

Fasting length and hay type effects on metabolic parameters in the horse

by

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Abstract

Several metabolic disorders in the horse have been associated with post-prandial fluctuations of blood insulin and glucose concentrations in response to the ingredient and/or chemical composition of feedstuffs consumed. The objectives of this study were to 1) quantify changes in plasma glucose, insulin and cortisol concentrations in response to different fasting intervals, and 2) evaluate the effects of consumption of warm-season and cool-season hays on those same parameters. Six mature geldings were utilized in a 2×3 factorial design experiment in which they were fed either warm-season (WS) bermudagrass hay (*Cynodon dactylon*) or cool-season (CS) tall fescue hay (*Lolium arundinaceum*) during each fasting treatment. The three fasting treatments consisted of no fast (NF) in which the horses were offered hay ad libitum hay throughout the night, a short fast (SF) in which the animals were offered hay until 2200 h with hay re-introduced at 0600 h, and a long fast (LF) in which the animals were offered hay until 2200 h and not re-offered hay until 0700 h. All horses received concentrate and additional hay at 0700 h. Following a 6-d adaptation period, on d 7, a 7-h serial blood draw was conducted in conjunction with the morning feeding. Area under the curve (AUC) was calculated by the trapezoidal method for plasma glucose, insulin and cortisol. Glucose, insulin and cortisol AUC were evaluated using PROC GLM. No significant differences in glucose, insulin or cortisol AUC were detected due to hay type, fasting length, or the interactions between the two. Peak concentration and time to peak concentration of metabolites were also analyzed

using PROC GLM, with no significant differences for glucose. There was a significant difference for time to peak insulin concentration between WS-LF and both the WS-NF ($P = 0.024$) and WS-SF ($P = 0.003$) groups. There were no differences among treatments in peak insulin concentration. There were no differences among treatments for cortisol peak values, but CS-SF and CS-LF differed ($P = 0.04$) for time to peak cortisol concentration. Individual differences were observed for cortisol and insulin for both AUC and peak concentrations. There was also a significant difference for time to peak and peak glucose concentrations between animals.

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List of Abbreviations

ACTH	Adrenocorticotropin
ADF	Acid detergent fiber
ATP	Adenosine triphosphate
AUC	Area under the curve
BCS	Body condition score
BW	Body weight
C	Celsius
CAM	Crassulacean acid metabolism
cm	Centimeter
CO ₂	Carbon dioxide
CP	Crude protein
CS	Cool season
d	Day
DE	Digestible energy
dL	Deciliter
DM	Dry matter
ECD	Equine Cushing's Disease
EGAD	Equine Grain Associated Disorders

EMS	Equine Metabolic Syndrome
FAO	Food and Agricultural Organization of the United Nations
G3P	Glyceraldehyde-3-phosphate
GI	Glycemic Index
GLUT	Glucose transporters
HCl	Hydrochloric acid
h	Hour
kg	Kilogram
L	Liters
LF	Long fast
mg	Milligram
min	Minutes
mL	Milliliter
mm	Millimeter
mo	Month
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NDF	Neutral detergent fiber
NF	No fast
NRC	National Research Council
NSC	Non-structural carbohydrates
PEPC	Phosphoenolpyruvate carboxylase
pg	Picogram

Rubisco	Ribulose – 1,5 – bisphosphate carboxylase/oxygenase
RuBP	Ribulose - 1,5-bisphosphate
SC	Structural carbohydrates
SDW	Structural dry weight
SF	Short fast
SGLT	Sodium-glucose co-transporters
TNC	Total non-structural carbohydrates
VFA	Volatile fatty acids
WHO	World Health Organization
wk	Week
WS	Warm season
yr	Year
μIU	Micro International Unit

Introduction

As equine nutrition evolves, there has been an emergence of commercial concentrate formulations. As these new concentrates become more readily available and convenient to use, many horse owners have placed more value on the concentrate portion of the diet, whereas the forage portion has become devalued. The change in the carbohydrate profile has led to many research undertakings to evaluate the concentrate portion, while not properly evaluating the forage component. Though the newer diets may be beneficial to some animals, for horses predisposed to metabolic illnesses, these diets may lead to the development of disease.

The type of concentrate is not the only practice in feedings of equids that has evolved. The actual feeding schedule itself has been changed from that of a normal grazing animal to one fed in several large portions throughout the day. Though the change in feeding practices accommodates a busy human lifestyle, it may not be the healthiest decision for the physiology of the horse.

In nature, horses can spend up to 65% of their time foraging (Waring, 1983). Due to the small stomach size of the horse (capacity of 8 to 15 L), the horse is best suited for eating almost continuously throughout the day. The small intestine is the main absorption site of soluble carbohydrates and functions as that of other domesticated animals (Hintz et al., 1971). Once forage passes into the hindgut, fermentation occurs, yielding VFA.

The main VFA produced are acetate, butyrate and propionate, which are used as a secondary supply for the energy needs of the horse.

This digestive strategy is optimized by the horse having a constant source of forage, which is rarely the case with equine management systems. Management systems have integrated daily fasting periods, with the horses receiving a pre-planned amount of forage and grain in a single feeding. These feedings may come once, twice, or three times daily, depending on the management style. These large allowances of feed lead to drastic changes in certain metabolic parameters multiple times daily.

Both fresh and stored forages contribute sugar and starch from the non-structural portion of the plant. Warm- and cool-season grasses are among the most commonly used forages in the equine diet. Both of these grasses contain simple sugars and starch; however, fructans are also present in cool-season grasses. Fructans are produced in cool-season grass and stored as an energy source, which are then utilized as needed when photosynthetic activity declines.

In the horse, blood concentrations of glucose and insulin are emerging as candidates in the development of many equine-related illnesses such as laminitis, equine metabolic syndrome and Cushing's disease. The emergence and subsequent understanding of these equine diseases has led to the further evaluation of equine feedstuffs and the role they play in altering equine metabolism.

To fully understand the physiological changes caused by diet, sugar, starch and fructan components must be examined to ascertain whether alterations in concentrations of these dietary carbohydrates influence the development of metabolic illness in predisposed animals. In conjunction with feed type, management styles also must be evaluated to examine the effects they have on the increase and decrease of the major blood-borne parameters including glucose, insulin and cortisol.

Literature Review

Horse Nutrition

To fully understand nutritional research in the horse, it is important to evaluate historical feeding practices compared with feeding practices of today. These changes over time have led to progress and also to new challenges in the horse nutrition industry. The following section describes feeding practices, the physiology of the digestive tract, and rate of passage through the equine digestive system.

Feeding Practices

The digestive system of the horse is designed for handling multiple small meals throughout the day. Feral horses are known to spend up to 65% of their time grazing on low-quality forage (Waring, 1983). They have been observed to eat as many as 17 small meals throughout the day (Laut et al., 1984). Through domestication, humans have altered the horse's natural eating pattern in order to make it more convenient to their daily lifestyle. This adaptation includes 2 to 3 feedings a day with restricted grazing time. For some horses, this type of management may lead to the gradual development of a host of metabolic problems.

Current feeding protocols revolve around a diet of hay and grain-based products, usually commercially formulated. These diets can sometimes be greater in caloric density than what is needed for an idle horse. Some owners misunderstand the amount of energy their horse needs and, when combined with lack of exercise, this leads to an increase in the prevalence of metabolic problems due to excessive caloric intake and reduced caloric expenditure. Some horses are more affected by this change in diet than others, which increases the risk of developing metabolic disease.

Digestive Physiology

To understand the risk of developing metabolic disease, the digestive physiology must be understood. The horse is classified as a hindgut fermenter with an approximate digestive system length of 30.5 m. Horse saliva is predominately water-based, with a pH varying between 8.6 and 9.1 (Meyer et al., 1985) and a very low amount of digestive enzymes (Varlout et al., 2006) in relation to their body size. Horses have a very small stomach capacity of approximately 8 to 15 L, or about 8% of the total digestive tract volume. This very small stomach enables a relatively small amount of digesta to be retained in the stomach for a short period of time in order to be broken down by HCl (Andrews et al., 1992). Bacteria may also play a role; however, to what extent is debatable (Varlout et al., 2007; de Fombelle et al., 2003). As the stomach becomes distended, pressure moves the digesta to the small intestine.

The small intestine is the main absorption site of soluble carbohydrates, and in this regard is similar in function to the small intestine of other domesticated animals (Hintz et al., 1971). The small intestine is a large proportion of the total digestive tract length, averaging 25 m in length. It comprises the duodenum, jejunum and ileum. Compared with other animals, the production of amylase, which breaks down amylose, is low (Kienzle and Radicke, 1993), but amylase production has the ability to be altered if concentrate in the diet is increased. Other factors that influence pre-ileal digestion include the source of starch, the processing of starch, amount of starch intake, the source and preparation of the roughage, and the individual horse (Meyer et al., 1993).

The next step of the process is hindgut digestion, which occurs in the cecum and the large intestine. In the hindgut, digesta is fermented by a mixture of protozoa, fungi and bacteria (Julliand, 1992). The hindgut length is relatively shorter than the small intestine at approximately 8 m, however, the capacity of the cecum is around 33 L, and the capacity of the large intestine is approximately 80 (Nickel et al., 1973) to 90 L (Pagan, 1990). The large intestine in the horse is 60%, whereas in the ruminant it is only 9% of total tract capacity (Pagan, 1990). However, the rumen is 33% more efficient in fermentation of substrate than the hindgut in the horse (Hintz, 1969). Though not as efficient as the rumen, the hindgut still produces VFA through microbial fermentation of NSC and SC. The VFA produced in the hindgut are an important source of energy for the horse by either being absorbed and used directly by cells, or sent to the liver for gluconeogenesis. The most common VFA produced in the hindgut are acetate, propionate

and butyrate, generally in a 70:20:10 ratio, respectively (Bergman, 1990). The addition of concentrate to the diet increases the production of propionate while having an inverse effect on the production of acetate (Hintz et al., 1971). The opposite is true when more forage is added to the diet (Mackie and Wilkins, 1988). Though the hindgut of the equid is not as efficient as the rumen, one study with ponies revealed that 30% of the DE intake was due to VFA production in the cecum (Glinsky et al., 1976). The horse's digestive system is constructed so that, if properly functioning, VFA generated from a diet of hay/concentrate mixture can produce 45 to 82% of total absorbed energy (Vermorel et al., 1997).

The complexity of the equine digestive tract is just one factor. The digesta must be moved through the digestive system at the correct rate in order for proper digestion to occur.

Rate of Passage

It is desirable for digesta to remain in the digestive tract for a greater amount of time, as it leads to an increase in the digestion and subsequent absorption of nutrients. However, timely movement of ingesta is imperative because slow movement can cause constipation leading to life-threatening conditions such as colic, whereas rapid movement minimizes absorption and results in poor nutrient uptake. Multiple factors play a role in

the rate at which feed passes through the digestive tract, including diet, water intake and exercise. Depending on digesta composition, particles are retained in different sections of the digestive tract for variable amounts of time.

Metayer et al. (2004) found that the amount of starch and quantity of diet played a role in how fast solid-phase gastric emptying occurred. These researchers assigned horses to a small-meal, low-starch diet; a small-meal, high-starch diet; or a large-meal, high-starch diet. All horses were rotated through each diet. The small meal with high starch emptied faster than the large meal with high starch. Fastest to empty, though, was the small meal with low starch. They concluded that larger meal size and a higher concentration of starch slowed gastric emptying rates on a percentage basis. Van Weyenberg et al. (2006) reported a transit time of only 2 to 6 h for the majority of gastric contents to be passed from the stomach to the small intestine. However, lack of feed (i.e., fasting) causes decreased digesta movement, which slows peristaltic motion and prevents the stomach from emptying completely. Fasting periods mechanistically slow the rate of passage due to the lack of distention of the stomach, which controls digesta movement from the stomach to the small intestine. Once digesta is moved from the stomach to the first section of the small intestine, the duodenum, transit rate increases to 30 cm/min (Van Weyenberg et al., 2006).

Digesta then passes from the small intestine into the cecum where fermentation occurs. Liquids and fine particles are diverted to the right dorsal colon, while the coarse

particles are separated and retained in the cecum, ventral colon and pelvic flexure (Drogoul et al., 2000). The cecum and ventral colon retain the digesta for the greatest amount of time. Undigested material can spend as much as 85% of total retention time in the large intestine (Wilfried, 1993). The digesta retention time may also increase with smaller particle size in the large intestine (Van Weyenberg et al., 2006).

Several non-dietary factors may influence MRT in the digestive tract. Light-breed horses have a shorter retention time compared with draft mares (Miraglia et al., 1992). Exercise may also affect MRT of digesta in the horse. Duren et al. (1992) reported a shorter MRT leading to decreased nutrient absorption because of decreased intestinal blood perfusion during exercise bouts in fasted ponies. Pagan et al. (1998) studied the movement of particulate markers in exercised or non-exercised Thoroughbred horses consuming forage only or a mixed grain/forage diet. The researchers observed that the forage diet had a more rapid rate of passage than the mixed grain/forage diet. These two diets were isoenergetic, leading to a greater DM intake of the forage diet. The researchers theorized that horses on the all-forage diet had increased water consumption and increased saliva output, both which may have played a role in the increased rate of passage. Exercise caused an additional increase in the intake of water, which in turn decreased retention time of the digesta in both exercised groups.

In certain sections of the GIT, alterations to MRT may occur due to feed particle size. Decreasing particle size by pelleting vs. chopping has been shown to increase

retention time of digesta, especially in the colon (Drogoul et al., 2000). Van Weyenberg et al., (2006) stated that when particle size was smaller, more water was absorbed, creating a more homogenous digesta leading to simultaneous solid and liquid progression through the digestive tract, slowing the rate of passage and allowing more time for absorption of nutrients. Taking into account those factors, MRT can range from 18 to 60 h (Van Weyenberg et al., 2006). During digesta transit, blood parameters also fluctuate and need to be evaluated.

Insulin, Glucose and Cortisol

Greater availability of commercially formulated equine concentrates has led to the horse often being fed a diet that meets or exceeds its nutritional needs for the physical demands it may face. However, with changes to the diet, adaptations may occur within the digestive system and, in turn, new problems may arise.

Many chronic illnesses and diseases in equids are related to malnutrition. Problems such as insulin resistance (Kronfeld et al., 2005), Cushing's disease (Beech and Garcia, 1985; Garcia and Beech, 1986), and laminitis (Pass et al., 1998; Pollitt and Davies, 1998) have all been associated with nutrition. Nutritional illnesses are so varied that Kronfeld (2003) described three different categories: Equine Syndrome X, EMS and EGAD. Equine Syndrome X is a grouping of metabolic disorders resulting from long-

term consumption of sweetened concentrates, which primarily consist of grains and molasses. Equine Metabolic Syndrome is characterized by mild lameness in mature, obese horses, usually in conjunction with elevated blood insulin levels, as first described by Johnson et al. (2002). A grouping of digestive disturbances involving rapid fermentation makes up the EGAD category. These problems combined are among the leading causes of death in horses. The commonality of the nutritionally caused illness has led to additional research primarily focused on understanding and altering the diet to minimize metabolic problems.

Insulin and Glucose

Carbohydrates are digested and absorbed in two main areas of the horse's GIT. Glucose and other simple sugars are absorbed in the small intestine following digestion of NSC. Volatile fatty acids are produced from the microbial fermentation of SC in the hindgut. Total tract disappearance of starch has been reported to exceed 90% in the horse (NRC, 2007).

The small intestine of the horse has the ability to break down α 1→4 and α 1→6 glycosidic linkages in starch via hydrolysis by amylase. Energy from glucose absorbed in the small intestine is more readily available than from VFA produced in the large intestine. When glucose is absorbed from the small intestine, it enters the blood stream

and is used largely for maintenance of body organs and tissues. In order for this to happen, glucose is moved from the brush border using SGLT1. The amount of SGLT1 can be modified through diet, which may be a key factor in glucose-related illnesses.

Dyer et al. (2009) investigated the expression of SGLT1 in horses fed various carbohydrate diets ranging from $< 1.0 \text{ g} \cdot \text{kg BW}^{-1} \cdot \text{day}^{-1}$ starch to $6.0 \text{ g} \cdot \text{kg BW}^{-1} \cdot \text{day}^{-1}$ starch. During the first period of their experiment, all horses were maintained on an all-forage diet for 3 mo. The second and third diets were fed for 1 mo each and consisted of 60% hay/40% grain and 40% hay/60% grain diet, respectively. Biopsies of the small intestine were taken during each period. After the switch to the high-grain diet, the expression of SGLT1 was 2 times greater in the jejunum and 3 to 5 times greater in the ileum, which showed that changes in diet can alter how glucose is transported in the small intestine of the horse.

For glucose to enter the blood stream and tissues, GLUT must be present to assist with glucose uptake. More than thirteen types of GLUT have been discovered and defined to date. Glucose is transported across the basolateral membrane via GLUT2 (Shirazi-Beechey, 1995), whereas, GLUT4 facilitates glucose uptake by skeletal and cardiac muscles with help from available insulin (Shepherd and Kahn, 1999; Gorovits and Charron, 2003). As blood glucose concentrations increase following ingestion of a meal, insulin concentrations should likewise increase. As insulin concentration increases, it facilitates translocation of GLUT4 to the cell surface, thereby allowing glucose uptake

into the cell. Manso Filho et al. (2007) also found GLUT4 played a key role in the uptake of glucose in the adipose tissue in the horse. Normal fasting blood concentrations in the horse range from 60 to 90 mg/dL and < 5 to 20 μ IU/mL for glucose and insulin, respectively (Ralston, 2002). Glucose and insulin concentrations increased from baseline values following ingestion of feed, peaked at different times depending on the physiological state of the equid and diet fed, and returned to their pre-prandial concentrations within 5 to 6 h following meal intake. In horses determined to be metabolically normal, fasting concentrations of blood glucose and insulin should be consistent; however, abnormal readings for more than 12 h may indicate a metabolic problem. Ralston (2002) advised that horses having concentrations greater than 250 mg/dL and 200 μ IU/mL for glucose and insulin, respectively, should be further investigated for metabolic illnesses.

Cortisol

The study of cortisol in relation to diet is one of the novel concepts being examined in equine nutrition research. Many questions are still being explored to establish the connection and mechanism of action of cortisol in relation to nutrient metabolism. In the early stages of cortisol research, it was thought that cortisol was a diurnally fluctuating hormone (James et al., 1970); however those theories have since been reconsidered. Also, cortisol in the horse is naturally released in pulses throughout

the day (Toutain et al., 1988). Multiple studies have proposed theories about the relationship of cortisol to metabolism, specifically in relation to glucose and insulin. There are divergent theories, including two on the manner by which diet influences cortisol and subsequently equine metabolism.

The first theory postulates that cortisol levels change post-prandially and in turn are affected by insulin and glucose. Glade et al. (1984) reported cortisol concentrations declined in weanlings after ingestion of meals providing either 80% or 160% of the recommendations for DE and CP (NRC, 1978). Conversely, Depew et al. (1994) observed an increase in cortisol concentration after ingestion of a meal, though the second phase of the same study showed deprivation or addition of feed had no effect on cortisol concentration. Cartmill et al. (2005) also reported that cortisol concentration was not affected by meals.

In humans, studies are being conducted to establish whether there is a relationship between diet and cortisol fluctuations. Van Cauter et al. (1992) tested the effects of the interval between meals in relation to glucose, insulin, cortisol and C-Peptide concentrations in male and female subjects. Meals were identical in composition (43% carbohydrate, 39% protein and 18% fat) and in calories, and were split equally into either 5 or 3 allotments for 6- and 12- h fast groups, respectively. Evening glucose AUC was greater than morning concentrations, irrespective of the interval between meals. However, the offering of food led to an increase in cortisol concentration. Insulin

concentrations increased from the morning to the evening meals, while cortisol declined during this period. Insulin concentration was greater in the evening, but there were no significant differences in maximum post-prandial insulin concentrations between morning and evening values. An inverse relationship was observed between insulin and cortisol concentrations from morning until evening, leading researchers to conclude that cortisol may partially moderate insulin response (Van Cauter et al., 1992).

The second hypothesis states that cortisol in horses is secreted in rhythmic patterns and may or may not be affected by environmental factors. DePew et al. (1994) found an increase in cortisol concentrations throughout the day, with the peak occurring in the afternoon hours followed by a decline until the nadir around 0800 h. Storer et al. (2007) found cortisol concentrations were greatest in the early morning hours and declined until approximately 2300 h.

There are also other factors that must also be taken into consideration when evaluating a hormone known for being influenced by external factors. Gender does not seem to play a role in cortisol concentrations or fluctuations (DePew et al., 1994). Housing environments also have been evaluated to ascertain whether they affected cortisol concentrations in horses. Irvine and Alexander (1994) evaluated cortisol concentrations in four different populations of horses maintained in different housing environments. The four treatments consisted of pasture-housed mares that were handled only during blood collection, mares confined to stalls, mares placed outdoors but not on

pasture, and 4 horses which were stabled and raced daily (Irvine and Alexander, 1994). Each of the four treatment groups had a specific blood draw schedule with varying lengths of time between blood collections. Horses in the undisturbed pasture group followed the circadian rhythm pattern, with greatest concentrations of cortisol during the dawn hours, followed by a decline throughout the day. The raced horses had a significant decline in cortisol throughout the duration of the serial blood draw, which returned to normal by the following morning. No patterns in circadian rhythm were found in horses in the other two treatment groups. The authors implied horses in an undisturbed atmosphere followed a circadian rhythm cortisol pattern; however, when external factors were added, stressors disrupted their normal patterns. The authors also implied the racing group had the same rhythmic blood cortisol concentrations patterns as observed in the undisturbed horses, which they suggested was due to adaptations from training or by the ability to adapt to constant change (Irvine and Alexander, 1994).

Storer et al. (2007) evaluated housing facilities as well in three groups. The three feeding and housing groups were ad libitum pasture, paddocked horses with ad libitum access to native grass hay, and stalled horses fed a hay and pelleted diet once daily. There were no differences in cortisol concentrations dependent on housing. A more recent study showed that adaptations to being isolated and stalled could affect the cortisol pattern in young Quarter Horses (Garey et al., 2010). Stull and Rodiek (2000) found certain stressful situations such as transportation for extended periods of time also increased cortisol concentrations.

Management

Management practices of horses may also alter the normal AUC pattern of glucose, insulin and cortisol. How long feed is withheld and the diet that is chosen need to be monitored to make sure they do not promote the onset of metabolic illness.

Fasting Length

Withholding feed, even for short periods of time, can alter the metabolism of a horse. Horses, as grazing animals, are not designed to have extended periods without feed. Nadal et al. (1997) conducted two studies on insulin and glucose concentrations of feed-deprived horses. In the first study, stallions were fed one of the following six diets on treatment days following a 21-h fast: (1) no feed; (2) pelleted diet meeting 82.5 % of the horse's CP requirement (NRC, 1989); (3) restricted intake with the pelleted diet fed at 25% of the amount offered to the previous group; (4) the pelleted diet and ad libitum access to water; (5) cracked corn at the same weight as the pelleted diet; (6) chopped alfalfa at the same weight as the pelleted diet. Water was withheld from all stallions on blood collection days with the exception of the group in which water was part of the treatment. Following blood collection, required maintenance amounts of feed and water were offered. Greatest insulin concentrations were observed in horses fed the pelleted diet with ad libitum access to water. Plasma glucose and insulin concentrations did not

increase when feed was withheld or when alfalfa was fed; however, an increase in plasma glucose and insulin concentrations was noted in the four other treatment groups. In the second experiment, horses were assigned to either a fasted or control treatment group. Horses in the fasted group had feed withdrawn 72 h prior to blood collection, whereas horses in the non-fasted control group were fed until 16.5 h prior to the blood collection. Blood was taken in 15-min increments beginning 30 min pre-prandial and continuing until 30 min after a meal of pelleted feed was offered at 0.3% of BW. Blood was then collected in 30-min increments for the final 270 min. A treatment \times time interaction occurred, and glucose increased in both groups post-feeding. Insulin concentrations increased similarly, and there were no significant differences between the control horses and the horses on the 72-h fast (Nadal et al., 1997).

Depew et al. (1994) conducted two experiments on the effects of feed deprivation on multiple blood parameters. The first consisted of a 19-h fast in which blood was collected for the final 4 h of the fast and continued 8 h after the introduction of a meal at 1200 h. The diet was formulated to meet 82.5% and 27.5% of the CP requirements (NRC, 1989) by pelleted concentrate and bermudagrass hay, respectively. The second experiment examined stallions which were either given or deprived of their normal noon meals. In the first experiment, glucose, insulin, glucagon and cortisol concentrations all increased after the horses were given their meal at 1200 h. The second experiment revealed post-prandial increases in prolactin, insulin, glucagon and glucose concentrations compared with pre-prandial concentrations. Cortisol concentrations did

not differ following feeding in the second experiment, indicating that cortisol concentration may not be coupled with changes in glucose and insulin.

In a study with donkeys, cortisol concentrations were evaluated in response to fasting (Forhead and Dobson, 1997). Six donkeys were subjected to 15- and 74-h fast. In each fasting period, the donkeys were given either saline or insulin 0.5 h prior to the serial blood draw. Blood was collected at varying intervals for the first hour followed by 0.5-h intervals for the following 7 h. The donkeys were then alternated and followed the same protocol either 2 d later or 1 mo later for the 15- and 74-h fast groups, respectively. Donkeys fasted overnight became hypoglycemic sooner than those fasted 74 h. Cortisol concentrations increased in 3 donkeys in the 15-h fast group after insulin was infused. Cortisol concentration was also significantly higher in the 72-h fast group than the 15-h fast. The increase in cortisol in both the 15-h and 72-h fast groups may indicate an insulin or glucose interaction with cortisol (Forhead and Dobson, 1997). However, the stress of the fast alone could play a role in increased cortisol concentrations.

Diet Effects

It is well known that diet affects the endocrine profile in the horse (Stull and Rodiek 1988; Powell et al., 2000; Depew et al., 1994). In humans, the GI has been defined as the incremental area under the blood glucose response curve of a 50-g

carbohydrate portion of a test food, expressed as a percent of the response to the same amount of carbohydrate from a standard food taken by the same subject (FAO/WHO, 1998). The GI technique has been adapted for application to different species. In human studies, the standard food is white bread, whereas oats commonly are used in equine studies. Though GI research in the horse is fairly recent, researchers have designed studies to produce a more comprehensive list and ranking of feedstuffs capable of altering blood glucose concentrations. The general horse-owning population currently may not be familiar with the GI concept and its application, but it may become more popular as the incidence of metabolic illnesses continues to increase. Some factors which play a role in determining GI values include meal size, proportion of hydrolysable carbohydrates, concentrations of fat and fiber, processing and digestibility of feedstuffs, rate of intake and rate of absorption in the GIT (Hoffman et al., 2003a). These factors make it difficult to assign an accurate GI for many feedstuffs. Though the use of the GI is fairly new in the horse, experimentation on dietary factors that alter blood parameters is not.

Hintz et al. (1971) investigated blood parameters and VFA concentrations in ponies fed diets of differing forage-grain ratios. The three diets included 1:0, 3:2 and 1:4 forage-to-grain ratios to provide 160 kcal of DE/kg^{0.75} of BW. The results failed to show a difference in blood glucose concentrations based on ration composition, but concentration of VFA increased with the greater percentage of forage.

Stull and Rodiek (1988) investigated several common equine diets to assess their effects on the blood glucose and insulin concentrations in the horse. The four diets were 100% alfalfa, 50% corn and 50% alfalfa, 100% corn, and 90% corn with 10% corn oil added. The horses also had ad libitum access to water. These diets were created to be isoenergetic, meeting 25 % daily DE needs, and were fed for a single treatment period in a Latin Square design experiment, allotting a 1-wk adaptation period between each diet. Blood was collected starting at 0800 h with feed being introduced at 0900 h. The intervals between blood collections were 15 min for the first 5 h and then every 30 min of the final 4 h. The researchers found no significant differences among the diets when comparing glucose and cortisol concentrations, however, insulin concentration differed between diets.

Powell et al. (2000) formulated two diets having a 70:30 and 40:60 roughage-to-concentrate ratio, respectively, that were fed at either 100% (NRC, 1989) or 70% of horses' DE requirement for moderately intense work, yielding 4 treatment groups. Total daily diet allotment was divided into three equally sized meals. Each diet was fed for an 8-d adaptation period followed by a feeding test consisting of 1.0 kg oats on d 9. Blood samples were collected pre-prandial, 30 min post-prandial and hourly for 7 h following the test meal. Horses consuming the restricted-intake, high-forage diet had increased glucose ($P < 0.05$) and insulin ($P < 0.01$) concentrations compared with the other three treatment groups. These findings supported the concept that a metabolic shift may have

occurred due to restricted feeding practices, and imply that the horse may be able to metabolically adapt following feed restriction.

Hoffman et al. (2003b) divided horses into three groups based on BCS: non-obese, moderately obese and obese. These horses, which had previously been adapted to a forage only diet, then were given two treatment supplements in a switchback design. A supplement that was fat and fiber-based or sugar and starch-based was fed in a 2:3 supplement: forage ratio in 2 meals for a total of 6.2 kg/d supplement per horse. The sugar and starch supplement had a greater GI than the fat and fiber diet. Horses in the non-obese group were more insulin-sensitive than obese animals. The researchers proposed that when horses become obese, they physiologically shift away from insulin-mediated glucose uptake and rely more on glucose-mediated glucose disposal (Hoffman et al., 2003b). Their results suggested that horses should not be fed a diet rich in starch and sugar and kept at a moderate body condition in order to minimize the chances of insulin resistance.

Storer et al. (2007) divided horses into three different housing locations for feeding and evaluated the effects on glucose and insulin. Researchers chose three protocols that are frequently used by horse owners, including pasture with ad libitum access to grass, a dry lot with ad libitum access to hay, and stalled with a once-daily hay and concentrate feeding. Each animal underwent a 5-d acclimation followed by 36-h blood collection period. Greater plasma glucose and insulin concentrations were observed

in pastured horses than in the other two treatment groups, and the most notable spikes in insulin and glucose concentrations were recorded in stalled horses following their single meal. The hay-only treatment group had no significant change in their blood glucose or insulin concentrations, and maintained the lowest and least variable glucose and insulin concentrations compared with the other treatment groups.

Not only the feed components, but also the form in which feedstuffs are offered, needs to be evaluated for effects on blood glucose concentrations. When a feed form is changed by processing, the digestibility of some nutrients may be changed. However, Andrew et al. (2006) evaluated feeding a single diet consisting of the grain and hay portions fed separately or both components in a pellet, slowing the rate of grain intake while increasing the ingestion rate of the forage portion and found no differences in blood glucose AUC between diets.

Metabolic Diseases and Related Conditions

A strong link has been found between endocrine hormones and the onset or progression of certain diseases and metabolic conditions in horses. Because certain feeding programs and diet compositions can cause long-term modifications of metabolic hormones released, diet has been implicated in the development of insulin resistance,

feed-associated laminitis and EMS. These illnesses are among the leading causes of metabolic-related horse death in the equine industry.

Insulin Resistance

Kahn (1978) stated that insulin resistance exists when insulin does not elicit a normal physiological response. This definition was later more clearly refined to describe insulin resistance as insulin insensitivity at the cell surface which regulates glucose availability inside the cell, or from insulin ineffectiveness due to disruption of glucose metabolism in the cell (Kronfeld et al., 2005). Insulin resistance is a common factor in many other metabolic diseases in the equid.

Several factors have been proposed as contributing to the development of insulin resistance. The most cited factor is obesity in both horses (Johnson, 2002) and people (Ferrannini et al., 1997). In mice, obesity has led to a reduced amount of GLUT4 protein in white adipose tissue and skeletal muscle, leading to the prediction of insulin resistance later in life (de Carvalho Papa et al., 2002).

Laminitis

Though there are several causative factors, many cases of laminitis are related to equine nutrition or diet in one form or another. Pollitt and Davies (1998) reported vasodilatation in hooves of horses diagnosed as laminitis-positive, as well as an increase in hoof temperature 12 to 32 h following a wheat slurry carbohydrate overload. Even in horses not diagnosed as laminitis-positive, a large dose of fructan could start the hoof separation process (Van Eps and Pollitt, 2006).

Bailey et al. (2007) investigated non-obese ponies predisposed to laminitis compared with non-obese control ponies fed a high-fructan pasture diet vs. a low-fructan hay diet. The control ponies maintained normal insulin concentrations and sensitivity when fed either diet and showed no signs of metabolic problems. The laminitis-predisposed ponies had greater insulin concentrations while on the pasture diet, but insulin concentrations decreased over time as they adapted to the hay diet. The researchers concluded that ponies predisposed to laminitis should be kept on a low-fructan diet to minimize the chance of becoming insulin-resistant. In a following study, Bailey et al. (2008) compared blood glucose and insulin concentrations in laminitis-prone ponies and control ponies grazing in different seasons in England. Laminitis-prone ponies grazing during summer months had symptoms of insulin resistance and pre-laminitis. These symptoms were not seen in the winter grazing months. The control ponies did not

show symptoms of laminitis or insulin resistance in either the summer or the winter grazing periods.

Cushing's Disease

Equine Cushing's disease is defined as the overproduction of ACTH due to a tumor in the intermedia portion of the brain (Schott, 2002). Estimates show that more than 38% of horses that have ECD also have greater insulin and glucose concentrations compared with a healthy horse (Garcia and Beech, 1986). McGowan et al. (2008) also found that horses with ECD have an altered metabolism, as these horses have a diurnal insulin concentration peaking at 1200 h that could not be altered by treatment for ECD. A lack in suppression of plasma cortisol in ECD animals could be an indicator for the disease (Dybdal et al., 1994).

Equine Metabolic Syndrome

Equine Metabolic Syndrome comprises a combination of symptoms and conditions, including insulin resistance, obesity, cresty neck, and probable multi-limb laminitis (Johnson, 2002). Walsh et al. (2009) divided 25 horses and ponies in to a control group, those diagnosed with EMS by plasma insulin concentration greater than 70 μ IU/mL with normal ACTH concentrations, or horses clinically diagnosed with ECD

based on plasma ACTH concentrations greater than 70 pg/mL. In all horses, plasma concentrations of ACTH, cortisol, insulin and serum concentrations of glucose and thyroxin (T₄) were measured. Both the EMS and ECD horses had greater insulin concentrations and more severe cases of laminitis than the control group based on the Obel grade of laminitis, ranging from 1 to 4. Horses classified as ECD were given 1mg pergolide mesylate daily to verify if, and how rapidly, ACTH concentration would decline due to the medical treatment. Horses in the EMS group were placed on a diet and exercise program that resulted in a decline in insulin concentrations along with laminitis severity. Diet and increased exercise routine led to a reduction in plasma insulin concentration that in turn was associated with a 0.7 ± 1.6 decrease on the Obel laminitis score. Horses classified as ECD and given pergolide had a trend toward decline in ACTH concentrations; however the data set was not complete, as 2 ponies died before completion of the study.

Forage

The specific types and proportions of carbohydrates present in forages must be accounted for to accurately assess the impact forage has on the nutrition of the horse as a whole.

Plant Physiology

Photosynthesis is the process by which atmospheric CO₂ is assimilated into carbohydrate for storage and support of physiological processes (e.g., growth) in the plant. It is a multistep process of gluconeogenesis that comprises two main reactions, the light-dependent reactions and the light-independent, carbon-assimilation reactions. Energy captured by photosynthesis is used by the plant to create the structural and the non-structural components of the plant.

There are three categories of CO₂-fixation pathways in photosynthesis: C₃, C₄ and CAM. Though the C₃ and C₄ pathways share a common step in the photosynthetic process, they differ in the fact that C₃ plants fix 3 carbons through a one-step process, whereas C₄ plants fix carbons through a two-step process. Plants more commonly belong to the classification of C₃ as opposed to C₄ (Ehleringer and Cerling, 2002).

An appreciation of the leaf structure is necessary to understand the differences in the carbon fixation process. The outer layers of the leaf consist of epidermal cells, and stomata are pores located in the epidermal layer that control gas exchange in the leaf (Waller and Lewis, 1979). Mesophyll cells are located between the epidermal cells and are categorized as either palisade or spongy. Palisade mesophyll cells are dense and are located above the spongy mesophyll layer, which is loosely packed. The mesophyll cells contain chloroplasts that are membrane-enclosed organelles containing a gel membrane called stroma that holds the stacks of thylakoids, known as grana.

The mesophyll cells surround bundle sheath cells in two very specific patterns. In C3 plants, bundle sheath cells are surrounded by mesophyll cells in a loose arrangement; in contrast, C4 plants have tightly packed mesophyll cells surrounding the bundle sheath. The bundle sheath cells are tightly positioned around the vein, which includes both the xylem and the phloem.

Photosynthesis

In the light-dependent reactions of photosynthesis, light is absorbed by the chloroplasts, specifically by chlorophyll in thylakoids. Two photosystems are present; Photosystem II oxidizes water into oxygen and hydrogen, followed by Photosystem I that utilizes electrons from oxidation of water to reduce NADP to NADPH. The products manufactured by the photosystems are then used for the carbon-assimilation, or light-independent steps, of photosynthesis.

During the light-independent reactions, glucose is formed. The process starts by CO₂ entering the stoma and air space within the cell. Once CO₂ is present, it is used in one of two ways, depending on the plant type. In C3 plants, CO₂ is taken into the mesophyll cells and the Calvin cycle begins. The Calvin cycle is split into 3 separate phases. The first is the carbon-fixation stage. Atmospheric CO₂ and RuBP combine to form 3- phosphoglycerate by action of the enzyme Rubisco (Furbank and Taylor, 1995).

This first intermediate is a 3-carbon molecule, which gives rise to the C₃ nomenclature. 3-phosphoglycerate is phosphorylated by ATP from the light-dependent reactions to yield 1,3-BPG. The second product of the light reaction, NADPH, reduces 1,3-BPG, a three-carbon molecule. Phosphoglyceraldehyde is then moved out of the mesophyll cell and combines with the equivalent of another G3P to synthesize glucose that is transported by the vein to be used by the plant. In the third phase of the Calvin cycle, RuBP is regenerated in a series of reshuffling, isomerization and phosphorylation reactions to keep the Calvin cycle proceeding for the production of G3P for glucose.

The second class of plants, C₄ plants, have a different process by which they first fix CO₂ molecules. In the mesophyll cells, phosphoenolpyruvate is formed from pyruvate by the enzyme pyruvate phosphate dikinase. This reaction is coupled with ATP from the light-dependent reactions and inorganic phosphate to phosphorylate pyruvate and inorganic phosphate. A cytosolic reaction in the mesophyll cells of C₄ plants uses the enzyme PEPC to bind phosphoenolpyruvate with diffused CO₂ to form the 4-carbon molecule oxaloacetate. The oxaloacetate is then converted to malate to allow for the movement of the compound into the bundle sheath where the next steps of the process occur. Once in the bundle sheath, the CO₂ is released by decarboxylation of malate for use in the Calvin cycle, and the resulting pyruvic acid is returned to the mesophyll cell where it is phosphorylated to regenerate phosphoenolpyruvate (Furbank and Taylor, 1995). The CO₂ delivered to the bundle sheath cells can then enter the Calvin cycle in the same way as in the C₃ plants. In the extra steps needed for C₄ plants, the equivalent of 2

high-energy phosphate bonds are used to drive this process. Once glucose is formed it may then be used for either a SC or NSC component of the plant. Though the C4 photosynthesis process requires more ATP, the minimization of photorespiration allows more energy to be conserved and allows photosynthesis to continue. This, in turn, allows for more biomass production in C4 plants (Xin-Guang et al., 2008).

Warm- and cool-season grasses

Both C3 and C4 plants have to photosynthesize to produce carbohydrates for plant growth and storage. As the name implies, warm-season or C4 plants grow primarily in warmer climates, thriving in temperatures around 30°C. Examples of these grasses are bermudagrass (*Cynodon dactylon*) and bahiagrass (*Paspalum notatum*). Cool-season or C3 plants thrive in temperatures ranging from 18 to 26°C. Examples include fescue (*Festuca* and *Lolium* spp.) and timothy (*Phleum pratense*). However, temperature is only one of many variables that contribute to optimum photosynthesis. Others include, but are not limited to: atmospheric CO₂ concentration, hours of sunlight (i.e., photoperiod), water availability, soil fertility, harvest management, and fertilizers used. During daylight hours, production of sugars for SC and NSC are at their greatest, with peak productivity mid-afternoon and declining through the night hours (Longland and Byrd, 2006). During the night hours, cellular activity shifts from production to usage of carbohydrates through respiration. This in effect is the opposite process, which allows for the plant to break

down stored energy in order to sustain itself throughout hours where sunlight is not available.

Storage forms of carbohydrate differ somewhat between C3 and C4 plants. Both C3 and C4 plants contain soluble starches and sugars; however, cool-season grasses also contain fructans. Sugars are mono- or disaccharides, whereas starches are α -linked polysaccharides. Fructans, also known as fructooligosaccharides, are β -linked fructose residues connected to a terminal sucrose residue at what would normally be the reducing end of the polymer.

Mechanistically, there is a difference in the process of NSC accumulation and storage between C3 and C4 grasses. Grasses that are classified as C3 produce fructans in vacuoles that are membrane-bound organelles also found in the cells of plants. Fructans are made by attaching fructose molecules with the enzyme fructosyltransferase, and ending the elongation with a glucose molecule on the terminal end. The fructans are translocated from the vacuoles to the stem for storage for later use (Longland and Byrd, 2006). This reserve, which can be used to produce glucose for maintenance, allows the plant to thrive when temperatures drop below optimum for long periods of time. Plants classified as C4 lack a transport mechanism to translocate starch for storage, limiting starch production. When starch is nearly saturated, a feedback mechanism activates and slows photosynthetic starch production. Photorespiration then slowly uses starch for energy, therefore allowing photosynthesis to slowly increase. Shifting between

photosynthesis and respiration causes the plants to have variable NSC concentrations throughout the day and growing season as they grow and use available energy.

Photosynthesis is greatest when the most amount of light is present, leading to peak production of glucose in the middle of the day. Production then steadily declines as respiration takes over throughout the night and early morning hours (Watts and Chatterton, 2004). Soil fertility also plays a role in the amount of NSC. When temperatures and sunlight are present photosynthesis occurs, but when plants lack proper nutrients for optimal growth, the plant will photosynthesize without the parallel upward plant growth, leading to excess NSC buildup (Longland and Byrd, 2006).

Fructan accumulation

Fructan accumulation is one of the differentiating factors between C3 and C4 plants. Chatterton et al. (1989) examined 57 C4 and 128 C3 plants to evaluate carbohydrate concentrations, and especially fructan accumulation in these 185 plants. Plants were compared in two different growing temperatures of 10°C/5°C and 25°C/15°C day/night temperatures. These plants were harvested after 4 weeks and analyzed for concentrations of TNC, fructan, sucrose, glucose, fructose and starch. It was interesting to note that fructan concentrations differed among species. Warm- and cool-season plants, and the interaction between species and temperature, were also different. The

response of cool-season plants grown in the 10°C/5°C temperatures produced a range from 0 to 455 mg/g SDW whereas warm-season ranged from 0 to 15 mg/g SDW. The mean fructan content of all cool-season plants grown at 10°C/5°C was 115 mg/g SDW and dropped to a mean of 12 mg/g SDW when grown at 25°C/15°C. Fructan accumulation for warm-season plants was 3 and 4 mg/g SDW for 10°C/5°C and 25°C/15°C respectively. Total non-structural carbohydrate was not different between cool-season and warm-season plants when grown at 25°C/15°C temperatures. There was a difference observed in TNC accumulation between the cool-season and warm-season species when grown at the 10°C/5°C that was due to fructan, as all other NSC accumulated at similar rates. The authors concluded warm-season grasses did not accumulate fructans, even though the results showed small fructan concentrations, which could be explained by the sucrosyloligosaccharides that may also produce those results. Finally, they noted genetic variation in TNC accumulation from plant to plant.

Objectives

The scientific literature and the lack of published equine research on effects of forage type and short term fasting on metabolic status were the grounds for design of the current study. As more metabolic issues arise in the horse, it is imperative to know what causes fluctuations in blood glucose, insulin and cortisol concentrations.

This study was designed with two objectives in mind. The first objective was to evaluate differences in post-prandial responses to a concentrate meal in horses provided hay at different time intervals in relation to the concentrate meal. The hypothesis was that offering ad libitum hay in the overnight hours preceding the concentrate meal would attenuate the post-prandial changes of circulating glucose, insulin and cortisol. It was further hypothesized that horses without hay overnight, and offered hay shortly before or at the same time as the morning concentrate, would experience more pronounced post-prandial glucose and insulin responses to the concentrate meal. Cortisol also was hypothesized to increase in horses deprived of hay for any length of time prior to the concentrate meal. Concurrently, cortisol was monitored to evaluate the interaction of stress to glucose and insulin changes.

The second objective of the study was to assess the post-prandial glucose, insulin and cortisol responses to warm- and cool-season hay types. Cool-season forage has the capacity to store fructan and therefore has greater capacity for NSC. Once consumed by the horse, the cool-season plants' greater concentration of NSC and simple sugars would

increase blood glucose concentrations, and in turn require a greater amount of insulin to clear glucose from blood. For this reason, it was hypothesized that a greater post-prandial response of glucose and insulin would be observed in the horses fed cool-season forage.

Materials and Methods

An experiment was conducted to determine the effects of fasting length and type of hay on blood concentrations of glucose, insulin and cortisol in idle horses. The research was conducted at Auburn University Horse Center between the months of September and November of 2009.

Horses

Six mature geldings ranging in age from 5 to 26 yr and of varying light-horse breeds were used. All animal procedures were approved by the Auburn University Institutional Animal Care and Use Committee.

Horses were independently evaluated weekly by two trained evaluators using the Henneke Body Conditioning Scoring System (Henneke et al., 1983). The two values were averaged for a weekly score. Because livestock scales were unavailable, BW was estimated weekly using the following equation: $BW (kg) = (\text{heartgirth}^2 \times \text{body length}) / (11,880 \text{ cm}^3)$ (Hall, 1971). Body length (cm) was defined as the distance from the point of the shoulder to the midpoint of the distance between the widest part of the stifle and the tail when viewed from the rear. Heart girth circumference (cm) was determined by placing the measuring tape behind the elbow, and passing it in a straight vertical line over

the withers and across the sternum. Mean BW and BCS were recorded for each horse prior to starting and upon conclusion of the study (Appendix 1).

Experimental Design

The experiment consisted of 2 hay types and 3 fasting treatments for a 2×3 factorial design with 6 treatment combinations. The fasting treatments consisted of no fast (NF), short fast (SF) and long fast (LF). The fasting treatments were designed according to common feeding practices. The NF treatment consisted of ad libitum access to hay throughout the night with no fast between the hay and the concentrate feedings. The second treatment, SF, received hay from the evening feeding that was removed at 2200 h and re-introduced at 0600 h, or 1 h prior to the concentrate feeding. The third treatment, LF, received hay from the evening feeding that was withdrawn at 2200 h and did not have it re-introduced until 0700 h, which coincided with the feeding of the concentrate. Two types of hay were offered. The WS hay was coastal bermudagrass (*Cynodon dactylon*) purchased from Dillard Farm in Reeltown, Alabama. The CS hay was Kentucky 31 tall fescue (*Lolium arundinaceum*), purchased from Kermitt Simmons of Jefferson, Georgia. Hays consisted of 2nd- and 3rd-cutting regrowth forage.

There were 6 treatment periods, each lasting 7 d. In the first treatment period, each horse was randomly assigned to 1 of the 6 treatments and systematically rotated

through each of the 6 treatments to complete the Latin Square (Appendix 2). Hay type was alternated weekly to avoid potential adaptation and carry-over effect.

Feeding

On d 1 through 5, horses were placed in 3.35-m × 3.35-m stalls at 1630 h for their 1700 h feeding of treatment hay and concentrate. All horses received 1.36 kg of a commercially formulated concentrate (Omolene 100, Purina Mills, Gray Summit, MO) (Appendices 3 and 4) per feeding. In each stall the horse had access to clean water and a trace mineralized salt block. Stall assignments were based on hay treatment such that horses changed stalls at the start of each period depending on their specific treatment combination. All hay was fed via hay bags with the exception of one animal that refused to consume hay unless it was placed in a trough. Horses were kept in stalls overnight. The following morning, horses were fed hay according to the hay protocol followed by the concentrate feeding at 0700. When each horse finished eating, it was turned out for the day. Four horses were turned out to pasture, which consisted primarily of dormant bermudagrass with ad libitum access to bermudagrass hay. The other two animals were kept in individual dry-lot paddocks and had ad libitum access to bermudagrass hay.

On d 6, all horses were stalled and fed according to the protocol. Following the 1700 h feeding, all horses were catheterized for serial blood draw the following day. Hay

was replenished through the night for horses in the NF group. The following morning, horses were fed as previously stated but were not turned out, as blood collections took place that morning (d 7). Following the serial blood draw, horses were turned out and that evening were re-assigned a new stall that correlated with their new treatment diet.

Blood Sampling

On d 6 of the period, horses were prepared for the serial blood draw to be conducted in conjunction with the morning feeding the following day. A small square over the jugular vein was clipped using a #10 blade to remove the hair. The shaved area was prepared with a 3 × 3 surgical scrub alternating Betadine[®] (Stanford, CT) and isopropyl alcohol. Once the area was disinfected, local anesthesia consisting of 1 mL 2% Lidocaine (Vedco, St. Joseph, MO) was injected subcutaneously where the catheter would be inserted. Once the catheter (Abbocath[®]-T Radiopaque FEP I.V. Catheter, Lake Forrest, IL) was inserted, a Macrobore extension set (Hospira 177.8 mm extension set with Option-Lok[™], Lake Forrest, IL) was attached and locked into place. Upon confirmation of successful catheterization, Loctite super glue (Dusseldorf, Germany) was applied to anchor the catheter to the skin, and 101.6 mm² gauze sponges provided padding between the catheter and Elastikon adhesive wrap (Johnson and Johnson, New Brunswick, NJ) which was applied around the neck over the catheter site. Only the end of the extension set was exposed for blood collection. The extension lines were flushed with

5 mL of 1% heparinized saline solution (0.9% NaCl) every 2 h until the start of the serial blood draw to prevent clotting of blood in the line. A flashlight was used to provide light for this procedure rather than turning on the barn lights in an effort to avoid startling and disturbing the horses.

For each blood sample collection during the serial blood draw, 5 mL of blood was drawn and discarded in order to clear the heparinized saline from the catheter and extension set. Immediately following the discarding of the blood, 25 to 30 mL were collected and divided into three blood collection tubes. All collection tubes had been pretreated with specific solutions depending on the assay to be performed: EDTA for cortisol, sodium heparin for insulin, and sodium fluoride for glucose (BD Vacutainer, Oakville, ON, Canada). After each collection the catheter and extension set were flushed with 5 mL of heparinized saline. The serial blood draw began at 0600 h and continued every 30 min until 1300 h, for a total of 15 blood samples per horse.

Following each blood collection, the tubes were immediately placed in ice water for 15 min followed by centrifugation. The cortisol and glucose samples were centrifuged using an IEC[®] Centra CL2 (Thermo Electron Corp., Milford, MA) for 10 min at $1,560 \times g$. Insulin samples were centrifuged using a Medifuge/Biofuge 13 (Baxter Corp., Mississauga, ON) at $2,500 \times g$ for 15 min. After centrifugation, plasma was extracted via pipette and placed into labeled micro centrifuge tubes which were frozen at -20°C for later analysis.

Upon completion of the serial blood draw, the catheter was removed and the site treated with Nitrofurazone Soluble Dressing (Squire Lab; Revere, MA) to prevent infection. The area was protected with a bandage of gauze and Elastikon to prevent contamination when the horses were turned out to resume their daily schedule. The bandage was removed at the 1700 h feeding, and the insertion site was inspected to ensure no hematomas had formed.

Blood Analysis

Glucose was assayed at Auburn University in the Department of Animal Sciences using an automated glucose and L-lactate YSI 2300 Stat Plus analyzer (YSI; Yellow Springs, OH). Duplicate analyses were conducted on every sample and the mean was used for statistical analysis. Glucose levels were averaged for each horse at every time period. Insulin and cortisol were assayed at Auburn University College of Veterinary Medicine in the Department of Anatomy, Physiology and Pharmacology using radioimmunoassay kit (Coat-a-Count, Diagnostic Products Corp., Los Angeles, CA) previously validated by Reimers et al. (1981). Samples were also run in duplicate or triplicate if needed, and the average was used for all statistical computing.

Feedstuff Sampling and Processing

One sample of each of tall fescue, bermudagrass and concentrate were taken each week and placed into individual 3.7 L resealable plastic bags. A sample of each round bale was taken when placed into the pasture. Upon completion of the study, the forage samples were dried at 60°C for approximately 30 h. The samples were ground with a Wiley Mill to pass a 1 mm screen for analysis. An aliquot of each grain sample was placed in a tared paper bag and dried at 60°C for 72 h for DM determination. Remaining grain samples were frozen and subsequently ground using commercial coffee grinders. The grain samples were ground and stirred numerous times to ensure total breakdown and sample consistency.

Feedstuff Analyses

Duplicate samples were placed in an oven at 60°C overnight, placed into zip-lock plastic bags with a desiccant, and air-equilibrated until analyzed. The Van Soest et al. (1991) method, using a heat stable amylase, was used in determining concentrations of NDF and ADF in bermudagrass hay, tall fescue hay, bermudagrass round-bale hay, and grain for each treatment period.

Total Non-structural Carbohydrates

A modified Weinmann (1947) method was used to determine the TNC concentration of the samples. The samples (ca. 0.20 to 0.5 g) were brought to a rolling boil in 0.05 N H₂SO₄ and digested for 1 h. The sample was then added to ice water and the pH was adjusted to approximately 4.5 by adding approximately 2.5 to 3.0 mL of 1.0 N NaOH. The pH of the samples was then corrected more closely to a pH of 4.5 by using small amounts of H₂SO₄ and NaOH, followed by the addition of 1 mL of amyloglucosidase (*Aspergillus niger*, Lot No. A 9913, Sigma-Aldrich, Inc., St. Louis, MO) solution. Samples then were placed in the oven for 1 h at 60°C, and then filtered into 250 mL flasks using 22 micron Whatman 541 filter paper (GE Healthcare, Little Chalfont, UK). After filtration, 2 mL of 0.1 N NaOH was added with deionized water to bring the mixture up to 250 mL. Ten milliliters of both the sample solution and Shaffer-Somogyi solution (AOAC, 1995) were added to a test tube, covered and boiled. The boiling was abruptly halted 15 min later when the test tubes were partially submerged in ice water. Two milliliters potassium iodide-oxalate solution and 5 mL of 1 N H₂SO₄ were added, followed after approximately 2 min by the addition of 5 mL of 1 N H₂SO₄. Sulfuric acid was added at intervals, rather than all at once, to prevent the contents of the tube from foaming over. To facilitate this process, 1 drop of an anti-foaming agent (Fastbreak) in a 1:100 dilution was added. Before titration with a thiosulfate solution, 1 mL of 1% starch solution was finally added to the test tube. Titration was complete when the solution in the test tube turned from a navy blue color to a translucent bright baby

blue. This was then compared to the blank with no sugars present. Final calculations were calculated by using the reducing sugar concentration multiplied by the dilution factor and divided by the sample weight. This was then multiplied by 100 for the TNC percentage. Duplicate analyses of all forages were performed on different days. Grain samples were analyzed multiple times on different days due to the wide range of values observed on one particular testing day.

Statistical Methods

A 2×3 factorial design was used with 2 hay types and 3 fasting periods. The statistical model included hay type, fasting period and the hay \times fasting interaction as independent variables for AUC, time to peak, and peak concentration for glucose, insulin and cortisol. Glucose, insulin and cortisol concentrations along with sampling times were integrated to calculate AUC using the trapezoidal method. The AUC was determined for each horse for each diet for all three of the blood parameters. Once generated, the blood AUC values for each blood metabolite were analyzed using the PROC GLM (SAS Inst. Inc., Cary, NC) with classes set to hay type and fasting period. Time to peak and peak concentration data were also analyzed using the PROC GLM (SAS Inst. Inc., Cary, NC) procedure. Means were separated using the PDIF function of LSMEANS.

PROC MEANS was used to analyze changes in mean BCS and predicted BW. Individual animal and period effects were analyzed using PROC MIXED. Significant differences were determined at $\alpha = 0.05$.

Results and Discussion

The experiment was conducted from September to November, 2009, in Auburn, Alabama. Pasture grasses were dormant, so bermudagrass hay was provided in the turnout paddocks as a forage source when blood was not being drawn.

All horses were in good health throughout the duration of the study and consumed the treatment diets as expected. One subject refused to eat hay unless it was placed in a feed trough and not in a hay bag according to protocol. No major injuries to the horses were experienced throughout the study, although two horses were treated for minor injuries. One subject developed a hoof abscess that eventually opened at the coronary band. The second horse incurred a small gash above its eye and had to be treated with phenylbutazone and triple antibiotic ointment; treatment with phenylbutazone was discontinued at least 24 h prior to the blood collection. It is important to note these minor injuries, as they could have affected blood cortisol concentrations for these horses. On rare occasion, one or two subjects had to be given xylazine (Vedco, St. Joseph, MO) to facilitate catheter placement; however, this sedative was given no sooner than 10 h prior to blood collection. On two occasions, an individual horse developed a problem with its catheter, requiring removal of the catheter before the serial blood draw was completed. In these instances, blood was collected via jugular venipuncture to complete the serial draw.

At the beginning of the study, mean BW of the horses was 523 kg and final mean BW was 529 kg (Appendix 1). There was no significant difference in the weight of the animals from beginning to end of the experiment. Mean BCS increased ($P = 0.015$) from 4.5 at the start to 5.1 at the completion, which may have occurred due to the lack of forced exercise throughout the length of the study. The fact that the BCS was different, while the BW was only raised by 6 kg was surprising. Both evaluators were trained how to BCS, however, the subjective nature of the score and human error could have caused the BCS difference.

No problems were observed with hay consumption due to differences in species; however, caretakers reported that horses appeared to consume the WS hay more rapidly than the CS variety, which may have been due the horses' familiarity with the bermudagrass hay, as it was their principal forage prior to the project and what was available in the pasture.

Feed Analyses

Laboratory analysis was conducted on samples of WS hay, CS hay and concentrate from every week of the study. A sample also was taken from the large round hay bale placed in the turnout area. Feed ingredients and guaranteed analysis for the concentrate portion of the diet are included in Appendices 3 and 4. Total non-structural

carbohydrate, ADF and NDF concentrations for WS and CS forage (DM basis) are reported in Table 1.

Table 1 . Total non-structural carbohydrate, ADF and NDF concentrations in warm- and cool-season forages (DM basis)

Week	Hay Type							
	Warm-season				Cool-season			
	DM ¹ ,	NDF ²	ADF ³	TNC ⁴	DM ¹	NDF ²	ADF ³	TNC ⁴
	%							
1	91.25	70.77	33.73	14.37	85.18	61.55	32.97	7.78
2	92.87	71.52	40.78	7.96	94.54	68.08	38.03	7.90
3	94.31	70.66	36.03	8.67	93.91	69.70	36.59	9.20
4	91.46	72.84	35.67	6.73	94.09	68.74	35.69	6.56
5	92.34	66.18	31.43	13.42	94.18	70.33	37.91	6.05
6	94.12	72.89	34.67	7.26	94.13	70.01	37.31	7.33

¹DM = Dry matter, as-fed basis.

²NDF = Neutral detergent fiber.

³ADF = Acid detergent fiber.

⁴TNC = Total non-structural carbohydrates.

There were no differences ($P > 0.05$) in chemical composition among concentrate samples from week to week. There also were no differences ($P > 0.05$) in TNC, ADF and NDF concentrations between the WS and CS hays across all periods. The fact that concentrations of cell-wall constituents in the WS and CS forages were not different was not surprising based on tabular values for NDF and ADF (Table 2) reported elsewhere.

Table 2. Tabular values for NDF and ADF concentrations (DM basis) in forages

Source	NDF ¹ (%)	ADF ² (%)
Arkansas Feed Database ³		
Bermudagrass hay	71.0	32.6
Tall fescue hay	66.5	37.9
Horse NRC ⁴		
Bermudagrass hay	73.3	36.8
Cool-season grass hay (mature)	69.1	41.6
Beef NRC ⁵		
Bermudagrass (fresh)	73.3	36.8
Tall fescue hay (mature)	70.0	39.0

¹NDF = Neutral detergent fiber.

²ADF = Acid detergent fiber.

³Arkansas Feed Analysis Database. 2008. Univ. of Arkansas, Coop. Ext. Serv., Little Rock. <http://feedanalysis.uaex.edu>.

⁴NRC. 2007. Nutrient requirements of horses. 6th rev. ed. Natl. Acad. Press, Washington, DC.

⁵NRC. 2000. Nutrient requirements of beef cattle. 7th rev. ed. Natl. Acad. Press, Washington, DC.

Glucose, Insulin and Cortisol Analysis

The following figures show mean glucose (Fig. 1 through 5), insulin (Fig. 6 through 10) and cortisol (Fig. 11 through 15) concentrations at each time point based on treatment. The figures are shown for both hay type and fasting length for all 3 blood parameters. Concentrate was introduced at 0700 h.

Glucose (Fig. 1 through 5) and insulin (Fig. 6 through 10) concentration response to treatments generally followed the expected pattern, peaking immediately after the introduction of the concentrate portion and starting to decline at 210 min. Cortisol concentrations in response to treatments are shown in Fig. 11 through 15. Cortisol concentration rose between 0700 and 0900 h in all treatments except NF for both the WS and CS hay types (Fig. 13). In those treatments, where the pattern was observed, there was an initial large peak followed by small peaks and valleys throughout the day, illustrating the rhythmic response of cortisol. Due to the rhythmic nature and response to stress, multiple cortisol samples need to be taken over time in order to get a more accurate characterization. In this study, all horses had 15 timed samples for each treatment combination, and AUC was calculated for each.

Individual horse values ranged from 33 to 140 mg/dL with a mean value of 80 mg/dL for glucose, insulin values ranged from 3 to 53 μ IU/mL with a mean value of 9 μ IU/mL, and cortisol values ranged from 53 to 259 nmol/L with a mean value of 154 nmol/L. Mean values were within the metabolically normal range. Concentrations greater

than 250 mg/dL and 200 μ IU/mL for glucose and insulin, respectively, as an indicator of metabolic illness, were not seen (Ralston, 2002). Mean cortisol values were within the normal range of 2.9 to 6.6 μ g/dL (80 to 182 nmol/L) (Dugat et al., 2010). Glucose and insulin concentrations returned to pre-prandial levels within 5 to 6 h.

Figure 1. Mean plasma glucose concentrations for the no-fast (NF), short-fast (SF), and long-fast (LF) groups when fed cool-season forage

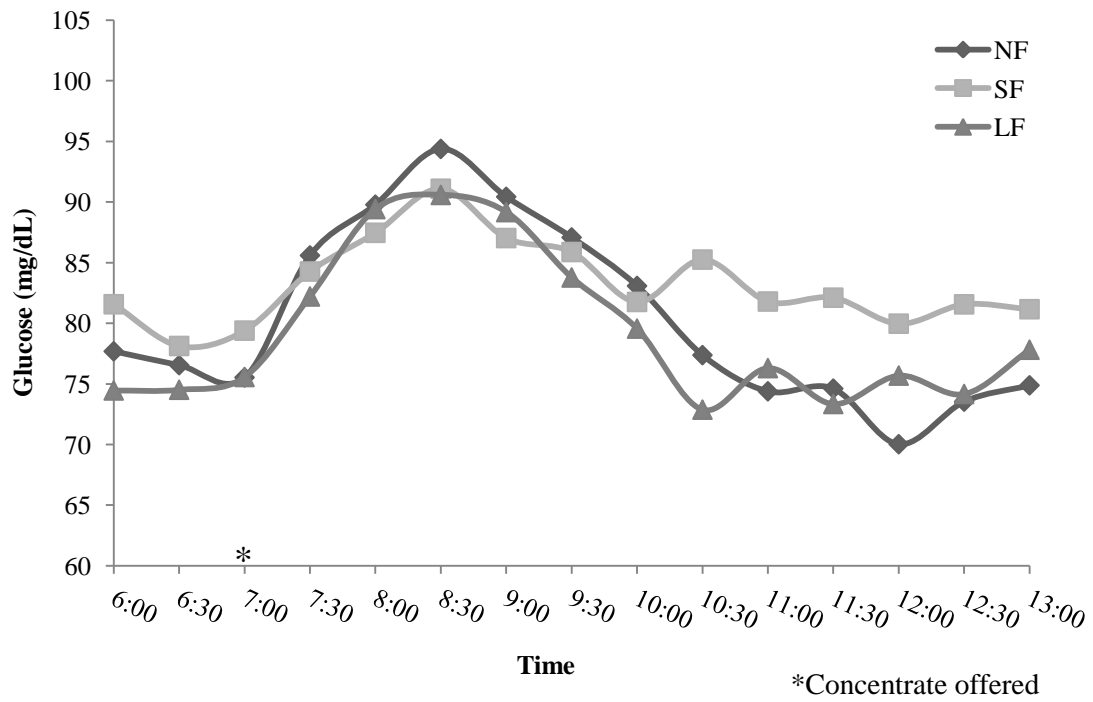


Figure 2. Mean plasma glucose concentrations for the no-fast (NF), short-fast (SF), and long-fast (LF) groups when fed warm-season forage

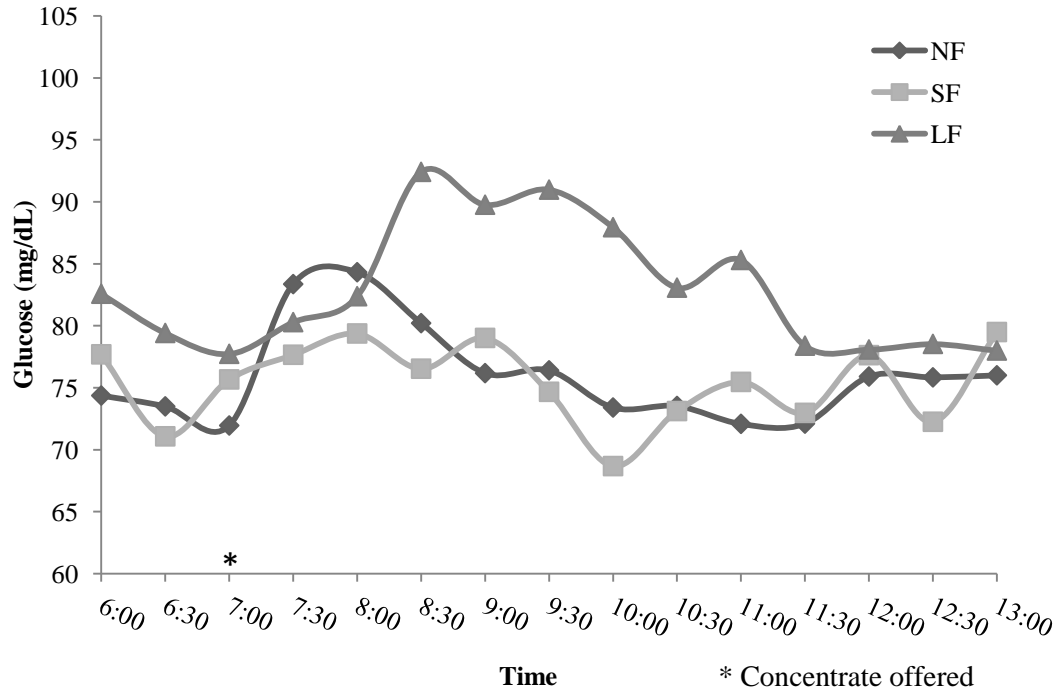


Figure 3. Mean plasma glucose concentrations for the no-fast group when fed warm-season (WS) and cool-season (CS) forage

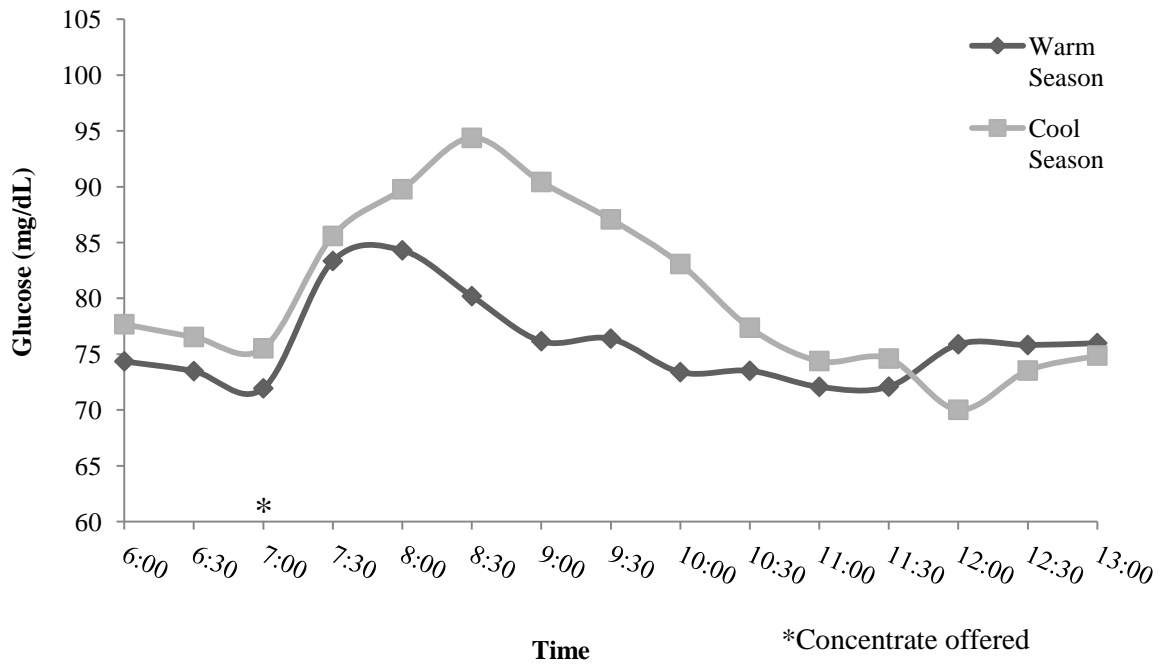


Figure 4. Mean plasma glucose concentrations for the short-fast group when fed warm-season (WS) and cool-season (CS) forage

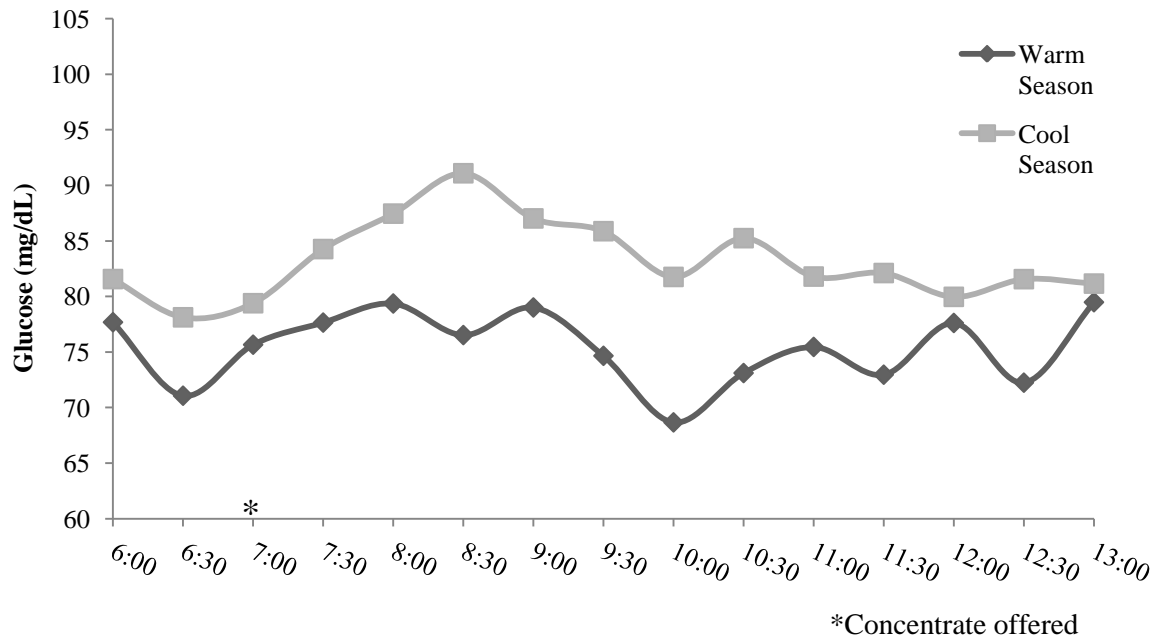


Figure 5. Mean plasma glucose concentrations for the long-fast group when fed warm-season (WS) and cool-season (CS) forage

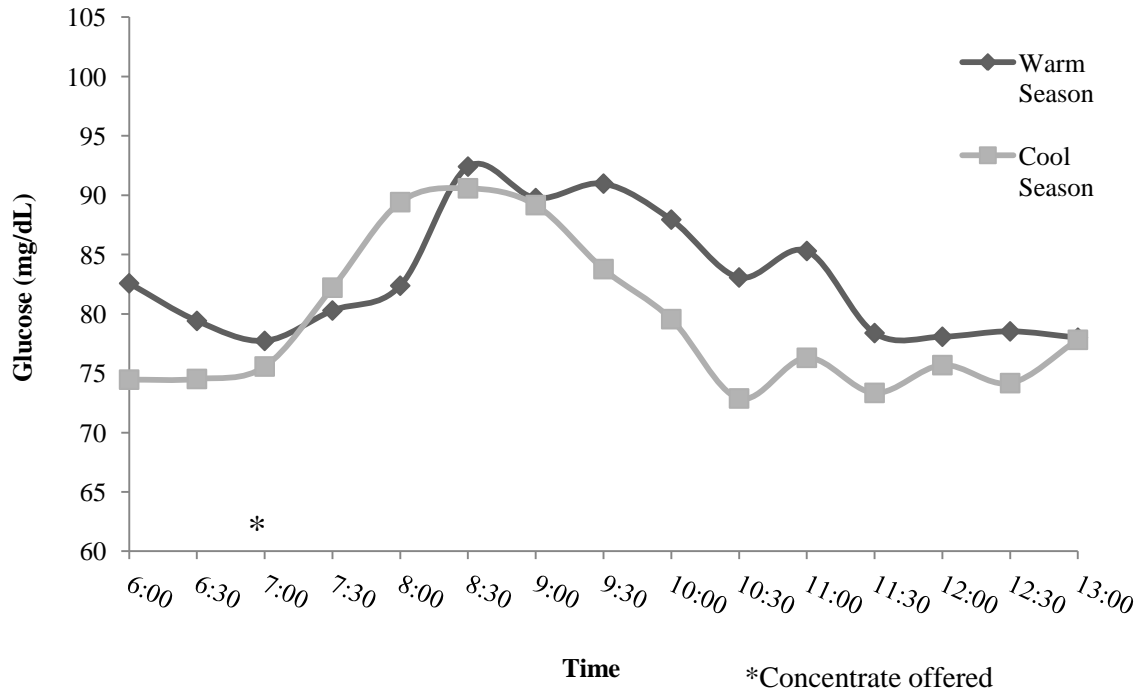


Figure 6. Mean plasma insulin concentrations for the no-fast (NF), short-fast (SF), and long-fast (LF) groups when fed cool-season forage

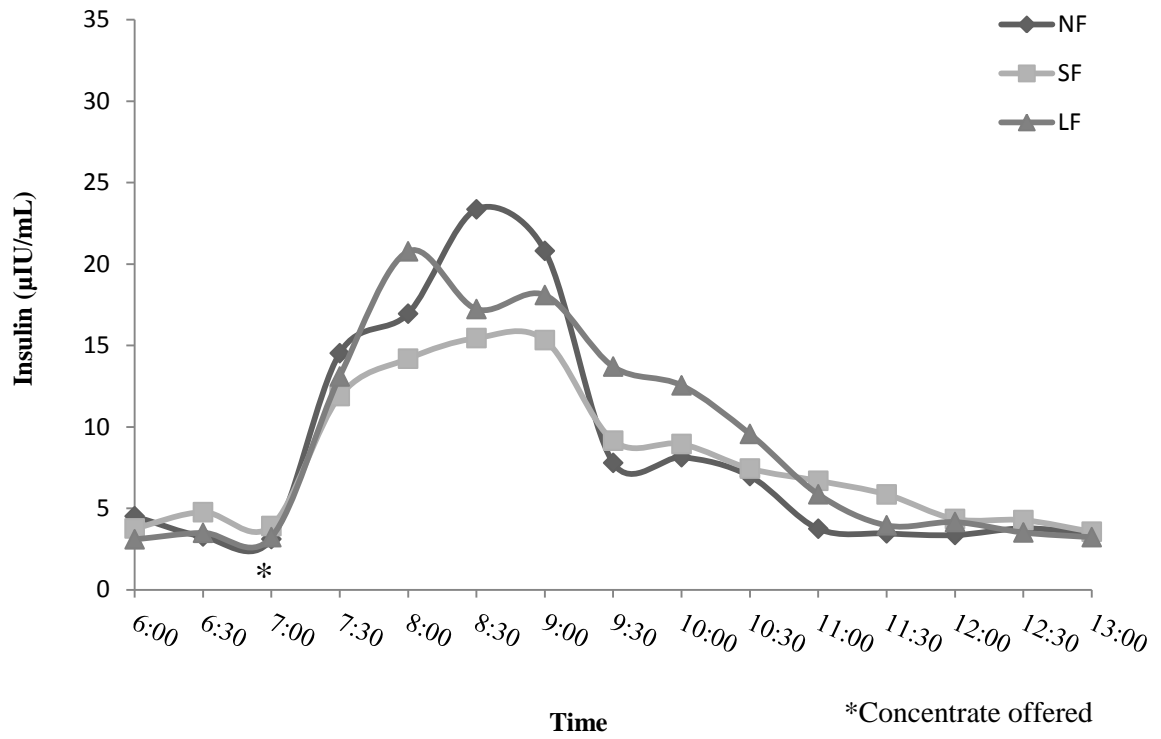


Figure 7. Mean plasma insulin concentrations for the no-fast (NF), short-fast (SF), and long-fast (LF) groups when fed warm-season forage

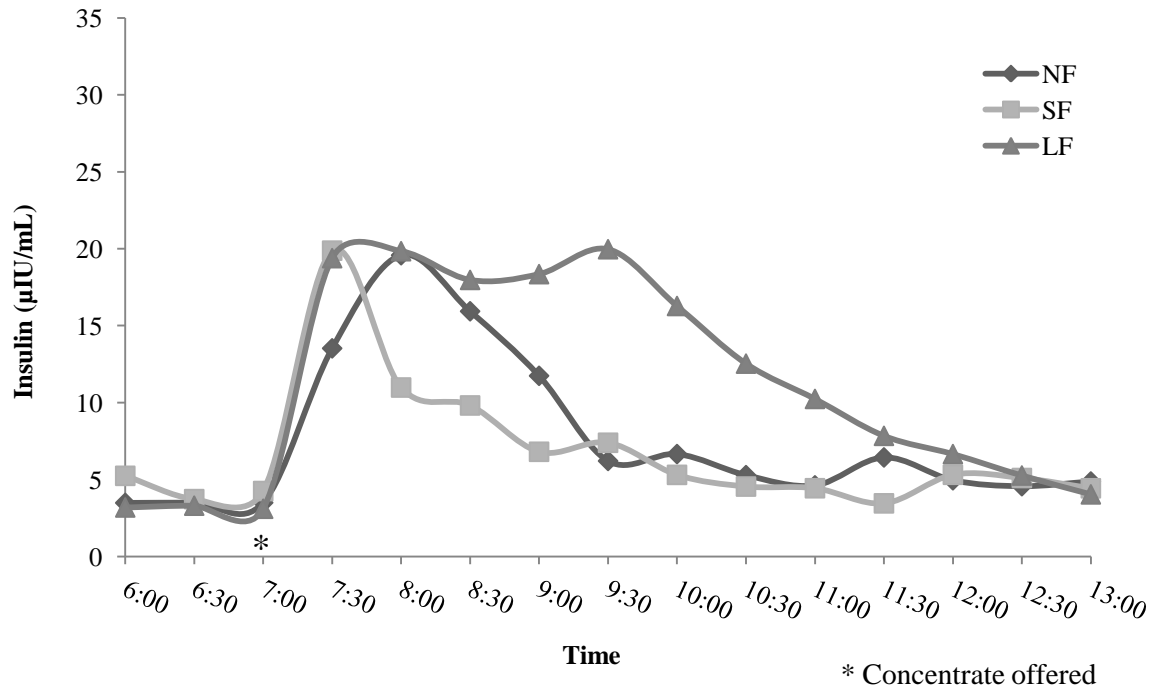


Figure 8. Mean plasma insulin concentrations for the no-fast group when fed warm-season (WS) and cool-season (CS) forage

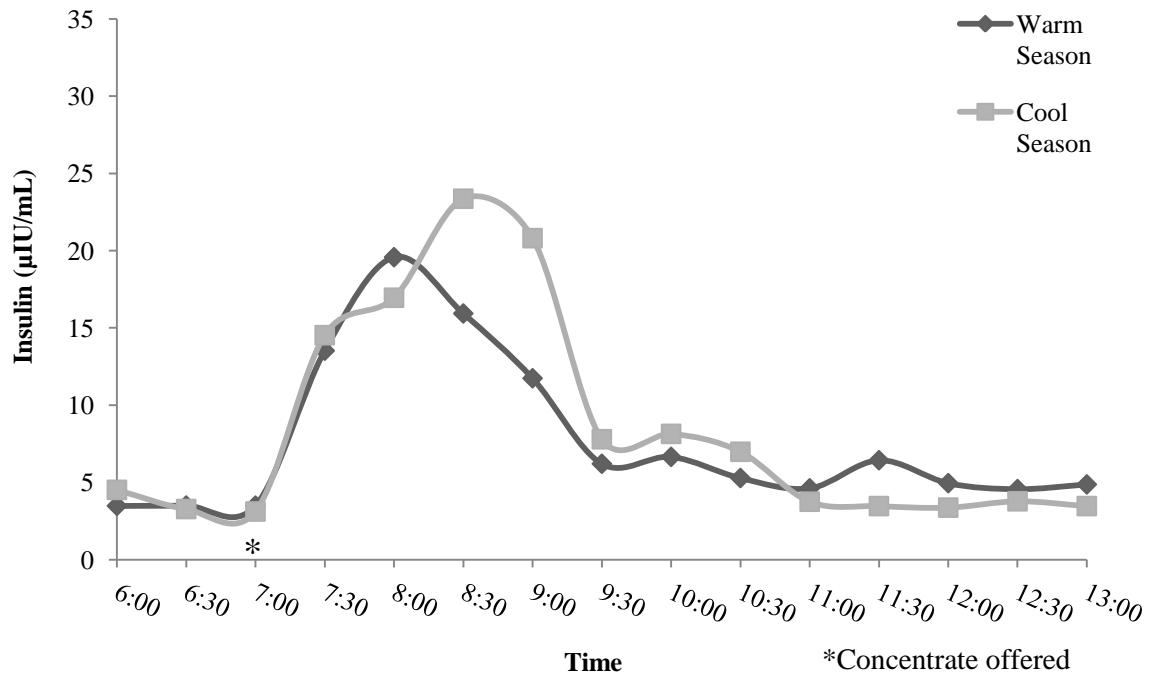


Figure 9. Mean plasma insulin concentrations for the short-fast group when fed warm-season (WS) and cool-season (CS) forage

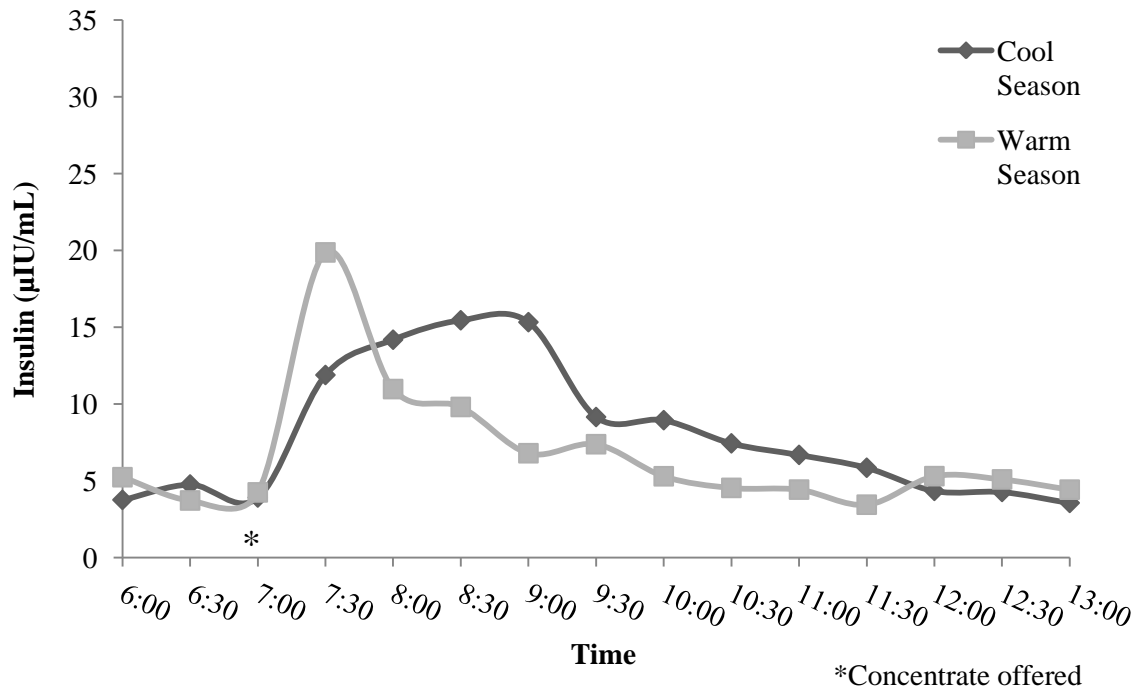


Figure 10. Mean plasma insulin concentrations of the long-fast group when fed warm-season (WS) and cool-season (CS) forage

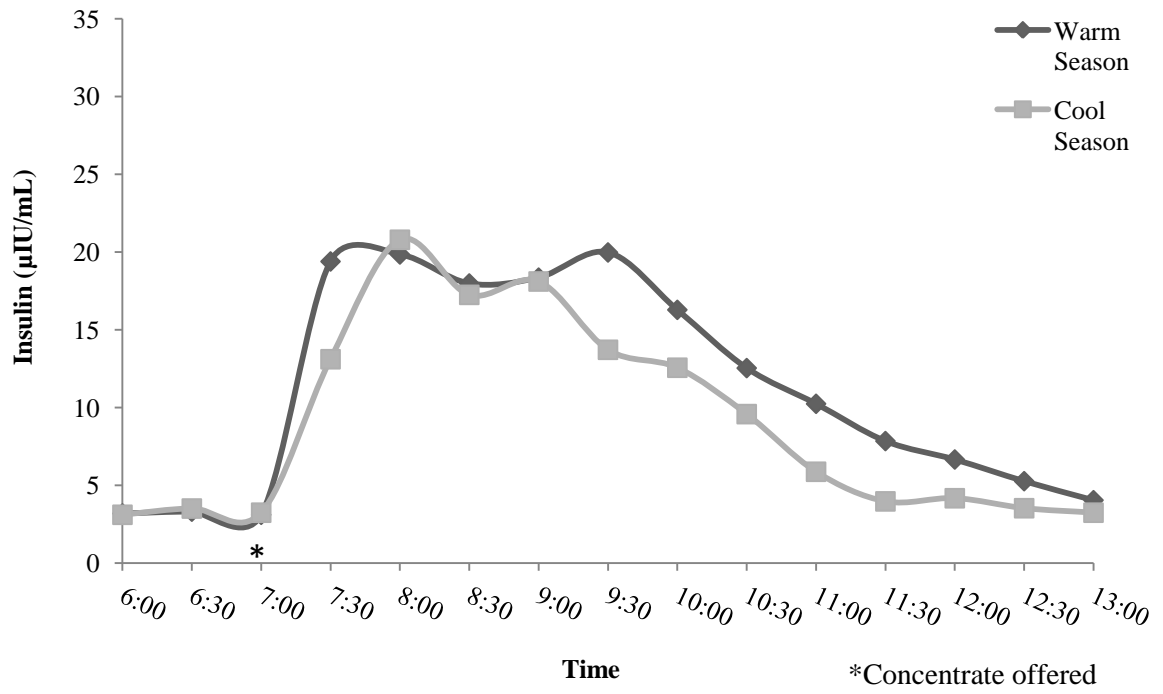


Figure 11. Mean plasma cortisol concentrations for the no-fast (NF), short-fast (SF), and long-fast (LF) treatment groups when fed cool-season forage

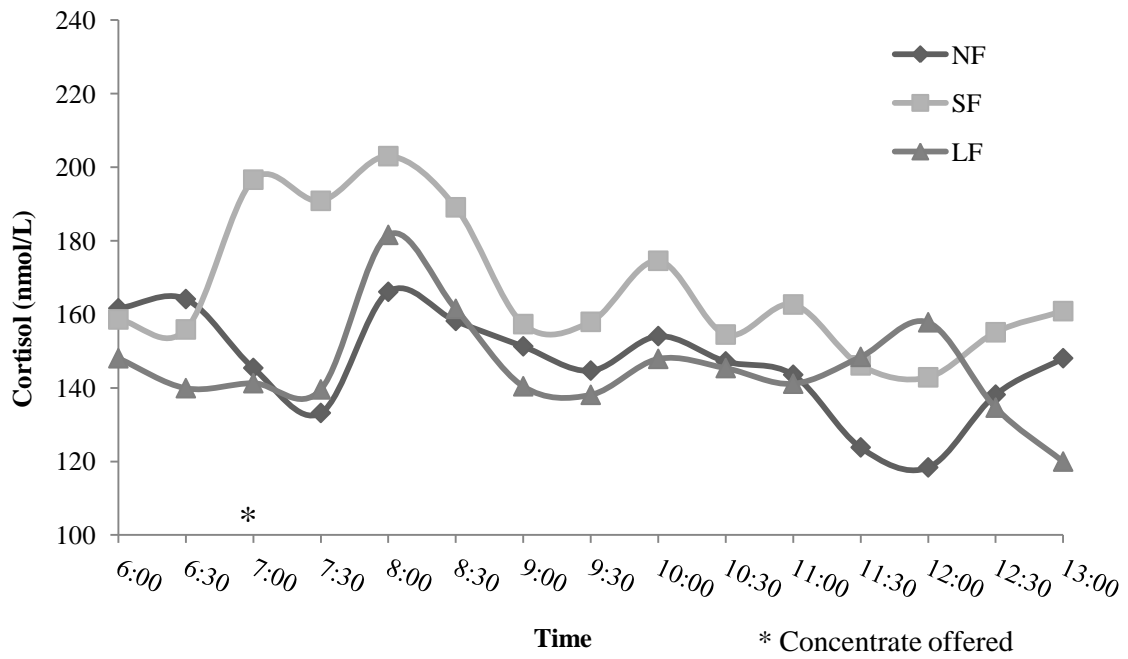


Figure 12. Mean plasma cortisol concentrations for the no-fast (NF), short-fast (SF), and long-fast (LF) treatment groups when fed warm-season forage

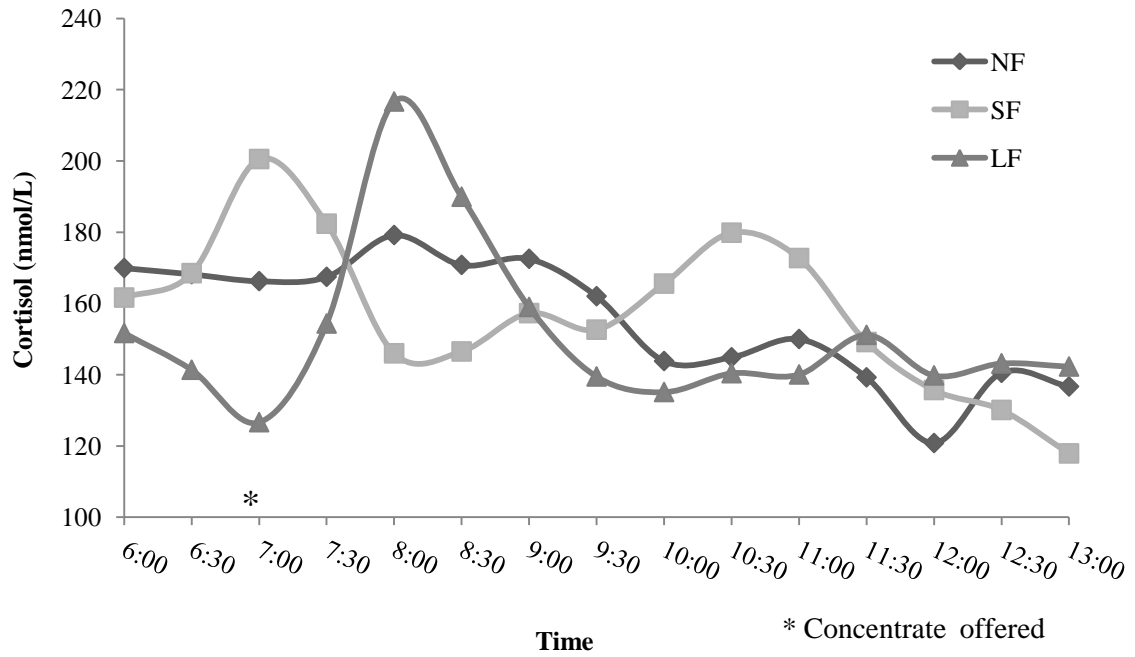


Figure 13. Mean plasma cortisol concentrations for the no-fast group when fed warm-season (WS) and cool-season (CS) forage

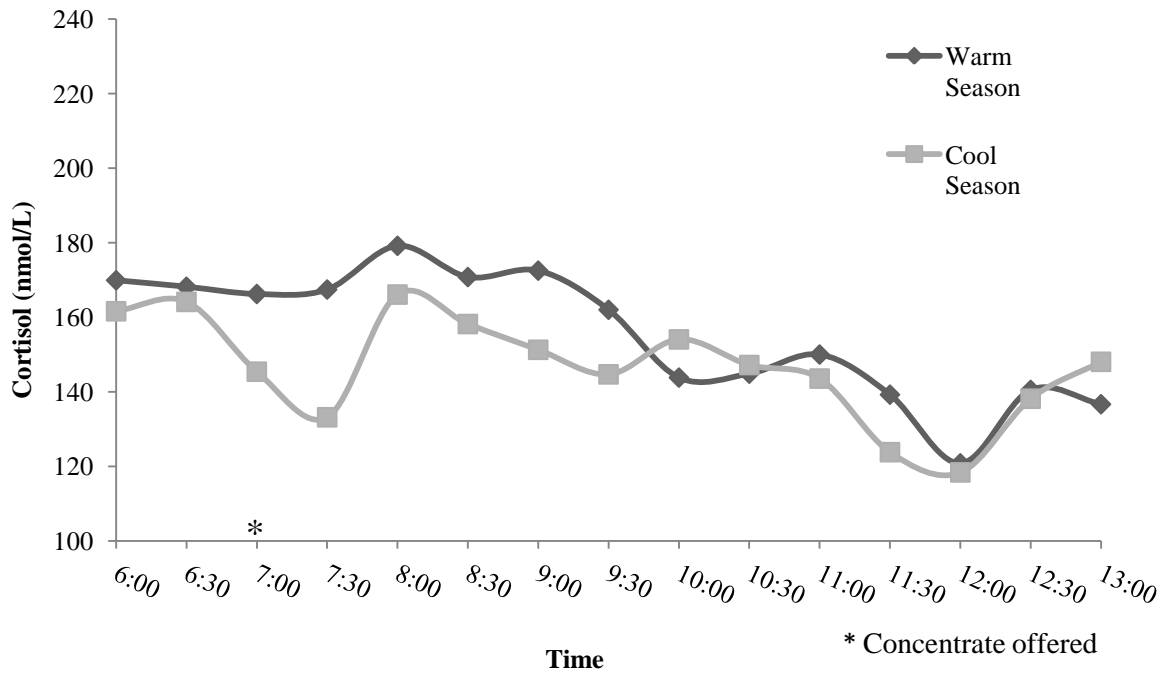


Figure 14. Mean plasma cortisol concentrations for the short-fast group when fed warm-season (WS) and cool-season (CS) forage

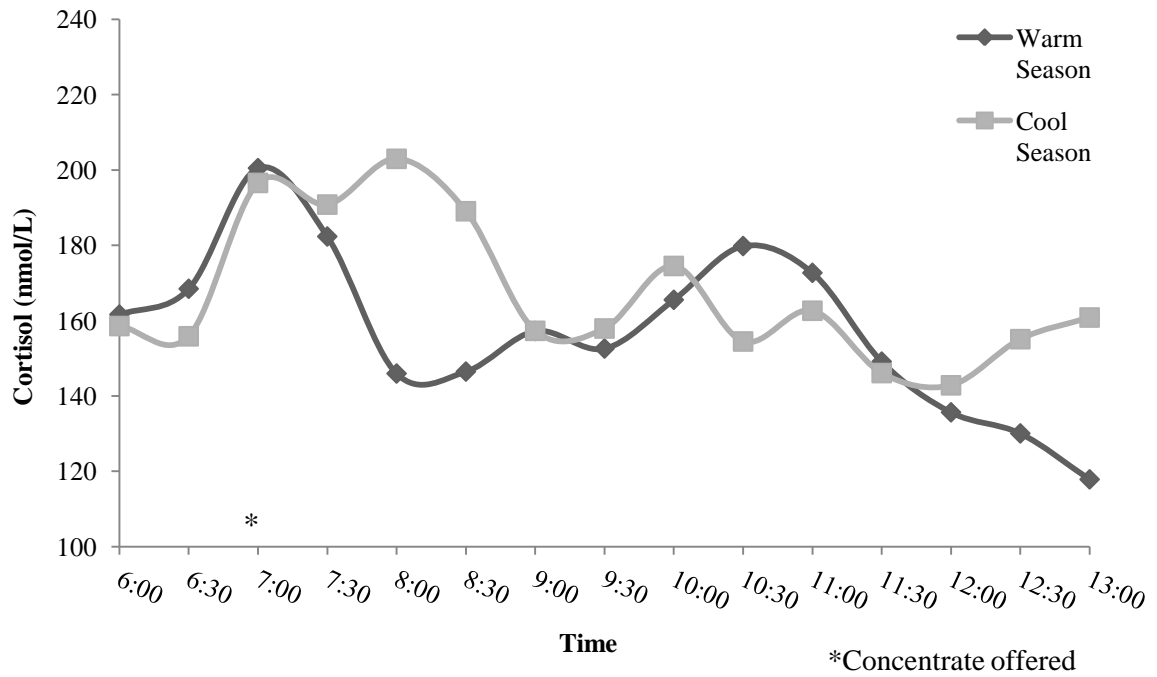
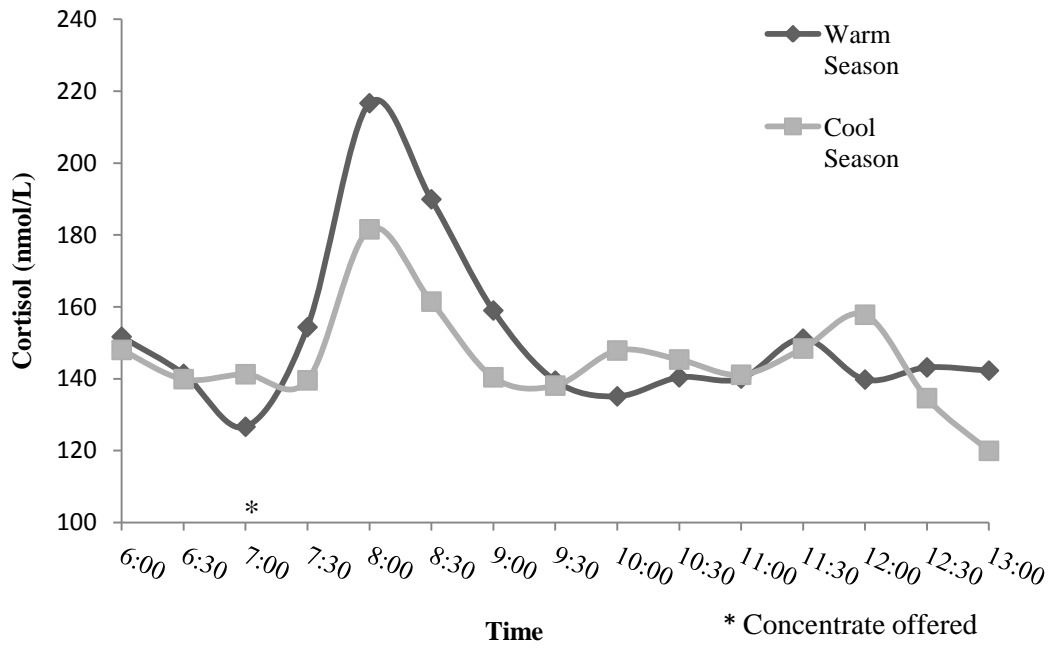


Figure 15. Mean plasma cortisol concentrations for the long-fast group when fed warm-season (WS) and cool-season (CS) forage



Analysis of AUC, time to peak, and peak concentrations were also evaluated in response to diet and fasting treatments. It was hypothesized that introduction of CS forage to subjects that had been adapted previously to WS forage would cause an increase in glucose and insulin AUC. This would be expected because CS forage has a higher amount of TNC than WS forage. Jensen et al. (2014) found the overall mean TNC concentration for a group of CS forages over 8 sampling dates to be 155.1 g/kg DM as compared to 70.4 g/kg DM for WS over those same 8 sampling dates. It would also be expected that the TNC concentrations would lead to increased intake. However, no differences were found in AUC for glucose (Table 3), insulin (Table 4) or cortisol (Table 5) during this study with respect to forage type, fasting length or an interaction between the two.

Table 3. Least-squares mean plasma glucose areas under the curve (mg/dL) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF), or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	31,914	32,993	32,453
SF	31,577	35,011	33,294
LF	34,935	33,399	34,167
Mean ²	32,809	33,801	

¹ SE = 982; n = 12.

² SE = 802; n = 18.

Table 4. Least-squares mean plasma insulin area under the curve ($\mu\text{IU}/\text{mL}$) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF) or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	3,320	3,701	3,511
SF	2,875	3,480	3,177
LF	4,929	3,974	4,452
Mean ²	3,708	3,718	

¹ SE = 721; n = 12.

² SE = 589; n = 18.

Table 5. Least-squares mean plasma cortisol area under the curve (nmol/L) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF), or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	65,378	61,310	63,344
SF	66,798	70,385	68,591
LF	63,733	61,563	62,648
Mean ²	65,303	64,419	

¹SE = 3,576; n = 12.

²SE = 2,919; n = 18.

Forage type may not have affected the AUC of the blood parameters because NDF, ADF and TNC concentrations in WS and CS forage did not differ from one another. Contrary to the stated hypothesis, fasting length did not alter AUC, which may have been due to the small 60 min difference in time between the SF and LF groups, making the animal's digestive system unresponsive to the change. The horses used in this study were previously conditioned to a twice-daily concentrate feeding with unrestricted forage access, so prior adaptation could be another reason why AUC for glucose, insulin and cortisol did not differ with the type of hay fed or fasting regimen, or show an interaction between the two. Feed particle size may have also played a role because the particle size was the same in both diets, possibly resulting in a similar MRT. If the MRT was similar, this could have led to comparable rates of carbohydrate digestion and absorption of glucose, failing to elicit changes in the AUC of blood glucose.

Dyer et al. (2009) found that quantity of starch ingested plays a role in SGLT1 expression. The expression of SGLT1 may not have changed in the current study because concentration of TNC was the same between the concentrate, WS and CS diets. If the expression of SGLT1 was not changed, the same amount of glucose could conceivably have been absorbed, with no changes to glucose AUC detected.

The fact that the AUC for cortisol was not affected by fasting or hay type may be consistent with James et al. (1970), who reported that cortisol concentration was affected by diurnal rhythm. However, the blood sampling protocol in the present study did not permit full characterization of the 24-h cortisol cycle to evaluate diurnal patterns. External stress factors between horses such as housing and turnout were minimized in this study because all horses were on the same strict daily schedule. However, individual horses may have a higher level of stress than others when being stalled.

Area under the curve for glucose did not differ by period or among horses. The study was based on metabolically normal horses, healthy and non-obese, so no differences in glucose AUC were expected independently from experimental treatments. Area under the curve for insulin was different ($P = 0.0004$) among horses, and tended to differ ($P = 0.058$) among periods, meaning that the amount of insulin needed to maintain baseline blood glucose values was different from horse to horse. Cortisol AUC was different ($P < 0.0001$) among individuals, which was not unexpected because individual horses have different stress-response thresholds and, in turn, different values of normal pulsating cortisol. However, most individual daily averages values lay within the reference range, with a few above (4 out of 36) and none below the range, suggesting the test subjects were not stressed throughout the study. There was no effect of period on

cortisol AUC, which was expected given that all horses were exposed to the same environmental factors from week to week.

Table 6 expresses time to peak glucose concentration with no significant differences found due to diet, fasting length, or the interaction between the two.

Table 6. Least-squares mean time to peak plasma glucose concentration (min) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF) or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	190	150	170
SF	200	180	190
LF	230	220	225
Mean ²	207	183	

¹ SE = 29; n = 12.

² SE = 24; n = 18.

Both the WS and CS treatments yielded similar values for time to peak glucose concentration (Table 6). Fasting length did not increase time to peak glucose concentration in the blood; thus, amount of forage in the digestive tract of the horse prior to consumption of concentrate did not appear to alter the post-prandial glucose absorption from the small intestine. Rate of passage was not evaluated, so whether MRT was affected by fasting or forage type is unknown.

There were no significant differences found due to forage type or fasting length for time to peak insulin concentration (Table 7). However, a fasting length \times forage type interaction ($P < 0.05$) was observed such that time to peak insulin concentration was greater for the LF than for the NF ($P = 0.024$) and SF ($P = 0.003$) treatments when WS forage was fed, but there were no differences among fasting treatments when CS forage was fed.

Warm season forage in conjunction with the LF delays insulin release, taking longer to reach peak concentration. If a delay occurs in insulin release, circulating blood glucose levels could remain high for a longer time, which could lead to metabolic illness in the future. However, all animals in the current study were in the metabolically normal range.

Table 7. Least-squares mean time to peak plasma insulin concentration (min) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF) or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	120 ^a	142	131
SF	100 ^a	135	118
LF	178 ^b	130	154
Mean ²	133	136	

^{a,b} Values in a column lacking common superscripts differ ($P < 0.05$) (SE = 17; n = 6).

¹ SE = 12; n = 12.

² SE = 10; n = 18.

A cephalic phase response may have occurred in horses in the LF group causing them to have a release of insulin in response to seeing and smelling WS treatment hay being given to the NF and SF groups. A cephalic phase response is the anticipatory physiological response related to food and feeding (Power and Schulkin, 2008). In humans, insulin secretion has been shown to increase within 10 min post-ingestion, prior to a rise in blood glucose. In the current study, if a small release of insulin was secreted, due to the cephalic response, there may have been a higher concentration of circulating insulin prior to an increase of glucose in the blood stream, slowing the release of insulin when blood glucose increased. If this were the case though, we would expect to see this interaction be present with regards to the CS forage as well.

A second theory is the consumption of WS hay prior to the concentrate feeding for horses in the SF and NF groups may have stimulated the secretion of insulin sooner than in horses in the LF group, leading to a shorter period of time to peak insulin concentration. However, the same was not observed for the CS group, as insulin concentration peaked at the same time point regardless of fasting length. Also, horses were observed to consume WS forage faster than the CS forage, which may also have played a role; however, forage consumption rate was not measured in the present study.

There was no difference in time to peak cortisol concentration between forage-type or fasting-period treatments. However, time to peak cortisol concentration was greater ($P = 0.04$) for LF than SF treatments when CS forage was fed, but there were no differences among fasting treatments when WS forage was fed.

Cortisol is a variable hormone that is pulsatile in nature and that may be altered by small stressors. The time to peak cortisol is highly dependent on what the horse views as stress, which would be different dependent on the horse.

Table 8. Least-squares mean time to peak plasma cortisol concentration (min) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF) or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	155	137 ^{ab}	146
SF	170	118 ^a	144
LF	130	237 ^b	183
Mean ²	152	164	

^{a,b} Values within column lacking common superscripts differ ($P < 0.05$) (SE = 40; n = 6).

¹ SE = 29; n = 12.

² SE = 23; n = 18.

Horses in the CS-LF had a longer time to peak cortisol concentration than the CS-SF group, but not CS-NF. The same response was not observed in the groups fed WS forage. The fact that the LF group was aware of the feeding of the SF group may have caused a shift in the release of cortisol due to being agitated, changing the time to peak cortisol concentration. However, there were no differences seen between the CS-LF and the CS-NF, which one would expect to see if fasting slowed cortisol time to peak. Also, if this were the case the same response should have been seen when WS forage was fed. Lastly, if the stressor of lack of feeding increased cortisol release, the peak cortisol value would also increase, causing a higher peak concentration of cortisol, and this did not occur.

The fact that the time to peak cortisol was so far past the feeding time and only happened in the CS-LF group shows that the delayed peak may have been caused by a stressor unrelated to feeding.

A significant individual horse difference was observed for time to peak glucose concentration, but not for time to peak plasma insulin or cortisol concentrations. There were no period effects for time to peak concentration for glucose, insulin and cortisol.

Peak concentrations for glucose (Table 9), insulin (Table 10) and cortisol (Table 11) were not different due to hay type, fasting length, or the interaction between the two.

Table 9. Least-squares mean peak plasma glucose concentration (mg/dL) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF) or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	88	96	92
SF	84	93	89
LF	97	97	97
Mean ²	90	95	

¹SE = 3; n = 12.

²SE = 3; n = 18.

Table 10. Least-squares mean peak plasma insulin concentration ($\mu\text{IU/mL}$) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF) or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	21	28	24
SF	20	21	20
LF	25	27	26
Mean ²	22	25	

¹SE = 5; n = 12.

²SE = 4; n = 18.

Table 11. Least-squares mean peak plasma cortisol concentration (nmol/L) for horses consuming warm- or cool-season hay in conjunction with the no-fast (NF), short-fast (SF) or long-fast (LF) treatments

Fasting treatment	Hay type		Mean ¹
	Warm-season	Cool-season	
NF	207	198	202
SF	208	231	219
LF	221	207	214
Mean ²	212	212	

¹ SE = 12; n = 12.

² SE = 9; n = 18.

Peak concentration values of blood metabolites were not affected by type of forage. Lack of difference in plasma concentrations of glucose (Table 9) between the forage types might be expected because the two forages had similar concentrations of NDF, ADF and TNC. Likewise, peak insulin concentrations (Table 10) might not be expected to differ between the forage treatments because plasma glucose concentrations, the stimulus for insulin secretion, were not different. Peak concentrations of cortisol (Table 11) would not be expected to differ, as stress is not normally related to hay type fed.

Fasting length did not affect peak glucose, insulin or cortisol concentrations. Fasting length would not be expected to alter peak glucose and insulin concentrations, as horses in this study were considered physiologically normal. The fact that peak cortisol concentrations were not affected by fasting length suggests withholding feed from horses, even while those around them are receiving feed, is not a great enough stressor to elicit an increase in cortisol. There were no differences seen in peak concentrations of glucose, insulin and cortisol due to an interaction between fasting length and forage type.

No differences were observed for peak concentrations for glucose, insulin and cortisol due to sampling period. Peak plasma glucose and insulin concentrations were different among individual horses. Metabolic differences in individual animals and how they process feed would cause differences in peak concentrations of blood glucose. The individual animal difference in peak insulin concentration would be expected with a

change in peak glucose concentration as insulin increases to lower glucose to baseline values. However, all horses were considered healthy and of moderate body condition throughout the study. None of the horses had a peak concentration of greater than 250 mg/dL and 200 μ IU/mL for glucose and insulin, respectively, and all were seen as metabolically sound. Individual peak cortisol concentrations varied by horse, and this may be attributed to differences in individual animal variation.

Conclusions and Implications

The first objective of this study was to investigate responses of plasma glucose, insulin and cortisol concentrations in horses to fasting periods and hay type. Under the conditions in the present study, post-prandial plasma glucose, insulin and cortisol concentrations were not altered by time of access to forage prior to the morning concentrate feeding. The hypothesis was that accessibility to hay over a longer period of time prior to concentrate feeding would attenuate the increase in glucose, insulin and cortisol values, minimizing peaks and valleys. The fasting periods in the present study may not have been different enough to elicit the hypothesized response.

The second objective of the study was to investigate how WS and CS forages might alter blood metabolites due to their different NSC components. It was hypothesized that CS forage would produce greater glucose and insulin peaks and valleys than WS forage. Forage type alone did not affect the post-prandial AUC values for either glucose or insulin. However, WS and CS forages in this study did not differ in concentrations of ADF, NDF or TNC.

There were some interactions between forage and fasting length that did occur. A difference was recognized in time to peak insulin between the WS-LF and both the WS-NF and WS-SF groups. If horses are fed a WS forage along with longer fasting periods, a slower release of insulin may occur, causing a longer time to peak, slowing the reduction of glucose from the blood stream. This could possibly predispose the horse to metabolic

illness over time, however, the slower time to peak was not large enough to change the AUC for either glucose or insulin and all horses fell within normal glucose and insulin ranges. More research is needed in order to fully understand the change in insulin time to peak and how it affects equine nutrition.

Differences were also detected in time to cortisol peak concentration between the CS-LF and the CS-SF groups. However, cortisol is a variable hormone with multiple fluctuations throughout the day and a small stressor can cause the curve to shift leading to a delayed peak, altering overall time to peak. Further investigation of cortisol with respect to the interaction between fasting length and hay type is needed.

Animal variability contributed to changes in individual peak glucose, insulin and cortisol concentrations. No period effects were seen in respect to AUC, time to peak or peak concentration for any of the blood parameters. Finally, there were differences observed for cortisol and insulin AUC and glucose time to peak concentration among individual horses.

Recommended future research would utilize physiologically normal horses fed WS and CS forages with widely differing ADF, NDF and TNC concentrations, and re-evaluate blood parameters, times to peak and peak concentrations.

A second proposed study would evaluate blood glucose, insulin and cortisol response using the same treatment structure as in the present study with horses that have

been diagnosed with insulin resistance.

The results of this study indicate that common hay feeding protocols and type of hay do not affect the AUC for glucose, insulin and cortisol in metabolically normal horses, which gives caretakers the ability to choose a feeding regimen that is most convenient for them. This study found CS and WS hay had the same effect on AUC of blood glucose, insulin and cortisol so either may be fed as part of a ration in a healthy horse; however, further research is needed in order to evaluate this. More research is needed to see if the same recommendations would be given to those horses currently suffering from metabolic problems.

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Appendix 1

Estimated Mean BW and BCS between beginning and end of the experiment

Horse	BCS- before	BCS-after	BW-before (kg)	BW-after (kg)
Laddie	4.5	5.25	586.61	594.96
Shamrock	3.5	4.25	541.00	530.38
Oliver	4.5	4.75	516.68	506.48
Dash	4.75	5.75	510.15	521.15
Whiz	4.0	4.5	394.89	410.63
Finch	6.0	6.0	590.38	610.35

Appendix 2

Feeding rotations for each of the animals throughout the duration of the study

Week	Whiz	Finch	Laddie	Shamrock	Oliver	Dash
1	NO FAST WARM SEASON	LONG FAST COOL SEASON	SHORT FAST WARM SEASON	NO FAST COOL SEASON	LONG FAST WARM SEASON	SHORT FAST COOL SEASON
2	SHORT FAST COOL SEASON	NO FAST WARM SEASON	LONG FAST COOL SEASON	SHORT FAST WARM SEASON	NO FAST COOL SEASON	LONG FAST WARM SEASON
3	LONG FAST WARM SEASON	SHORT FAST COOL SEASON	NO FAST WARM SEASON	LONG FAST COOL SEASON	SHORT FAST WARM SEASON	NO FAST COOL SEASON
4	NO FAST COOL SEASON	LONG FAST WARM SEASON	SHORT FAST COOL SEASON	NO FAST WARM SEASON	LONG FAST COOL SEASON	SHORT FAST WARM SEASON
5	SHORT FAST WARM SEASON	NO FAST COOL SEASON	LONG FAST WARM SEASON	SHORT FAST COOL SEASON	NO FAST WARM SEASON	LONG FAST COOL SEASON
6	LONG FAST COOL SEASON	SHORT FAST WARM SEASON	NO FAST COOL SEASON	LONG FAST WARM SEASON	SHORT FAST COOL SEASON	NO FAST WARM SEASON

Appendix 3

Ingredient composition (in descending order) of Omolene 100

Ingredient
Whole Oats
Wheat Middlings
Cracked Corn
Cane Molasses
Coarse Barley
Wheat Flour
Calcium Carbonate
Soybean Oil
Ground Soybean Hulls
Salt
L-Lysine
Citric Acid
Propionic Acid
Choline Chloride
Vitamin E Supplement

Appendix 4

Guaranteed Analysis of Omolene 100

Crude protein	Min	10.0%
Crude fat	Max	4.50%
Lysine	Min	0.60%
Crude fiber	Min	10.00%
Calcium (ca)	Min	0.90%
Calcium (ca)	Max	1.20%
Phosphorus	Min	0.45%
Copper (cu)	Min	35PPM
Selenium (se)	Min	0.60PPM
Zinc (zn)	Min	140PPM
Vitamin a	Min	3000IU/LB
Vitamin e	Min	100IU/LB
