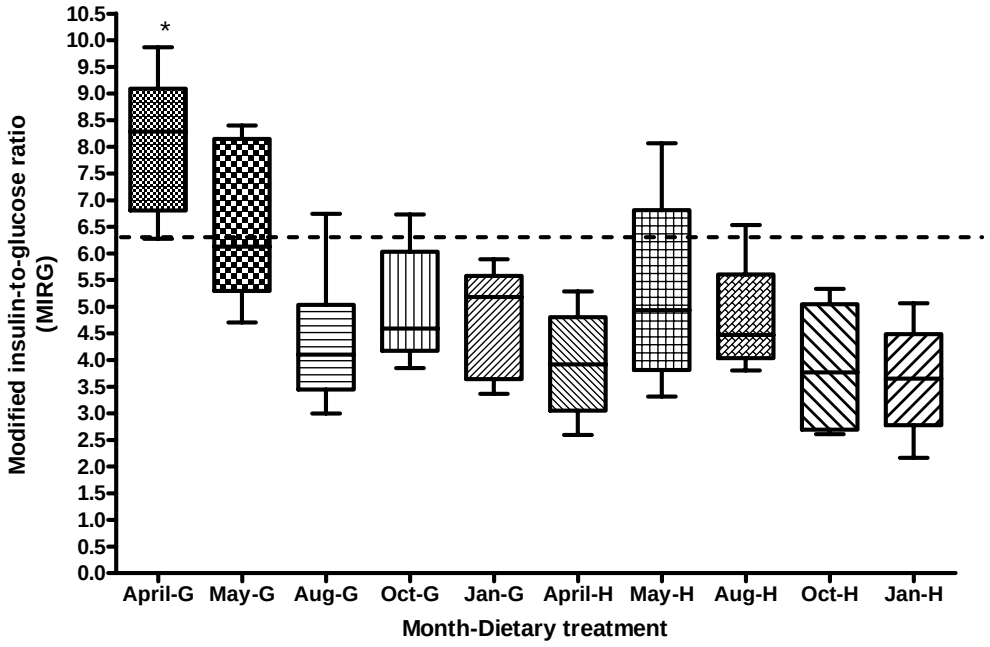

Figure 6.2. Calculated MIRG for grazing horses (G) and horses fed hay (H) in April, May August, October and January. Dashed line represents cut off point for criteria identifying compensatory β-cell secretory response (MIRG > 5.6 $mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$).

CHAPTER SEVEN
SUMMARY AND IMPLICATIONS

The purpose of this series of studies was to identify possible risk factors for equine laminitis in pastures and horses. It was a pioneer study in examining the environment-plant-animal interface and implications with laminitis and insulin resistance. The healthy Thoroughbred horses used in this study were physiological models for identifying relationships between metabolic responses to carbohydrate profiles forages that can provide information for other breeds and classes of horses.

The first study (April 2005) illustrated that carbohydrate profiles in spring pasture plants varied throughout the day, and these changes were influenced by changes in the environment, particularly by the shift in ambient temperature from day night to day and intense sunlight. The entire series of studies provided a glimpse into the complicated interactions between plants and their environment, and how they may affect digestion and metabolism in grazing horses, providing direction for future research.

These studies were unique in that they compared analytical methods for NSC between laboratories. One lab being commercially available to horse owners (LAB1), and the other utilizing new enzymatic techniques for more exact quantification of NSC fractions, particularly fructans (LAB2). Overall, the two labs were in agreement for estimation of forage NSC and starch. An unexpected outcome was the low fructan and relatively high simple sugar (glucose, sucrose, and fructose content). While validation of the enzymatic technique is needed, it is groundbreaking evidence that fructan content of forages may vary widely from location to location (depending on environmental conditions) and between species. These studies, particularly the April 2005 study,

indicate that simple sugars rather than, or in addition to, fructans may be important in the pathogenesis of the metabolic and digestive disorders (e.g. laminitis) that occur in grazing horses

These studies were the first to identify a link between pasture carbohydrates and metabolic and digestive responses in grazing horses. Grazing horses were affected on a daily and seasonal basis by the fluctuating carbohydrate content of the pasture forage, as was made apparent by the range of insulin concentrations across the months. The range of insulin concentrations and the proxies for insulin resistance and β-cell response observed in these studies provides insight into identifying realistic cut off-points for identifying insulin resistance in equines. Besides the glycemic and insulemic responses observed in these studies, there are undoubtedly other aspects being affected by pasture carbohydrate content, including hindgut fermentation and grazing behavior. Digestive factors measured indicated rapid fermentation taking place in grazing horses in the spring which is a risk factor for laminitis. Grazing behavior was observed in these studies, but more complex systems for quantifying grazing behavior and forage intake are needed.

Ultimately, this work identified risk factors in pastures and horses for the development of laminitis. Through collecting more data on general patterns of NSC fluctuation and profiles in forages, and insulin concentrations in grazing horses, we can improve the management of horses at risk for developing laminitis. Identifying when there are heightened risks in the environment, pastures, and horses enables the development of innovative feeding and management strategies that avoid these risk factors, and hence, reducing the incidence of laminitis in predisposed equines. The goal is that in the future there will be set cut off points for each of these risk factors to determine

pre-laminitic carbohydrate profiles in the pastures, and pre-laminitic metabolic profiles in the animals themselves. Since laminitis is a disease that can not be treated with 100% recovery to soundness, it is important for research efforts to focus on avoidance and prevention.

APPENDIX I
Pairwise comparisons of pasture NSC and environmental variables

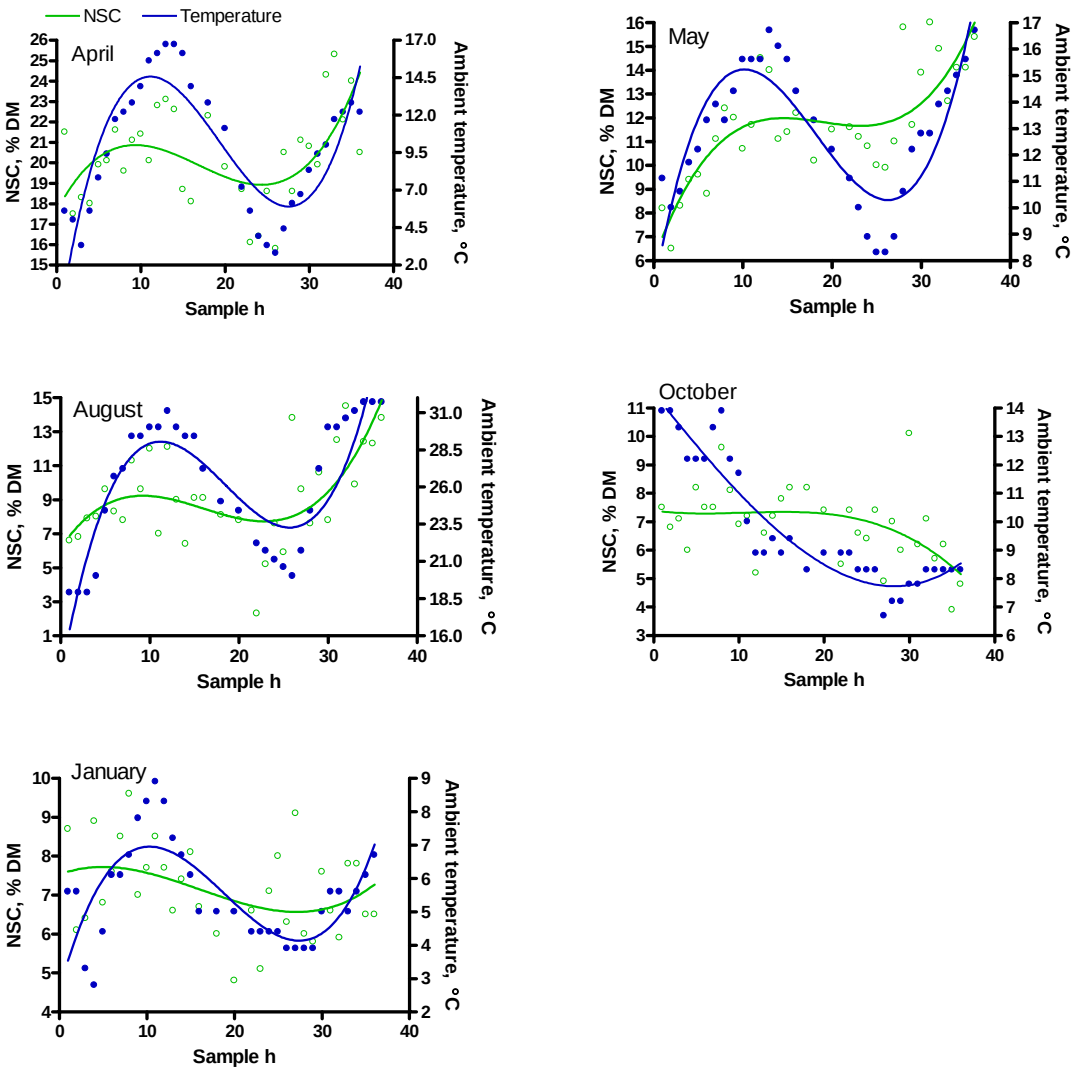

Figure A.1. Pair-wise comparisons of pasture forage nonstructural carbohydrate (NSC, % DM) (LAB1) and ambient temperature (ºC) (n = 258).

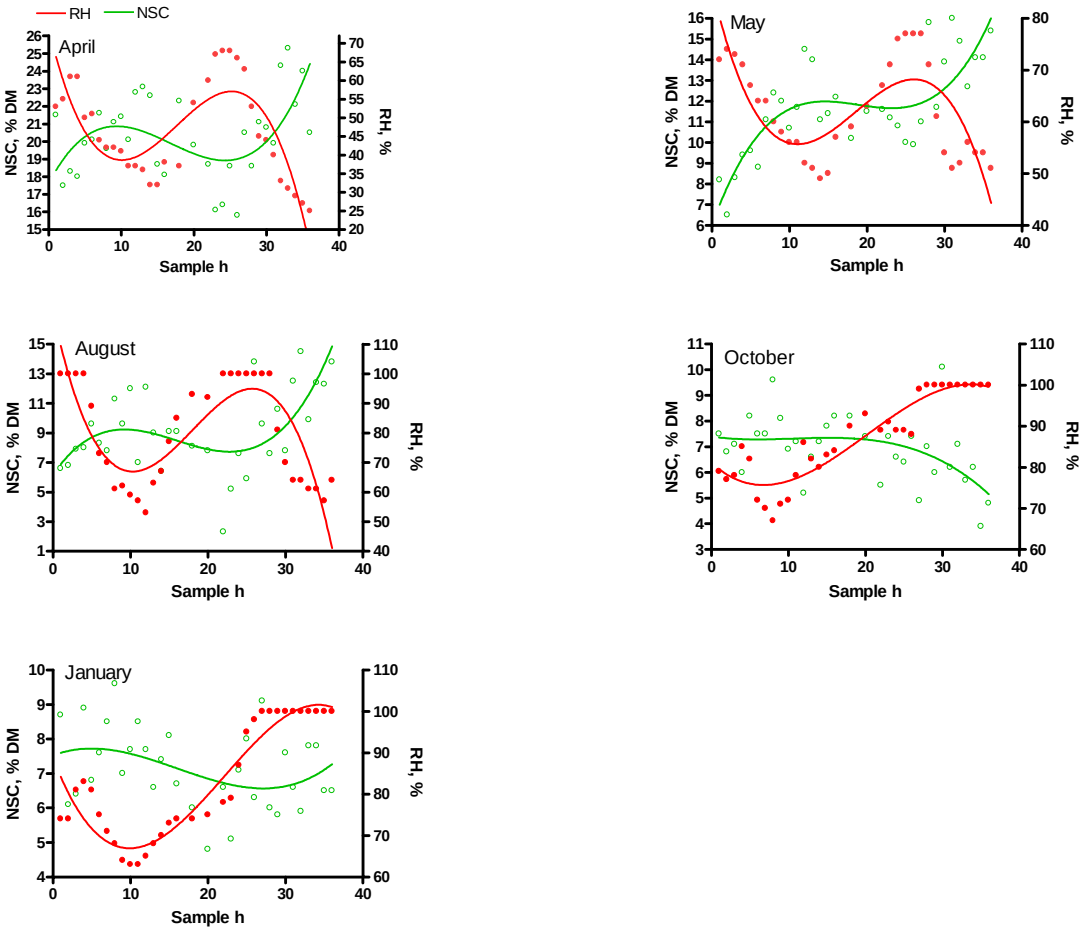

Figure A.2 Pair-wise comparisons of pasture forage nonstructural carbohydrate (NSC, % DM) (LAB1) and relative humidity (RH, %) (n= 258).

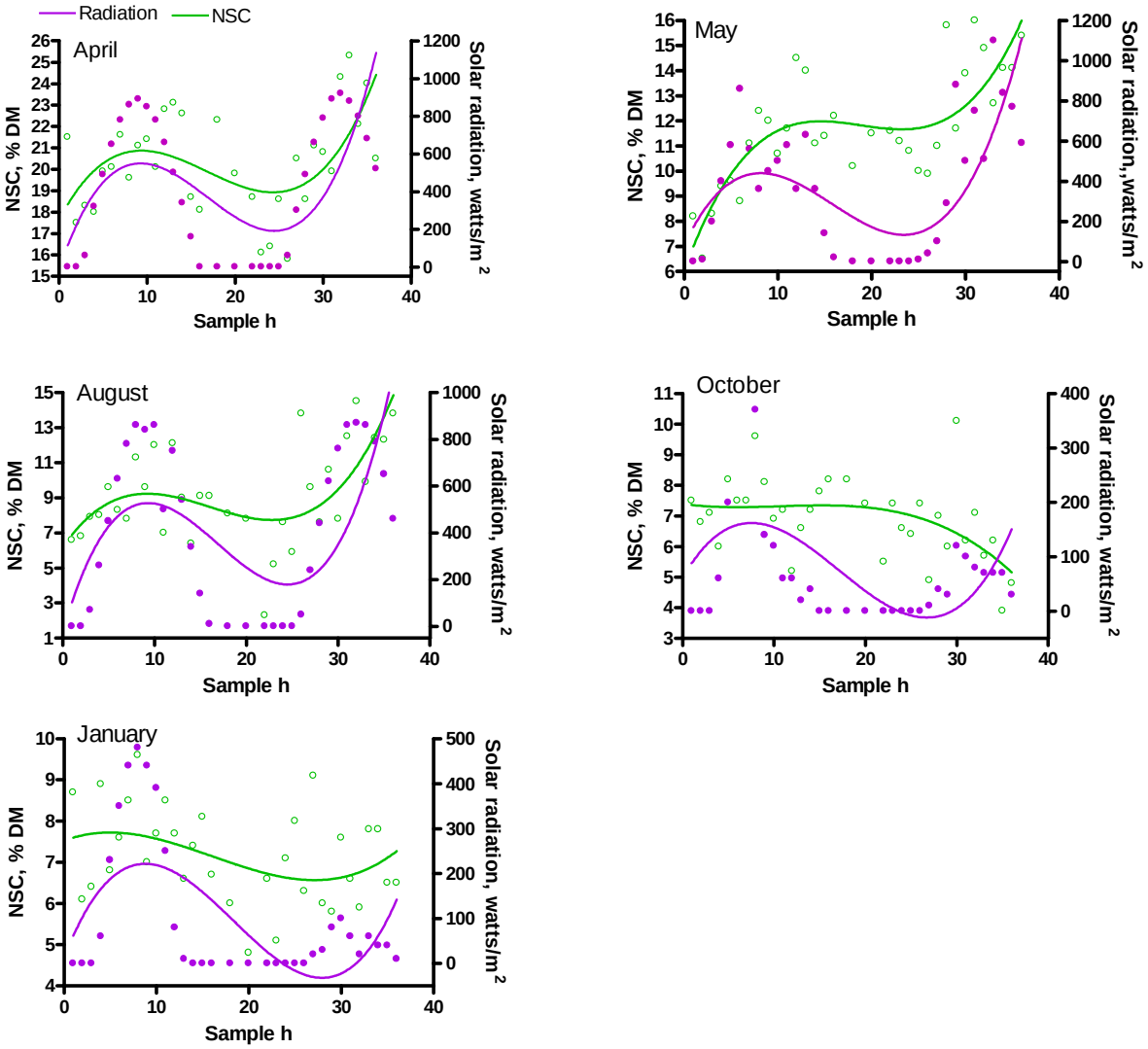

Figure A.3. Pair-wise comparisons of pasture forage nonstructural carbohydrate (NSC, % DM) (LAB1) and solar radiation (watts/m^{-2}).

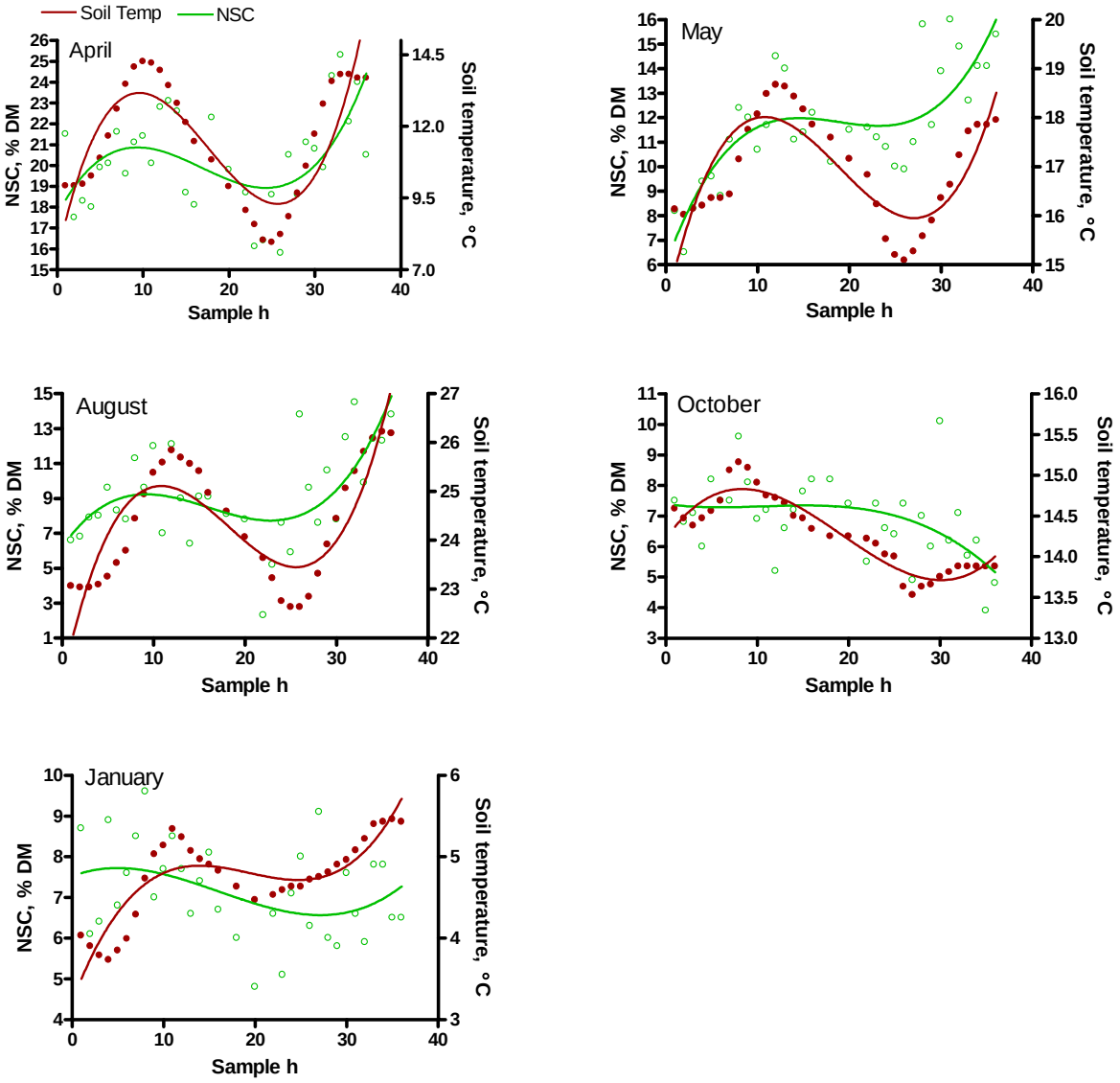

Figure A.4. Pair-wise comparisons of pasture forage nonstructural carbohydrate (NSC, % DM) (LAB1) and soil temperature (ºC) (n=258).

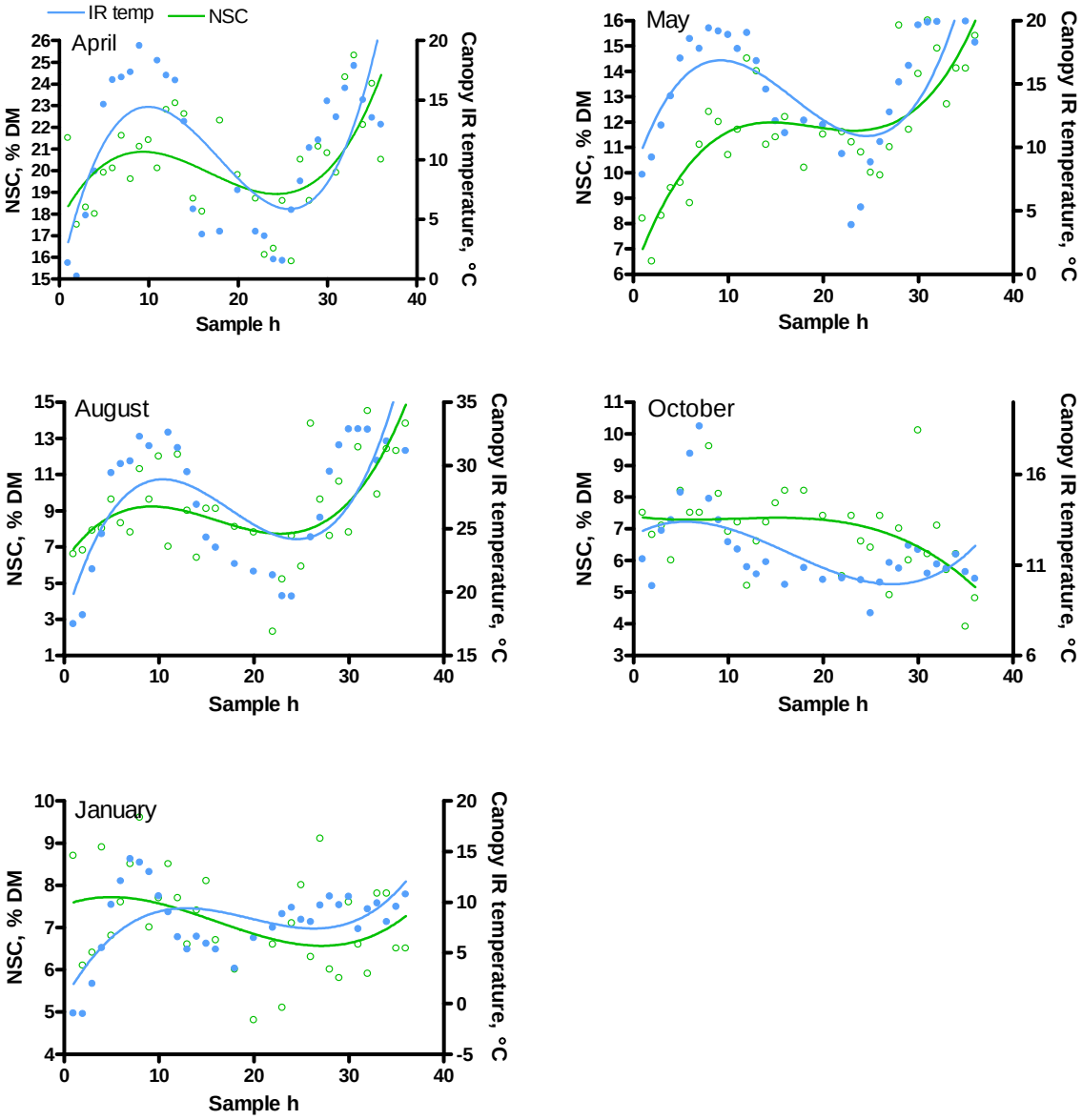

Figure A.5. Pair-wise comparisons of pasture forage nonstructural carbohydrate (NSC, % DM) (LAB1) and canopy infrared (IR) temperature (ºC) (n=258).

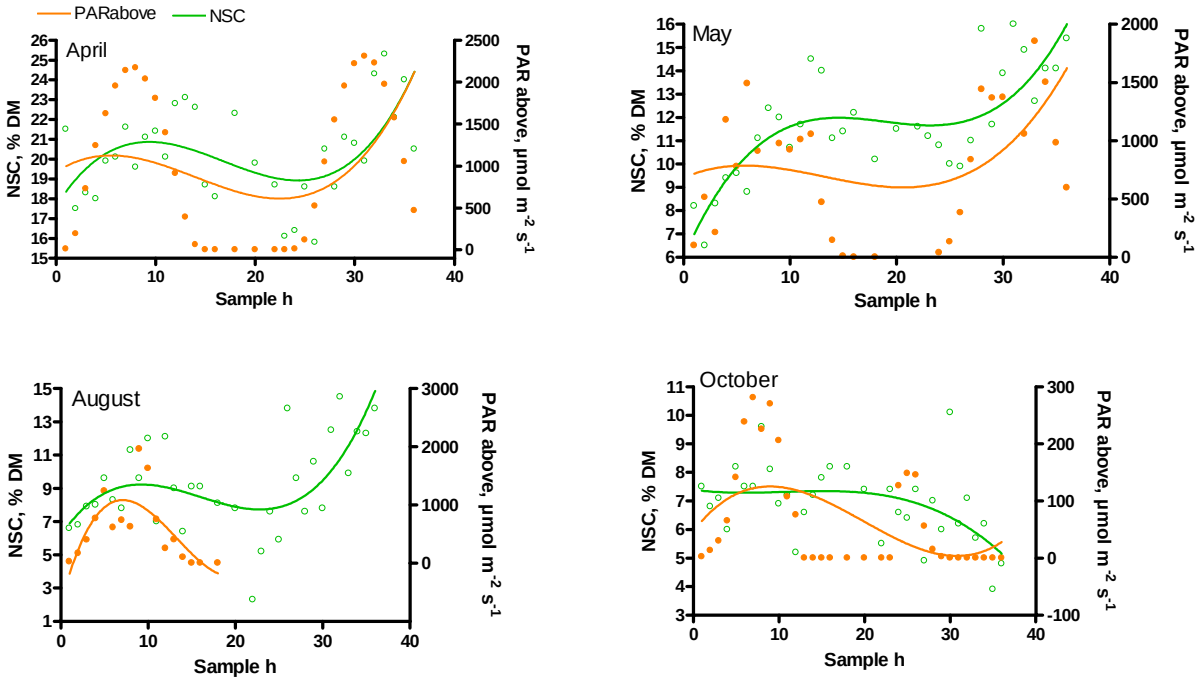

Figure A.6. Pair-wise comparisons of pasture forage nonstructural carbohydrate (NSC, % DM) (LAB1) and photosynthetically active radiation (PAR) above the canopy (µmol m^{-2} s^{-1}) (n=258). (No data for d 2 in August and no data available for January 2007.)

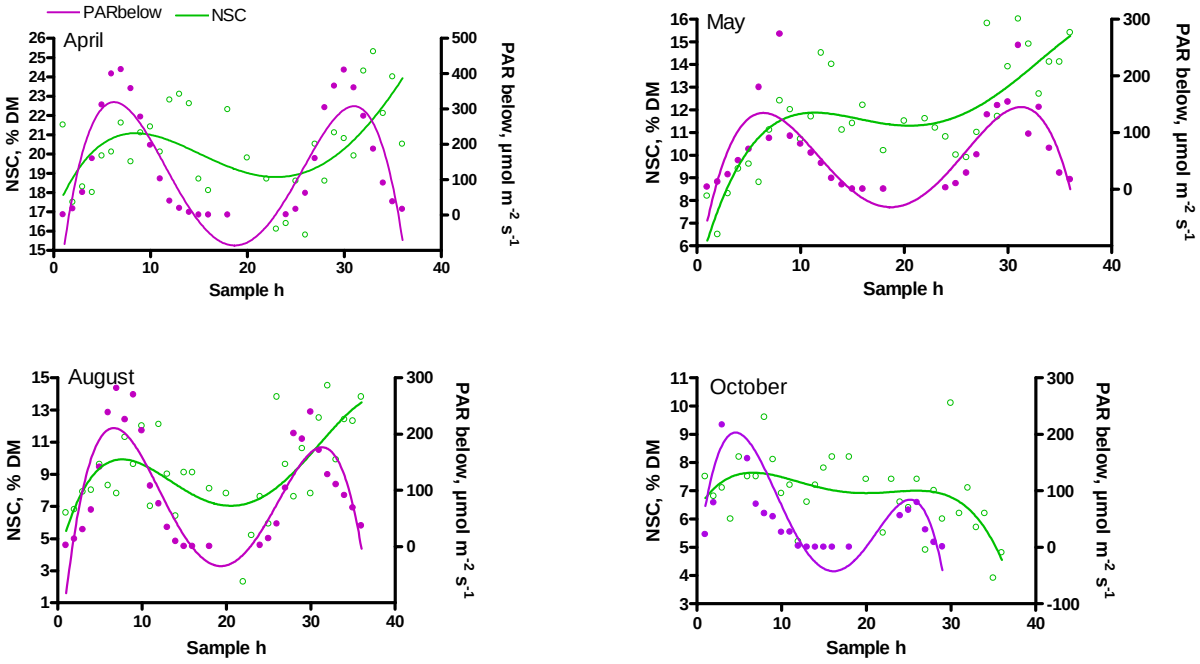

Figure A.7. Pair-wise comparisons of pasture forage nonstructural carbohydrate (NSC, % DM) (LAB1) and photosynthetically active radiation (PAR) below the canopy (ºC) (n=258). No data available for January 2007.

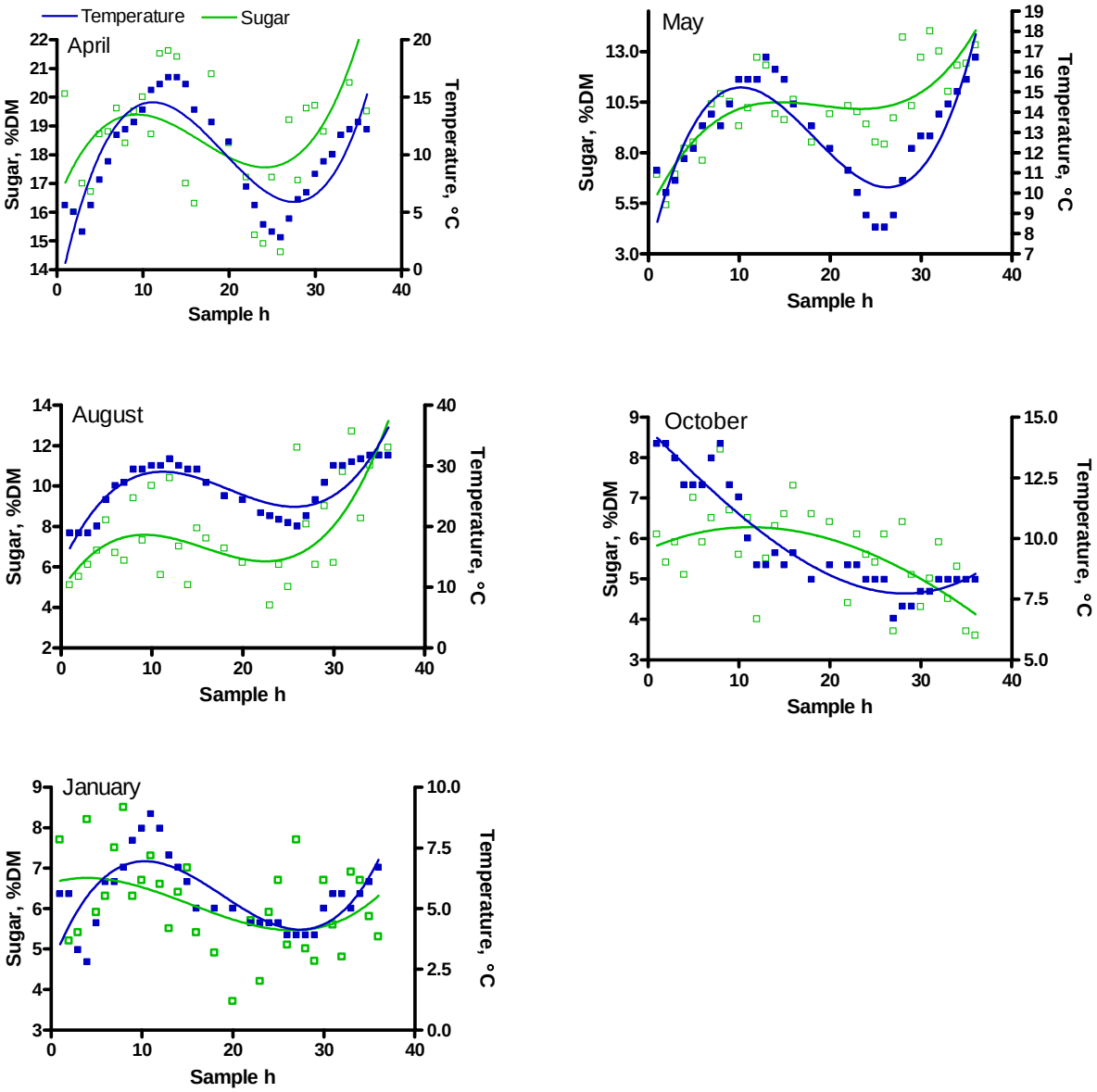

Figure A.8. Pair-wise comparisons of pasture forage sugar (% DM) (LAB1) and ambient temperature (ºC) (n=258).

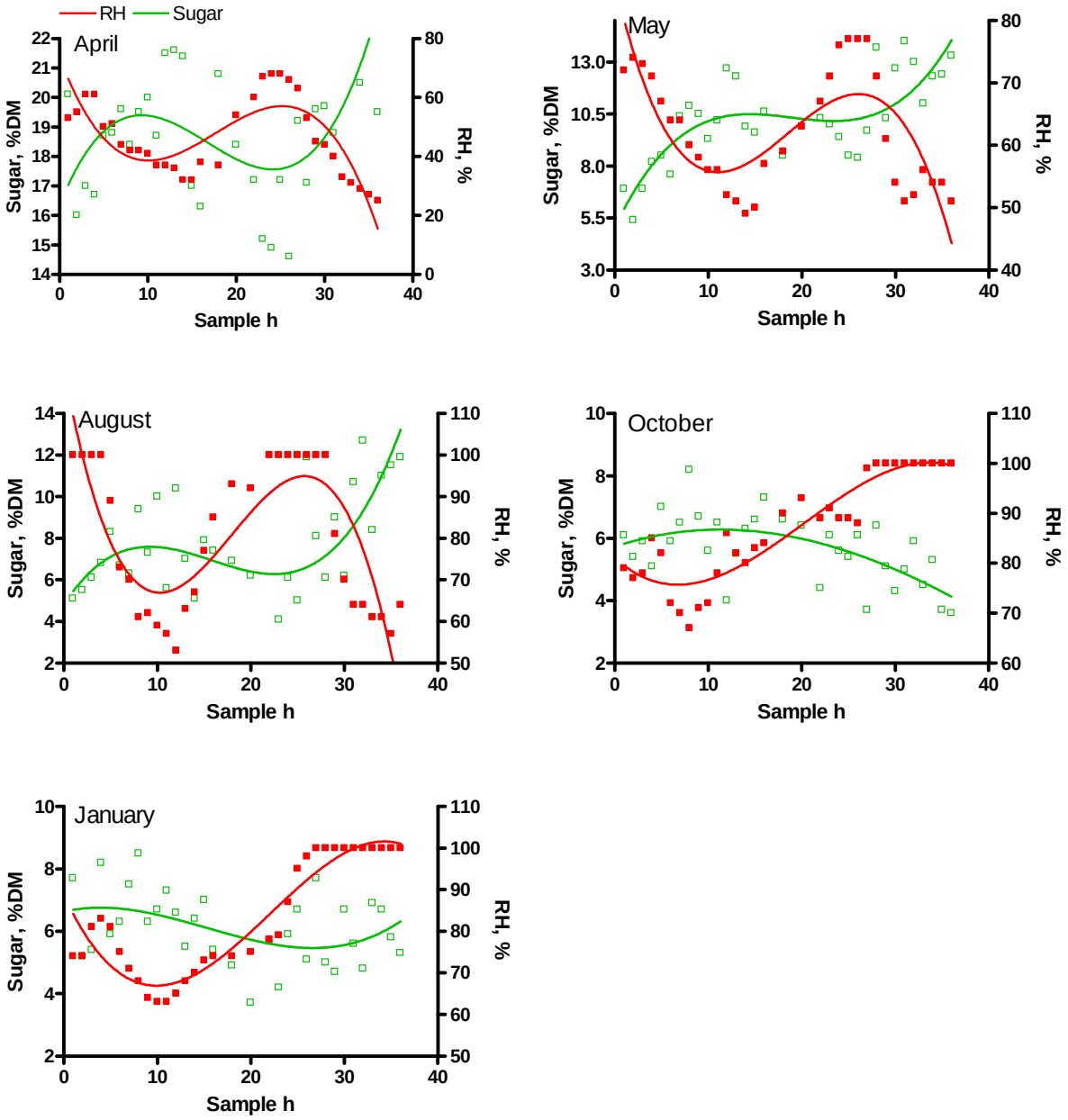

Figure A.9. Pair-wise comparisons of pasture forage sugar (% DM) (LAB1) and relative humidity (RH, %) (n= 258).

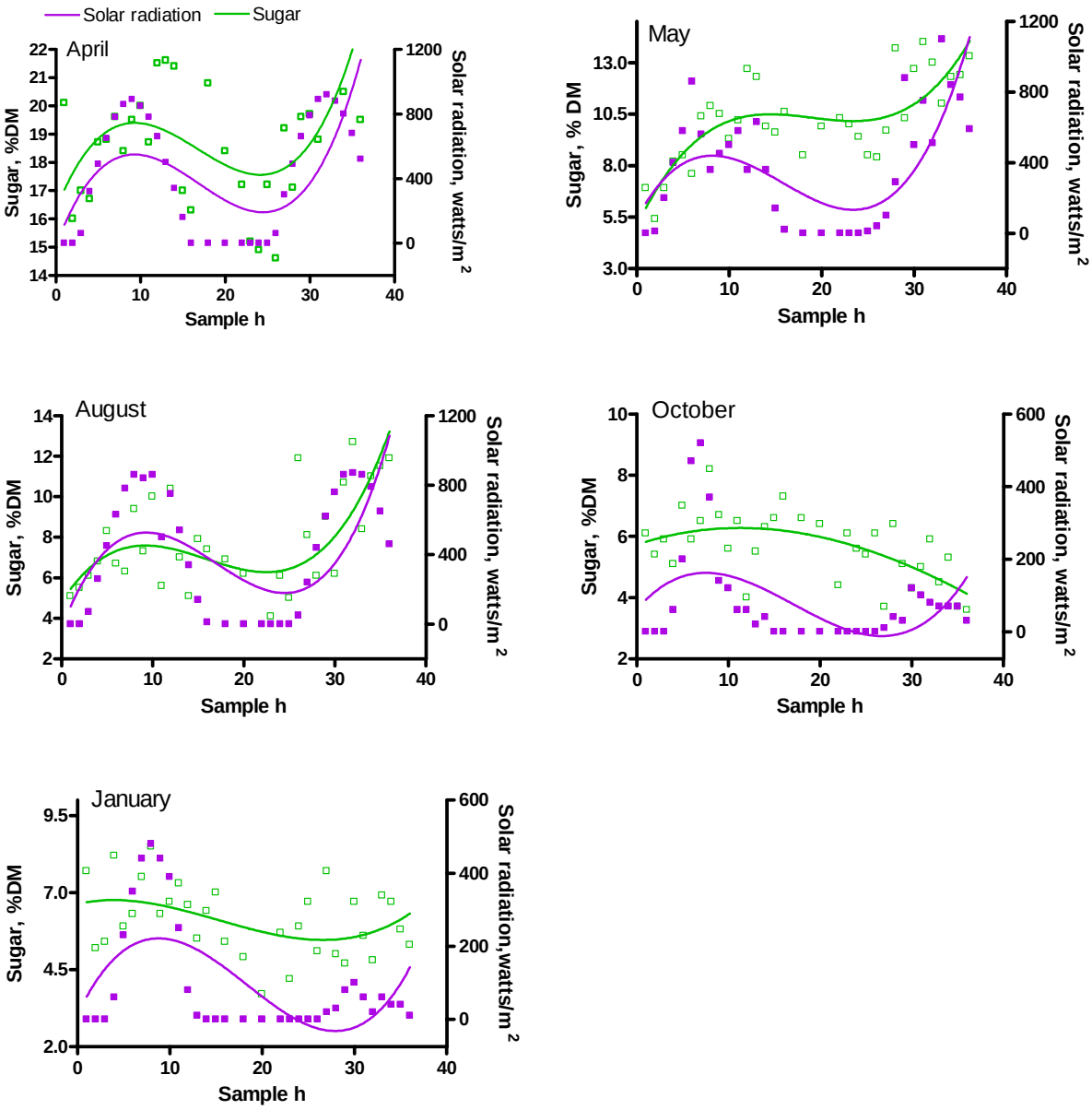

Figure A.10. Pair-wise comparisons of pasture forage sugar (% DM) (LAB1) and solar radiation (watts/m^2) (n=258).

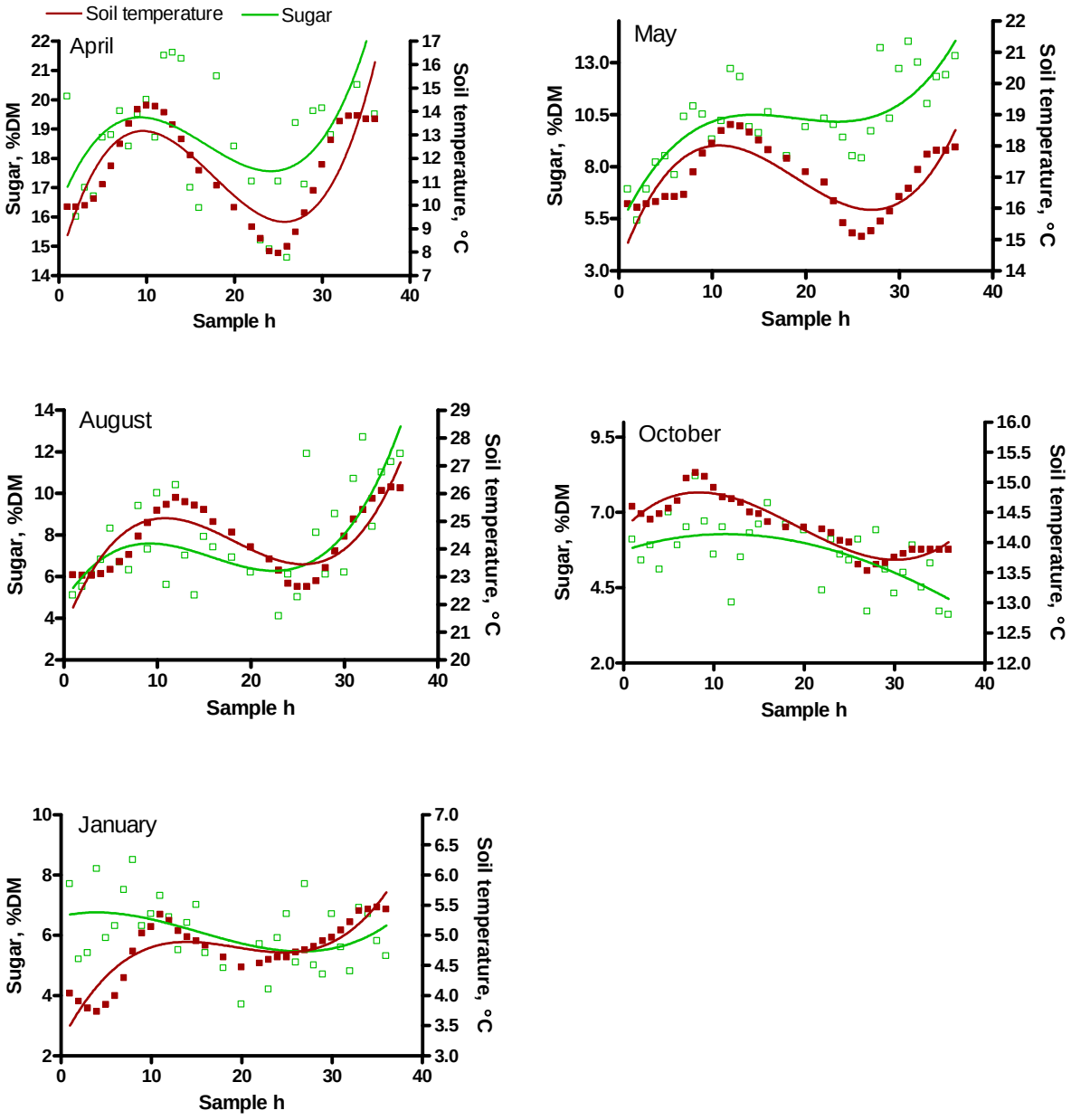

Figure A.11. Pair-wise comparisons of pasture forage sugar (% DM) (LAB1) and soil temperature (ºC) (n = 258).

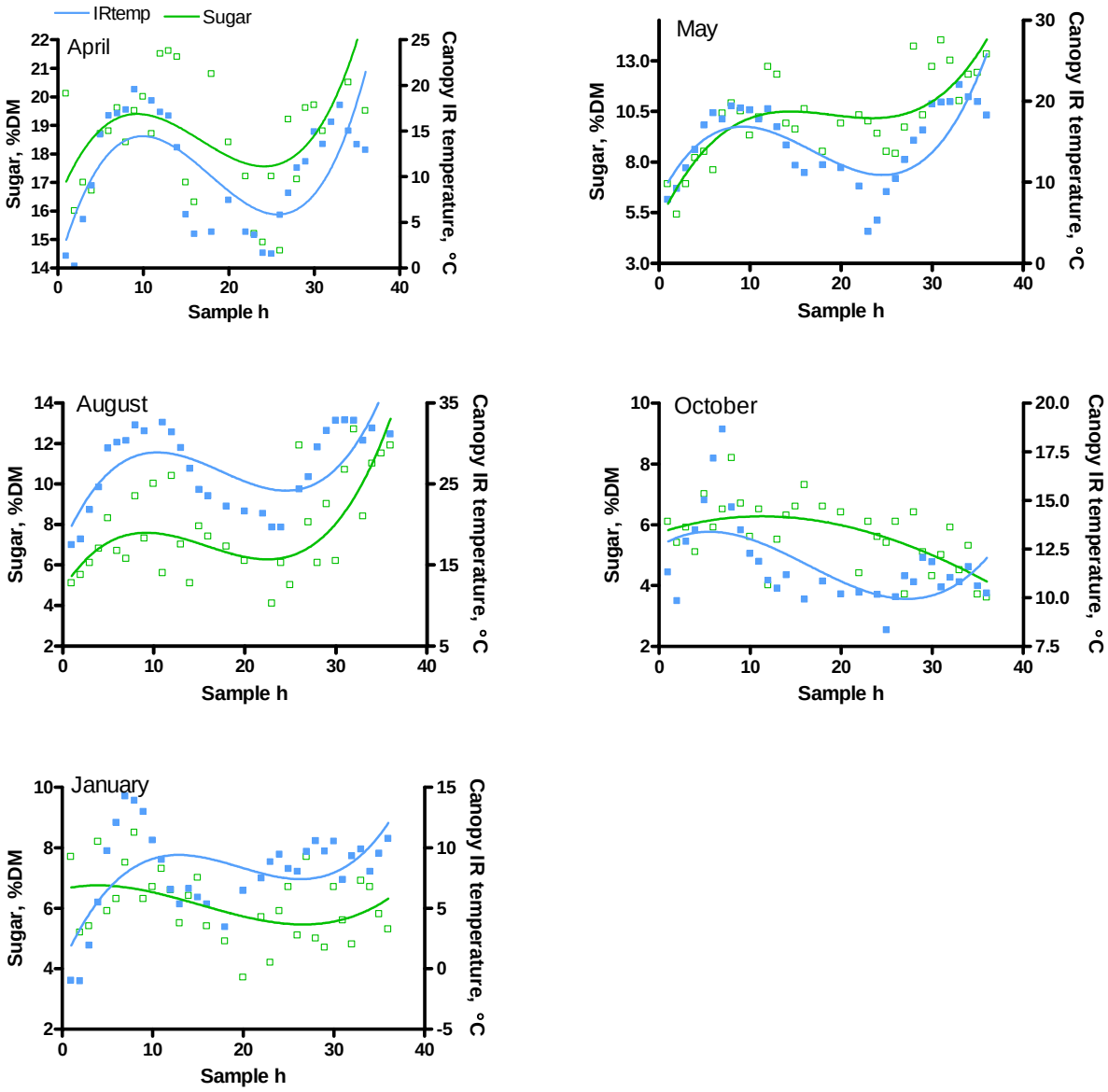

Figure A.12. Pair-wise comparisons of pasture forage sugar (% DM) (LAB1) and canopy infrared (IR) temperature (ºC) (n=258).

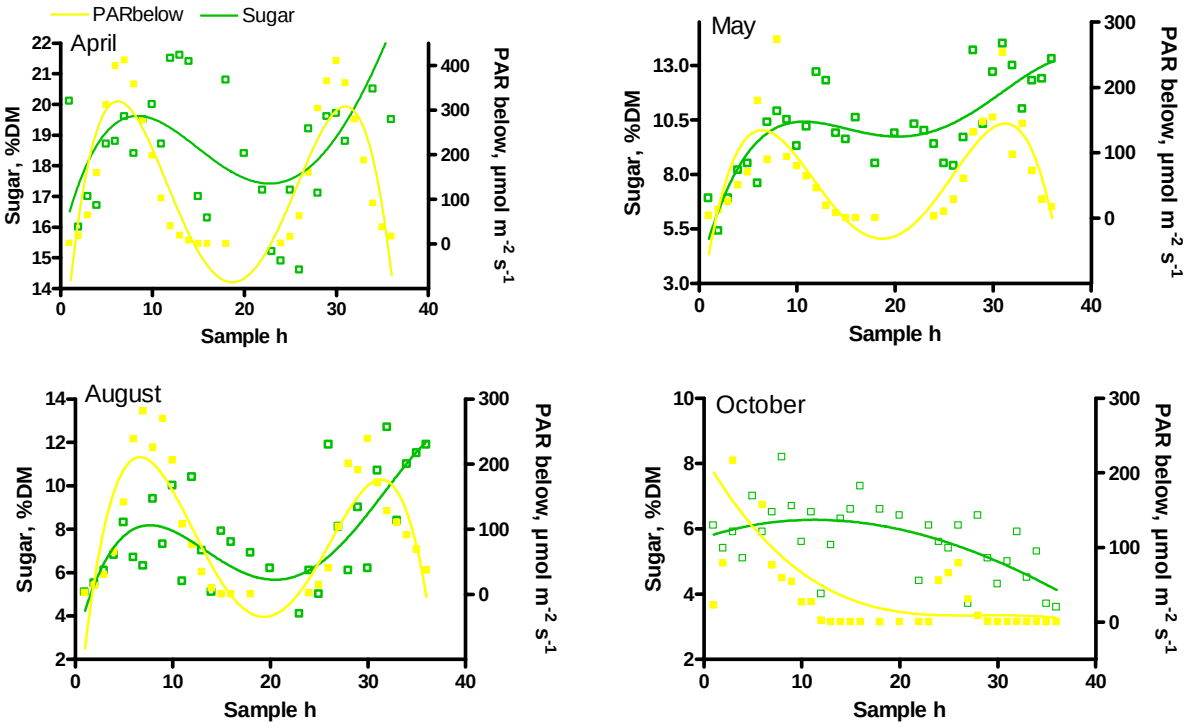

Figure A.13. Pair-wise comparisons of pasture forage sugar (% DM) (LAB1) and photosynthetically active radiation (PAR) below the canopy (µmol m^{-2} s^{-1}) (n=258).

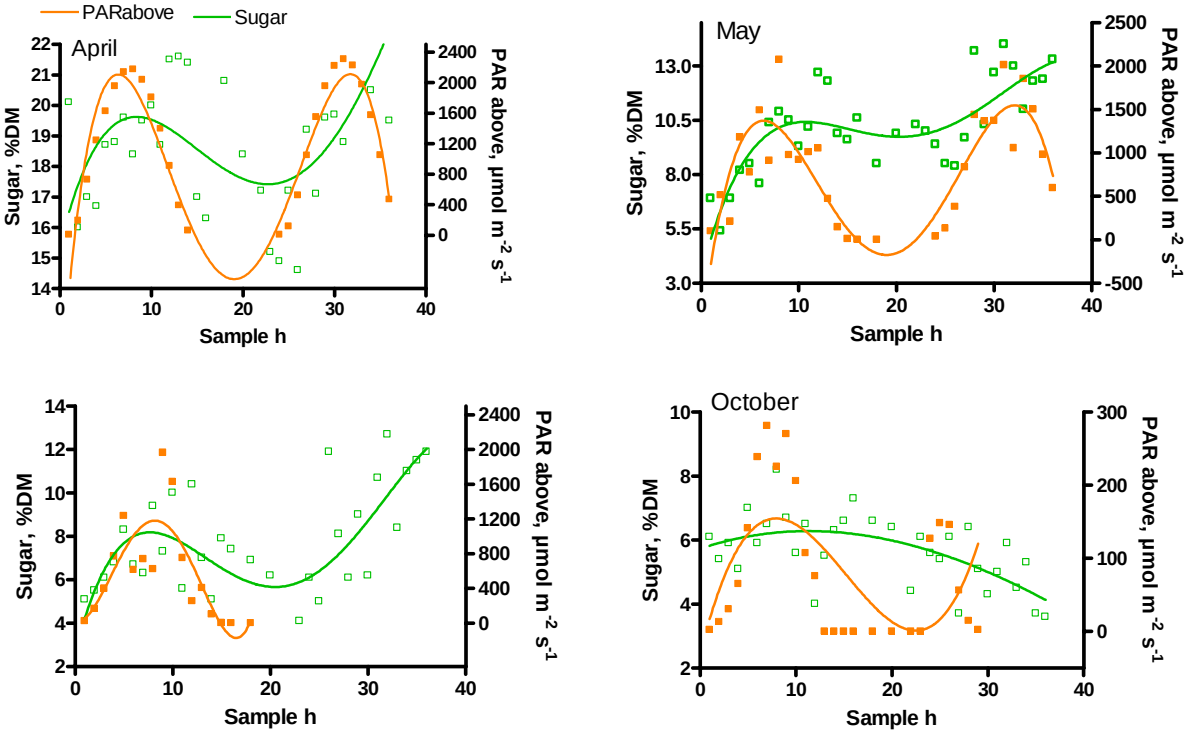

Figure A.14. Pair-wise comparisons of pasture forage sugar (% DM) (LAB1) and photosynthetically active radiation (PAR) above the canopy (µmol m^{-2} s^{-1}) (n=258 for all months except August where n = 21). (No day two data for August and no data available for January 2007.)

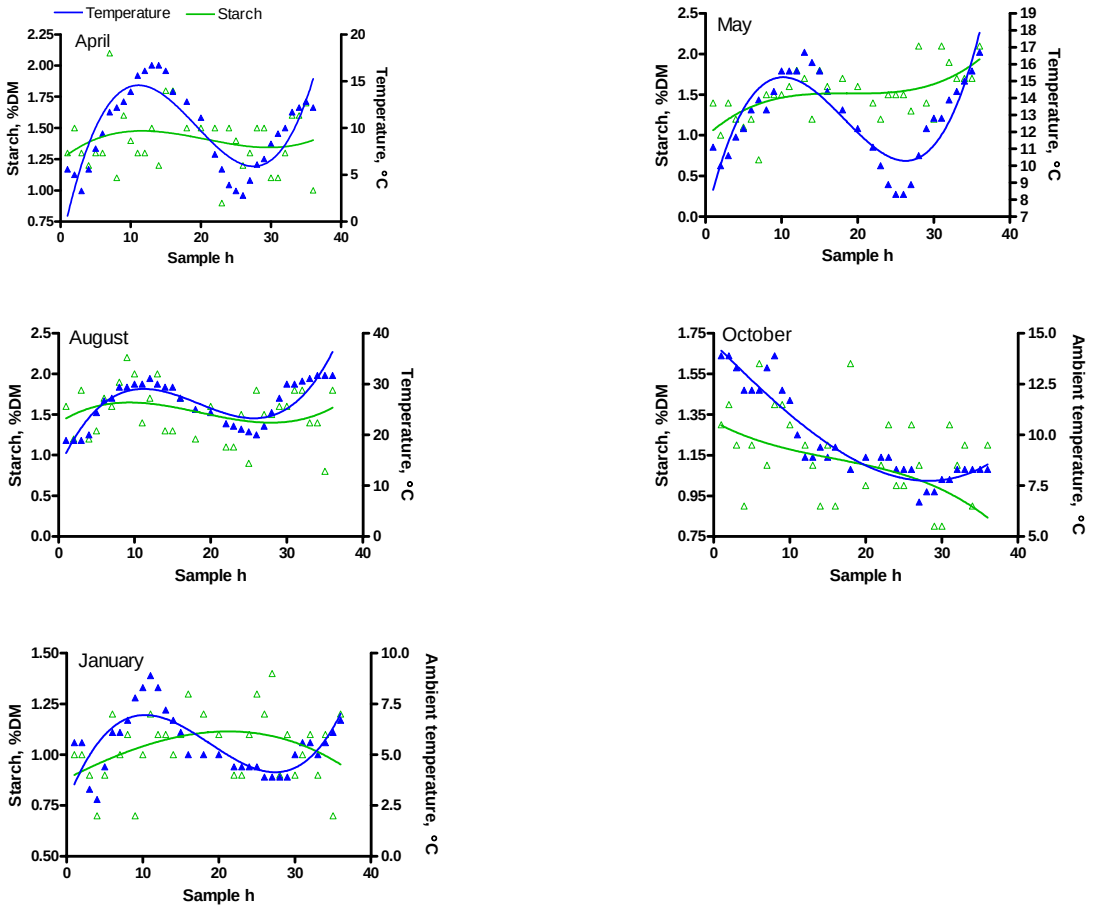

Figure A.15. Pair-wise comparisons of pasture forage starch (% DM) (LAB1) and ambient temperature (ºC) (n=258).

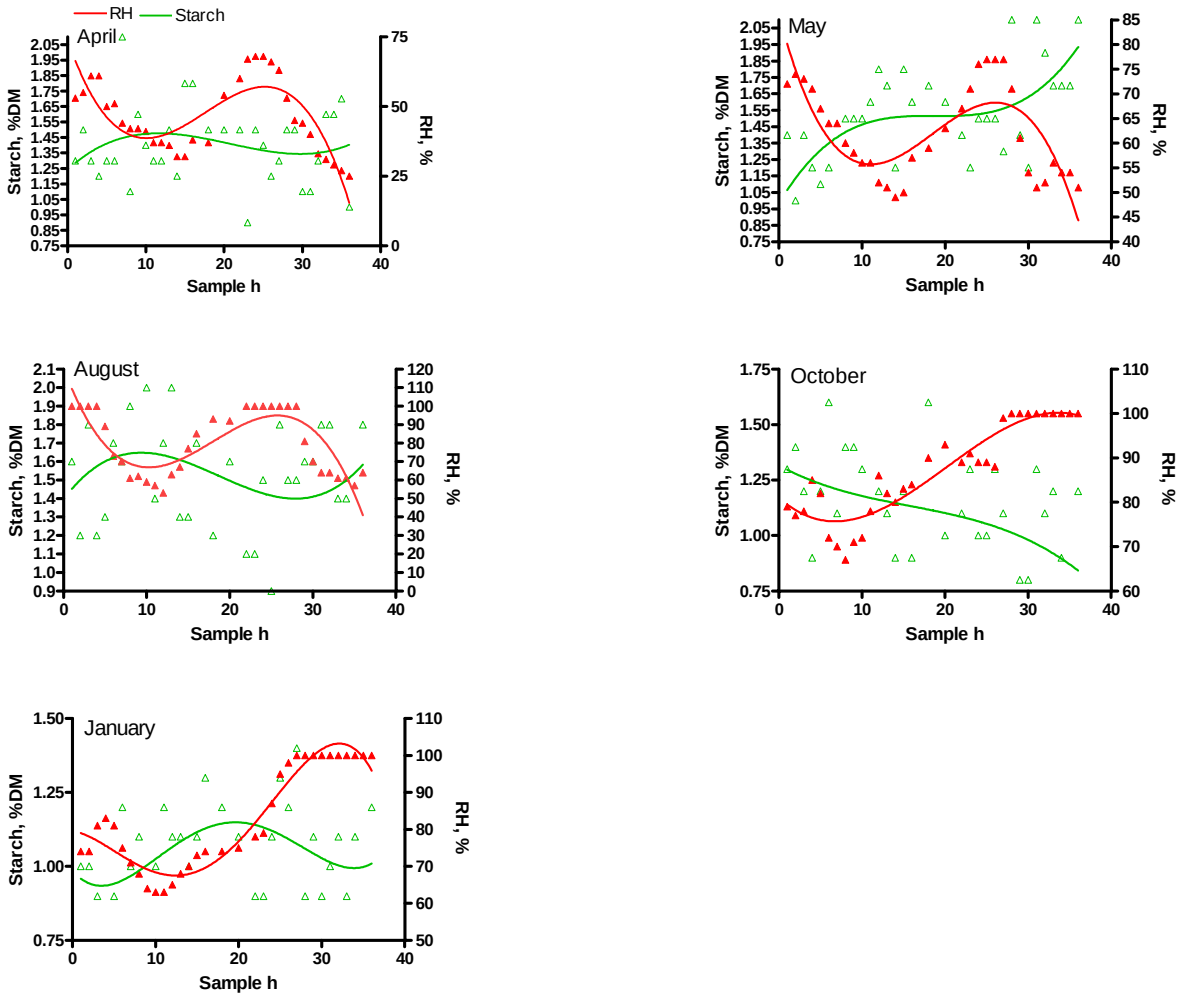

Figure A.16. Pair-wise comparisons of pasture forage starch (% DM) (LAB1) and relative humidity (RH, %) (n= 258).

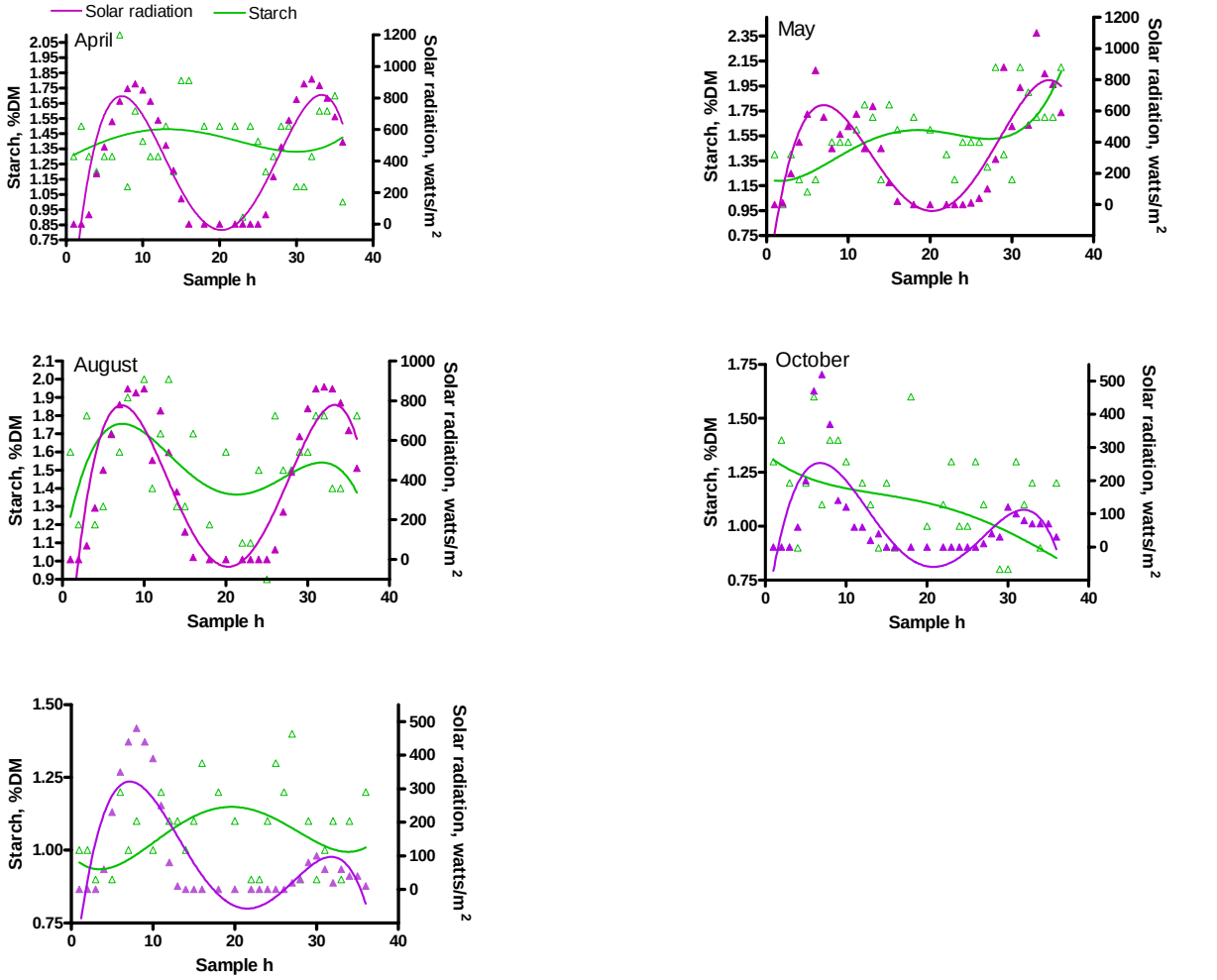

Figure A.17. Pair-wise comparisons of pasture forage starch (% DM) (LAB1) and solar radiation (watts/m^2) (n=258).

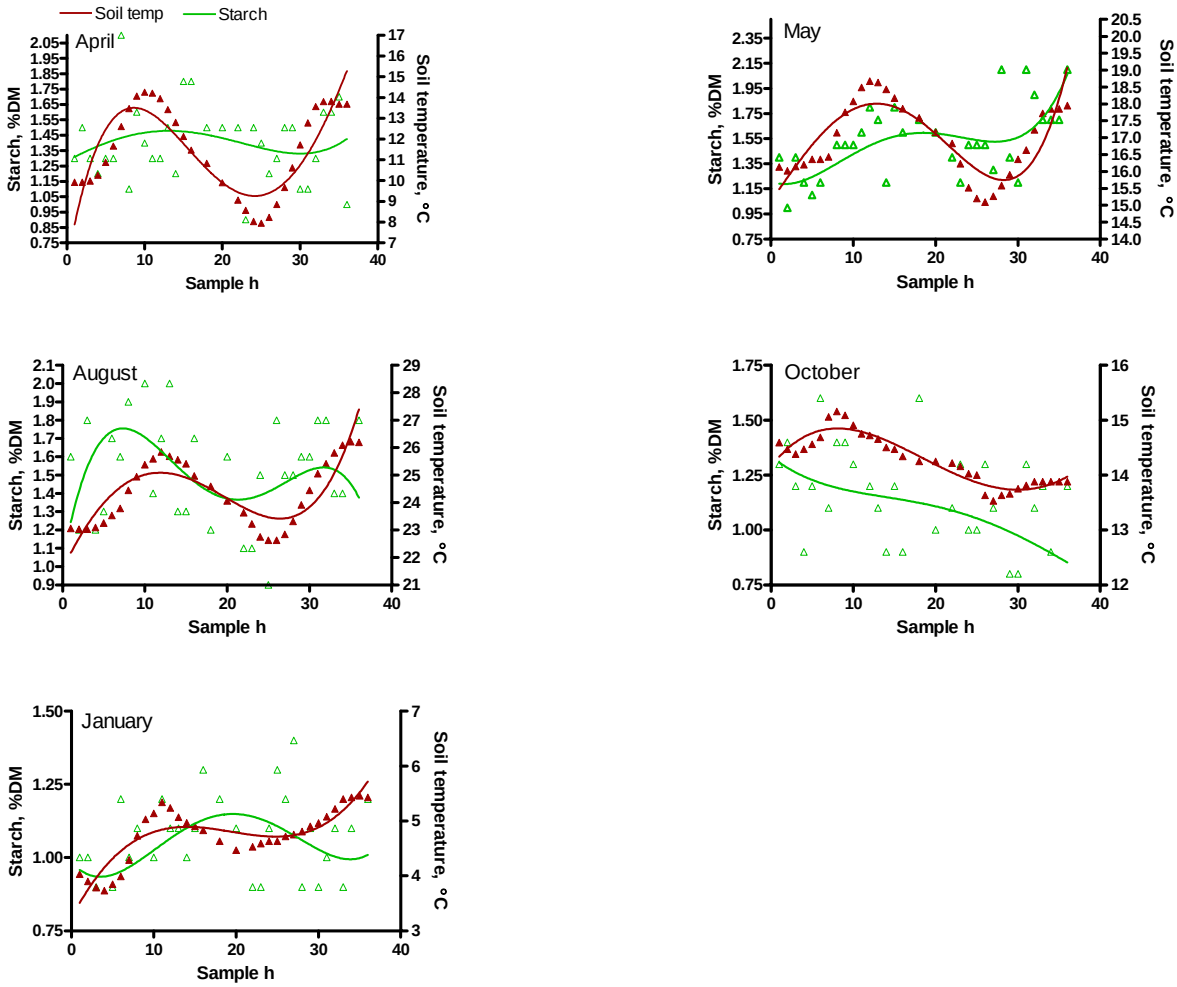

Figure A.18. Pair-wise comparisons of pasture forage starch (% DM) (LAB1) and soil temperature (ºC) (n = 258).

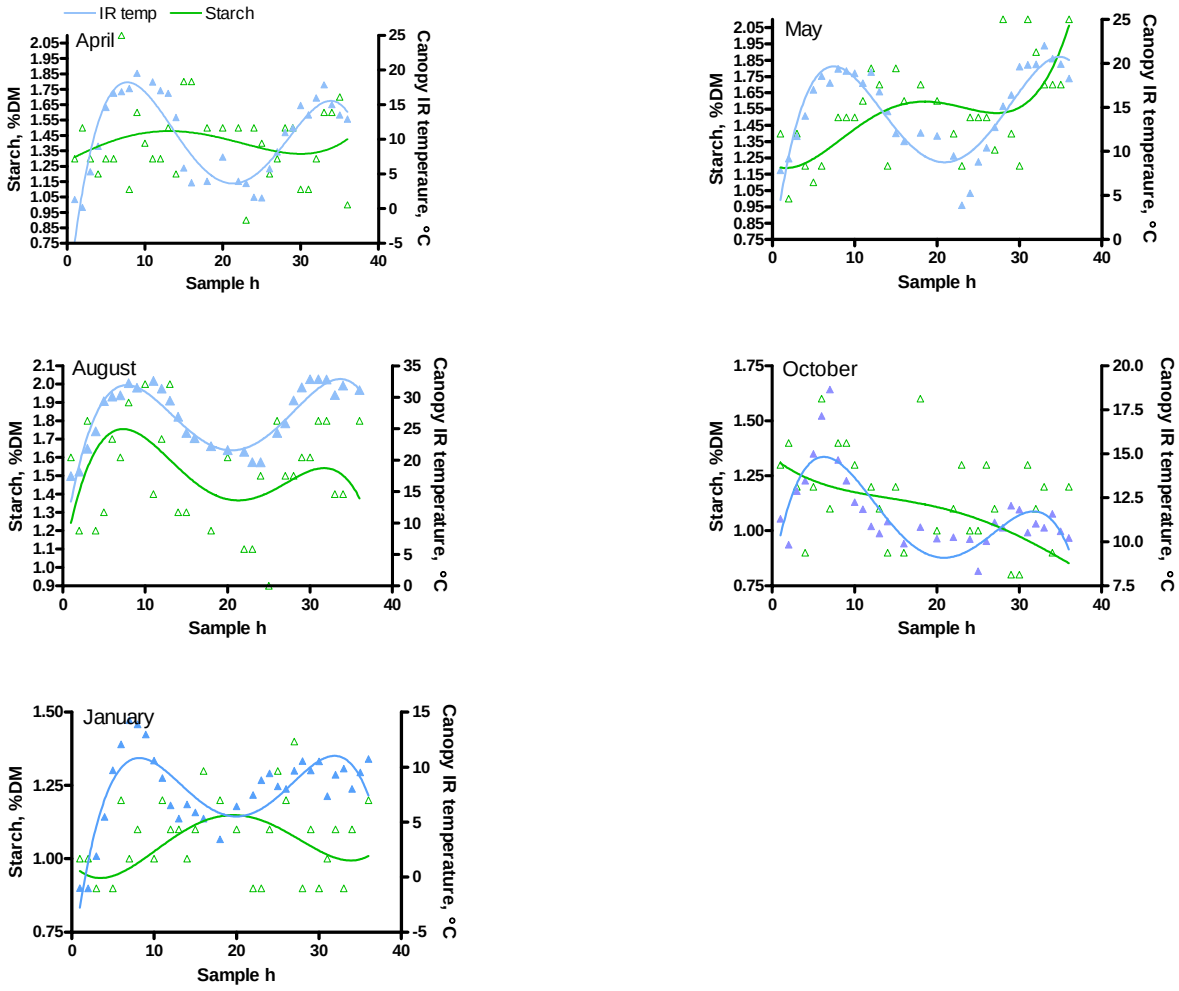

Figure A.19. Pair-wise comparisons of pasture forage starch (% DM) (LAB1) and canopy infrared (IR) temperature (ºC) (n=258).

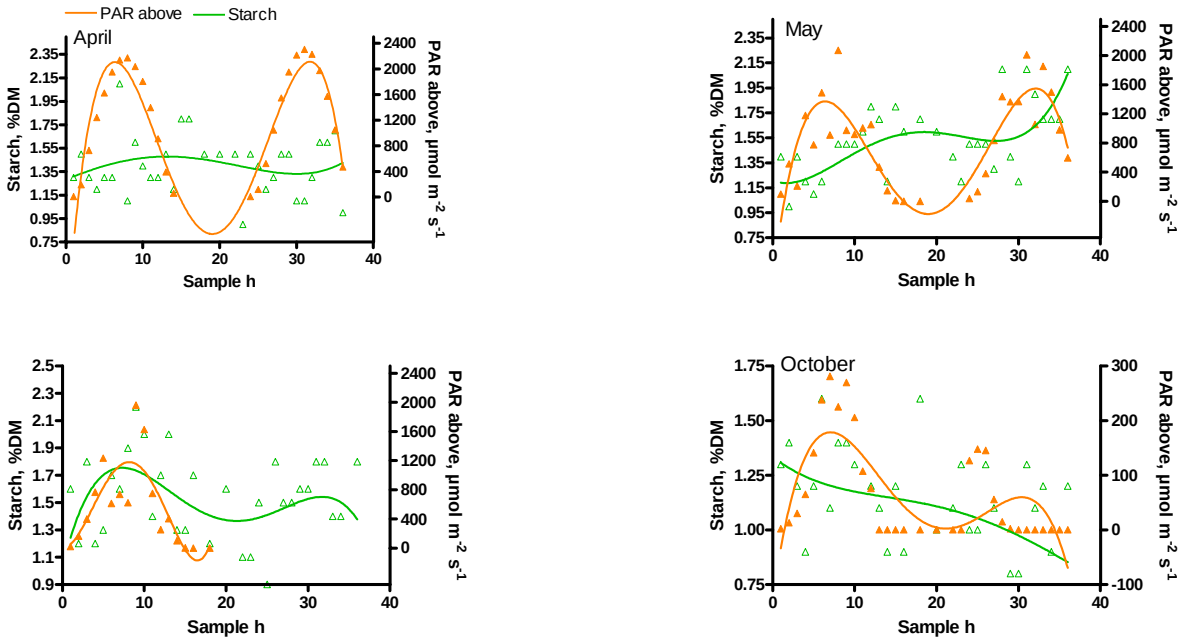

Figure A.20. Pair-wise comparisons of pasture forage starch (% DM) (LAB1) and photosynthetically active radiation (PAR) above the canopy (µmol m^{-2} s^{-1}) (n=258 for all months except August where n = 21). (No day two data for August and no data available for January 2007.)

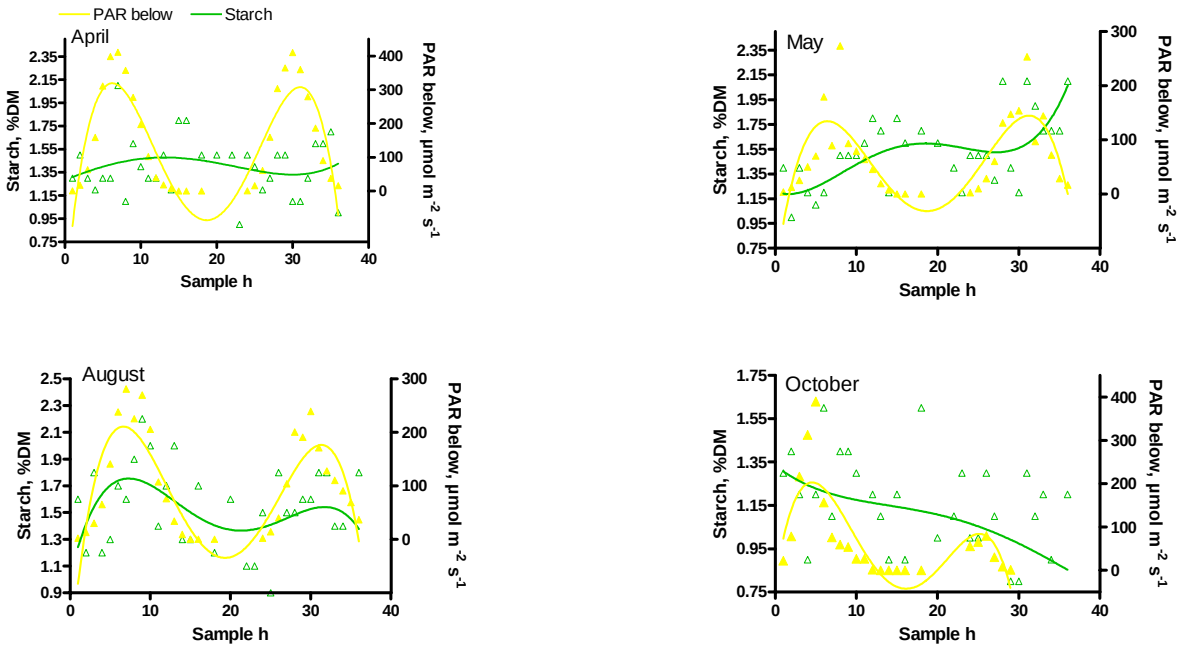

Figure A.21. Pair-wise comparisons of pasture forage starch (% DM) (LAB1) and photosynthetically active radiation (PAR) below the canopy (µmol m^{-2} s^{-1}) (n=258).

APPENDIX II

Validation of the chemiluminescent assay for plasma insulin

The chemiluminescent assay for plasma insulin concentration in horses was validated at the MARE Center by Staniar et al. 2006 (unpublished data). This insulin assay utilizes mouse monoclonal anti-insulin alkaline phosphatase conjugate and paramagnetic particles coated with mouse monoclonal anti-insulin antibody. A chemiluminescent substrate, Lumi-Phos 530, was added to the reaction vessel, and light generated by the reaction was measured with a luminometer. The photon production is proportional to the amount of conjugate bound to the solid support. The interassay CV for this insulin assay was 5.2 % and intraassay CV was 1.5 %. Accuracy and precision were examined in a line of identity plot (Figure 1) of insulin measured in 55 samples (range, 1.9 to 267 mIU/L) by a previously validated radioimmunoassay (Coat-A-Count Insulin, Diagnostic Products Corp., Los Angeles, CA) and the current chemiluminescent immunoassay.

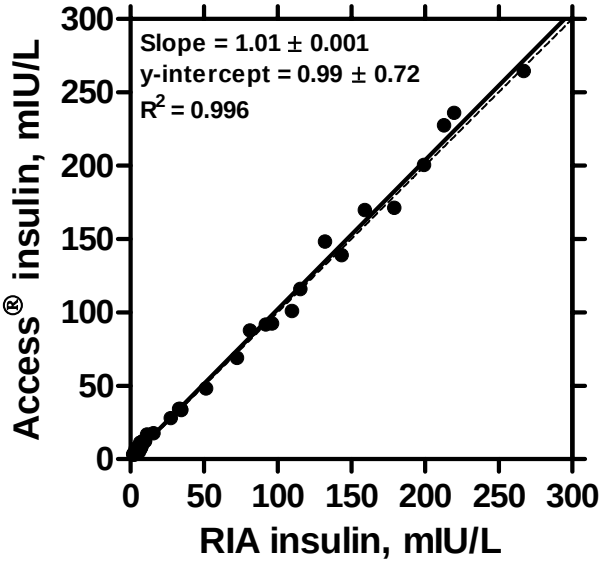

Figure A.22. Line of identity plot for the validation of the insulin chemiluminescent immunoassay using the Access® Ultrasensitive Insulin, Beckman Coulter, Brea, CA. The dotted line represents the line of identity and the solid line and equation represent the linear regression fit to the data. (Staniar et al, 2006, unpublished data).

APPENDIX III
Tables of individual horse values for proxies

Table A.1. Basal and peak glucose (mg/dL) values for all horses during each monthly trial.

	April 2005		May 2005		August 2005		October 2005		January 2006	
Horse	Basal	Peak	Basal	Peak	Basal	Peak	Basal	Peak	Basal	Peak
1	141.8	151.1	121.3	131.3	95.4	110.5	98.1	101.4	98.3	108.6
2	110.4	121.4	117.8	119.6	96.2	112.2	95.8	103.1	99.7	104.5
3	126.1	133.1	107.8	115.8	94.9	102.5	89.1	97.6	90.8	97.0
4	114.9	129.9	115.0	132.5	128.2	159.2	94.1	103.4	94.4	101.2
5	104.5	121.5	106.1	113.9	91.4	104.1	87.9	99.2	96.4	101.0
6	111.3	131.3	112.0	125.2	90.92	100.1	105.1	121.4	101.7	112.3
7	103.2	114.6	108.5	120.1	89.3	103.9	86.5	93.7	97.6	101.5
8	105.6	118.8	101.2	117.0	90.6	102.3	86.3	95.6	95.8	106.6
9	108.4	125.2	105.5	116.1	91.9	113.0	90.6	94.8	99.5	107.2
10	108.2	124.0	112.7	124.9	94.7	112.8	93.5	98.9	97.1	105.4
11	109.7	120.5	115.6	126.4	97.1	105.9	95.6	100.6	99.9	103.8
12	99.33	108.8	114.9	135.7	93.7	102.7	90.7	102.7	92.2	98.84
13	113.1	125.2	106.7	132.2	93.7	99.7	94.3	107.5	95.7	116.1
14	106.1	121.1	99.11	113.9	100.1	108.6	99.8	118.6	113.3	123.3

Table A.2. Basal and peak insulin (uIU/mL) values for all horses during each monthly trial.

	April 2005		May 2005		August 2005		October 2005		January 2006	
Horse	Basal	Peak	Basal	Peak	Basal	Peak	Basal	Peak	Basal	Peak
1	101.0	241.0	39.5	60.32	15.4	51.5	16.3	27.1	12.2	36.0
2	54.7	143.8	35.0	47.2	10.4	38.1	12.9	35.8	12.7	20.6
3	46.5	113.7	19.3	31.0	10.5	19.3	7.9	27.6	11.7	23.3
4	54	118	29.7	47.1	14.8	31.5	8.0	18.5	11.9	24.9
5	28.2	80.4	16.6	33.5	6.6	20.1	11.6	23.9	11.5	18.9
6	21.4	56.7	12.9	23.3	6.6	15.6	9.9	27.7	6.9	11.7
7	18.2	52.1	16.8	25.3	4.6	18.0	6.5	12.4	7.2	13.4
8	22.8	63.2	13.4	28.8	6.5	12.8	7.6	14.9	6.6	12.8
9	30.3	73.4	16.5	34.4	8.5	22	10.3	22.8	11.2	24.3
10	17.9	33.5	14.1	23.3	5.1	13.4	6.9	13.3	7.2	11.8
11	11.03	20.6	17.2	26.3	9.7	13.9	9.7	19.2	6.7	15.2
12	4.54	11.2	8.4	14.8	8.0	12.6	3.8	20.0	2.9	6.9
13	8.84	22.4	10.4	28.1	6.9	9.1	4.5	16.9	7.4	15.2
14	13.59	32.9	21.6	37.6	16.2	21.8	12.3	38.4	14.5	28.0

Table A.3. Proxies for RISQI ($[mU/L]^{-0.5}$). and MIRG ($mU_{insulin}^2/[10 \cdot L \cdot mg_{glucose}]$) for individual horses during each monthly trial.

	April 2005		May 2005		August 2005		October 2005		January 2006	
Horse	RISQI	MIRG	RISQI	MIRG	RISQI	MIRG	RISQI	MIRG	RISQI	MIRG
1	0.10	0.18	0.16	8.4	0.25	6.7	0.25	6.7	0.29	5.4
2	0.14	9.9	0.17	8.3	0.31	5.0	0.28	5.9	0.28	5.5
3	0.15	8.3	0.23	6.6	0.31	5.1	0.35	4.6	0.29	5.9
4	0.14	9.4	0.18	8.0	0.26	4.4	0.35	4.2	0.29	5.7
5	0.19	8.8	0.25	6.1	0.39	3.8	0.29	6.2	0.29	5.4
6	0.22	6.8	0.28	4.7	0.39	3.8	0.32	4.2	0.38	3.4
7	0.23	6.8	0.24	6.0	0.47	3.1	0.39	4.1	0.37	3.7
8	0.21	7.6	0.27	5.6	0.39	3.8	0.36	4.6	0.39	3.6
9	0.18	8.7	0.25	6.1	0.34	4.6	0.31	5.4	0.30	5.0
10	0.23	6.3	0.27	5.0	0.44	3.0	0.38	3.8	0.37	3.7
11	0.30	4.3	0.24	5.6	0.32	4.7	0.32	4.7	0.39	3.4
12	0.47	2.6	0.34	3.3	0.35	4.3	0.52	2.6	0.59	2.1
13	0.34	3.5	0.31	4.3	0.38	3.8	0.47	2.8	0.37	3.9
14	0.27	5.3	0.21	8.1	0.25	6.5	0.29	5.3	0.26	5.1

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