Resistance Training and the Effect of Feeding Carbohydrates and Oils on Healthy Horses and Horses with Polysaccharide Storage Myopathy

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DEDICATION

This dissertation is dedicated
to my long-suffering husband,
Richard Brostrom, who supported me
both emotionally and financially through the last four years.

And to my son,
Julian Borgia, who overcame
cancer with courage and dignity.

ABSTRACT

Type 1 Polysaccharide Storage Myopathy (PSSM) is a heritable glycogen storage disease found in many breeds that is managed with low carbohydrate, high fat diet and regular exercise. The purpose of these studies was to better understand different modes of exercise and to further study dietary management of this disease. The central hypotheses of this thesis are:

1) Resistance training as provided by a water treadmill increases fitness parameters and metabolic properties of muscle tissue;
2) feeding forage with nonstructural carbohydrate (NSC) concentration >11% increases blood insulin and glucose concentrations compared to lesser NSC;
3) feeding a short odd carbon chain fat reduces muscle damage with exercise in PSSM horses.

The first investigation examined the effect of resistance training via the use of a water treadmill. Healthy unfit horses performed a standardized exercise test on a conventional treadmill before and after training on an underwater treadmill for 4 weeks to measure relative fitness and the effect on muscle enzyme activities and metabolite and substrate concentrations found in gluteal and superficial digital flexor (SDF) tissue. No increase in fitness or difference in muscle enzyme activity, or concentrations of metabolite or substrates was detected with training. SDF had lower oxidative capacity and substrate concentrations when compared to gluteal tissue.

The second investigation examined the effect of feeding hay with differing amounts of carbohydrate to healthy Quarter Horses (QH) and QH with PSSM. Hay with high and low NSC concentrations was fed to randomly assigned horses over a period of 5 days. The diets were then reversed after a 7 day washout in which medium NSC hay was fed. The insulinemic response to the high NSC forage diet were higher compared to the low NSC diet for both the control and PSSM horses.

The third investigation studied the effect of feeding hay with differing amounts of carbohydrate to healthy QH and Thoroughbreds (TB). A baseline FSGIT was conducted on both groups, after which hay with different NSC concentrations was fed over a period of 5 days in a crossover block design similar to the previous study. FSGIT results showed that QHs were more insulin sensitive and had a faster rate of Sg (glucose mediated glucose disposal) than TB. QHs secreted more insulin in response to hay > 17% NSC than hays of lower NSC, and that QHs secreted more insulin than TBs in response to higher NSC hay meals. Of note, all QH were mares, all TB were geldings, so gender, along with breed differences, could be a factor in the results.

The fourth investigation examined the effect of feeding odd carbon (C7) short chain oil (triheptanoin) and other more conventional feeds to healthy and QH with PSSM with exercise. Both groups were fed isocaloric diets of triheptanoin (TH) and corn oil (CO) and exercised daily on a treadmill for 3 weeks, then performed a 15 min exercise test on the last day. These horses were also fed high carbohydrate and a commercial high fat diet. The high NSC and TH diets resulted in muscle damage with exercise indicated by

plasma creatine kinase activity. The high NSC diet increased citrate and lactate concentrations compared to the other diets. The high fat and corn oil diets increased the amount of serum non-esterified fatty acids available. Long chain fat supplementation and consistent exercise remain the best choice for management of PSSM.

In conclusion, resistance training as provided by water treadmill use following manufacturer's protocol for tendon rehabilitation did not produce an increase in cardiocirculatory fitness. Feeding hay with NSC of 17% produced an increase in insulin secretion in normal horses compared with hay of lower NSC concentrations. There may be a difference in how TB and QH respond to a glucose challenge test, but the confounding factor of breed remains unexplored. Feeding an odd carbon short chain fat to type 1 PSSM horses increased muscle damage and exercise intolerance.

TABLE OF CONTENTS

CHAPTER SIX

FUTURE DIRECTIONS

LIST OF TABLES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

† PSSM SIGNIFICANTLY DIFFERENT FROM CONTROLS FOR ALL HAY TYPES COMBINED

§ HC SIGNIFICANTLY DIFFERENT FROM LC AND MC WHEN DATA FROM PSSM AND CONTROLS ARE COMBINED

[A] DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES WITHIN ROWS

MEAN (± SD) AREA UNDER CURVE FOR WHOLE BLOOD GLUCOSE AND PLASMA INSULIN RESPONSE AND PEAK VALUES FOR GLUCOSE (G) AND INSULIN (I) AS RECORDED FOR PSSM AND CONTROL HORSES OF QUARTER HORSE BREEDING. SIGNIFICANCE IS SET AT $P<0.05$.

[1] DIFFERENT NUMBERS INDICATE SIGNIFICANT DIFFERENCES FOR CORRESPONDING VALUES WITHIN COLUMN.

[A] DIFFERENT LETTERS INDICATE SIGNIFICANT DIFFERENCES FOR CORRESPONDING VALUES WITHIN ROW.

CHAPTER FOUR

NUTRIENT ANALYSIS OF HAYS USED IN THIS TRIAL. VALUES REPRESENT SINGLE ANALYSES PERFORMED AT EQUI-ANALYTICAL LABORATORIES. NDF (NEUTRAL DETERGENT FIBER), ADF (ACID DETERGENT FIBER), WSC (WATER SOLUBLE CARBOHYDRATE), ESC (ETHANOL SOLUBLE CARBOHYDRATES). *THE FRUCTAN CONTENT REPRESENTED HERE WAS

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER FOUR

CHAPTER FIVE

MEAN ± SD PERCENTAGE OF INDIVIDUAL EXERCISE TARGET COMPLETED BY HORSES EACH DAY WHILE FED THE TRIHEPTANOIN (WHITE SQUARES), CORN OIL (WHITE CIRCLES), AND HFLS (BLACK TRIANGLES) DIETS (A) AND MEAN PLASMA CK ACTIVITY IN SAMPLES OBTAINED 4 HOURS AFTER EXERCISE IN HORSES WHILE THEY CONSUMED THE GRAIN (BLACK SQUARES), TRIHEPTANOIN, CORN OIL, AND HFLS DIETS (B). IN PANEL A, THE INDIVIDUAL EXERCISE TARGET FOR EACH OF 8 HORSES WAS CALCULATED AS THE MEAN AMOUNT OF TIME EACH HORSE COULD EXERCISE WHILE BEING FED THE GRAIN DIET. MEAN PERCENTAGE OF EXERCISE TARGET COMPLETED WAS CALCULATED AS THE MEAN OF THE AMOUNT OF THE DAILY EXERCISE COMPLETED EACH DAY DIVIDED BY THE INDIVIDUAL EXERCISE TARGET AND MULTIPLYING THE QUOTIENT BY 100 FOR EACH OF THE OTHER 3 DIETS. HORSES COMPLETED SIGNIFICANTLY ($P < 0.05$) LESS OF THEIR EXERCISE TARGET WHILE BEING FED THE TRIHEPTANOIN DIET THAN WHILE BEING FED THE CORN OIL OR HFLS DIETS; NO OTHER SIGNIFICANT DIFFERENCES WERE DETECTED AMONG THE OTHER DIETS. IN PANEL B, LOG-TRANSFORMED PLASMA CK ACTIVITY WAS SIGNIFICANTLY ($P < 0.05$) HIGHER FOR THE TRIHEPTANOIN DIET THAN FOR THE CORN OIL OR HFLS DIETS; NO OTHER SIGNIFICANT DIFFERENCES WERE DETECTED AMONG THE OTHER DIETS. PLASMA CK ACTIVITY FOR THE HFLS DIET REPRESENTS RESULTS FOR ONLY 7 HORSES. DAY 0 = FIRST DAY ON WHICH THE COMPLETE AMOUNT OF THE SPECIFIED

Abbreviations

AIRg	Acute insulin response to glucose
ADP	Adenosine diphosphate
AMP	Adenosine monophophate
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUCg	Area under the curve for glucose
AUCi	Area under the curve for insulin
BPM	Beats per minute (heartrate)
BW	Body weight
CAC	Citric acid cycle
CK	Creatine phosphokinase
CO	Corn oil
CS	Citrate synthase
DI	Disposition Index
ER	Exertional rhabdomyolysis
FSIGT	Frequently sampled IV glucose tolerance test
G6P	Glucose-6-phosphate
GLUT 4	Glucose transporter 4
GYS1	Glycogen synthase gene
HAD	3-hydroxyacyl CoA dehydrogenase
HC	High nonstructural carbohydrate
HFLS	High fat, low starch
IMP	Inosine 5′-monophosphate
IR	Insulin resistance
LC	Low nonstructural carbohydrate
LDH	Lactate dehydrogenase
MC	Medium nonstructural carbohydrate
MH	Malignant hyperthermia
NEFA	Non-esterified fatty acid
NSC	Non structural carbohydrate
PAS	Periodic acid Schiff's
PSSM	Polysaccharide Storage Myopathy
QH	Quarter Horse
RER	Recurrent exertional rhabdomyolysis
SC	Subcutaneous
SDF	Superficial digital flexor
SET	Standardized exercise test
Sg	Glucose mediated glucose disposal
Si	Insulin sensitivity
TH	Triheptanoin
V_{200}	Velocity when heartrate reaches 200 beat per minute
WSC	Water soluble carbohydrate

CHAPTER ONE

LITERATURE REVIEW

INTRODUCTION:

The research contained within this thesis encompasses studies of the effect of diet on blood glucose and insulin responses of healthy horses and horses with polysaccharide storage myopathy (PSSM), the effect of varying chain lengths of fat on PSSM horses exercise response, and the effect of water treadmill training on equine skeletal muscle.

Recent advances in equine dietetic research show that the severity of several diseases can be minimized through appropriate diet and exercise regime. Evidence is available to justify avoidance of high-glycemic, high starch concentrates to decrease the risk of acute digestive disturbances associated with rapid fermentation (Ralston and Baile 1983; Ralston 2007), and chronic metabolic disorders associated with insulin resistance (Geor and Harris 2009; Treiber et al. 2006). Further, diets that substitute oil and fiber sources for dietary starch have been shown to reduce exercise intolerance (Borgia et al. 2010; Duren et al. 1999; Harris et al. 1999; McKenzie et al. 2003; Pagan et al. 2002; Pratt et al. 2006; Ribeiro et al. 2004; Zeyner et al. 2006) and benefit athletic performance of horses with PSSM (Firshman et al. 2003; Ribeiro et al. 2004; Valentine et al. 2001).

Dietary fat serves as an alternative energy source as a means to reduce the exercise intolerance associated with PSSM, but little is known about the impact of carbon chain length of the fed fat. Similarly, previous feeding studies have not determined whether non structural carbohydrate (NSC) content of hay should also be considered when designing diets for this disease or horses with insulin resistance (Firshman et al. 2003; Harris and Geor 2009; McKenzie et al. 2003; Ralston 2007; Ribeiro et al. 2004). There

has been little scientific research documenting the glycemic/insulinemic response to fed hay containing different concentrations of NSC (Hoffman et al. 2001; Longland and Byrd 2006), and no research on the effect of different concentrations of forage NSC comparing healthy horses of different breeds.

The effect of conventional land treadmill training on fitness is well-know (Brojer et al. 2006; Byrd et al. 1989; Davie et al. 1999; Essen-Gustavsson et al. 1989; Essen-Gustavsson et al. 1999; Eto et al. 2003; Geor et al. 2002; Goodyear et al. 1990; Gottlieb et al. 1989; Gottlieb-Vedi and Lindholm 1997; Hodgson et al. 1990; Hyyppa et al. 1999; Johnston et al. 1999; Lindner et al. 2009; Lindner et al. 2010; McCutcheon et al. 2002; Schuback and Essen-Gustavsson 1998; Sinha et al. 1993; Tyler et al. 1998; Valberg et al. 1989; Valberg et al. 1993; Valberg et al. 1999; Wijnberg et al. 2008) (Bayly et al. 1983; Rose et al. 1983; Rose et al. 1988; Thomas and Fregin 1981; Valberg et al. 1985); however, less is known about the effect of resistance training as provided by the use of water treadmills. Water treadmills are currently popular and are being used to train horses. Only a few studies explored biomechanics and heart rate in water treadmills (Nankervis and Williams 2006; Voss et al. 2002), and there are no studies of muscle metabolic properties to indicate this modality's effectiveness.

Resistance training in particular increases the workload on the muscle without increasing exercise speed and may in so doing provide a means to quickly enhance muscle oxidative capacity ((Tokuriki et al. 1999). It is thought that flexor tendon injury may be due to flexor muscle fatigue, contributing to fetlock joint hyperextension and tendon damage

(Gottlieb et al. 1989; Gottlieb-Vedi et al. 1996; Gottlieb-Vedi and Lindholm 1997). A water treadmill provides resistance training on flexor tendon muscles, which might reduce the risk of tendon injury.

Thus, the studies in this thesis will serve to better define appropriate fat supplements and hay types for horses with PSSM, as well as serve to determine whether water treadmills may provide a useful means of resistance training in horses.

I. ENERGY METABOLISM IN EQUINE SKELETAL MUSCLE

Muscle Structure and Composition: Normal skeletal muscle fibers are arranged in fascicles surrounded by connective tissue called the perimysium. Within the fibers is a regular array of contractile proteins, called filaments. These proteins are responsible for the mechanical work resulting in muscle contraction and relaxation. The contractile filaments are configured in structures called fibrils, which are then arrayed into fibers (Wortmann 2000).

Myofibrils have repeated sarcomere units of aligned myofilaments that form visible alternating dark and light bands; the dark band is referred to as the A band, and the light band as the I band. The I band length varies with the state of contraction because the filaments of the I band slide into the A band with contraction and out of the A band with relaxation. In the middle of the I band is a thin dark band called the Z line. A thin M line is present in the middle of the A band and is bound by pale H zones on either side. The A band and two I bands between two Z lines comprise the sarcomere, the basic unit of skeletal muscle fibers, which is 2.5 to 3.0 µm in length(Engel and Franzini-Armstrong 1994). Muscle fibers grow by adding sarcomeres at the myotendon junction (Wortmann 2000).

The dark A band is comprised primarily of myosin filaments which are thicker than the actin filaments found in the I band (15-18 µm vs. 7-10 µm). Thin filaments are composed of two F-actin strands. Other proteins are also present including creatine kinase in the M

line. The proteins troponin and tropomyosin are present in the I band. These proteins work in concert with calcium to regulate muscle contraction (Ebashi et al. 1969). Tropomyosin is arranged along the length of the thin filaments, within the grooves of the two F-actin strands. Troponin is globular in shape and composed of three subunits: TN-1 (troponin inhibitory component), TN-T (tropomyosin-binding component), and TN-C (calcium-binding component) (Engel and Franzini-Armstrong 1994). The Z line includes α-actinin (Engel and Franzini-Armstrong 1994). The giant protein titin is anchored at the Z line and can reach to the M line (Wortmann 2000). Titin and the protein nebulin, found in the I band, and other M- and Z-line proteins form a complex scaffolding that maintains the organization of the sarcomere during contraction and relaxation.

The external envelope of the muscle fiber is the sarcolemma. The sarcolemma is composed of two layers. The inner layer is the plasma membrane of the muscle fiber and the outer layer is the adjacent basal lamina (Wortmann 2000). Within a muscle, the fibers are functionally grouped into motor units. A motor unit consists of all fibers innervated by an individual motor neuron. The fibers within each motor unit have common histochemical and electrophysiological properties (Wortmann 2000).

Fiber Types: Individual muscles are composed of mixtures of fiber types categorized based on differences in speed of contraction. Fibers can be divided into two types based on the difference in their speed of contraction (fast or slow). Slower contracting fibers are red in color, reflecting a higher concentration of the oxygen-carrying protein myoglobin. These fibers also contain higher activities of enzymes involved in aerobic

metabolism, larger lipid stores, and more mitochondria. They are relatively fatigue resistant and energy efficient than other fiber types. In contrast, the faster contracting fiber types fatigue more quickly, and have a higher content of glycogen than slow-twitch fibers (Wortmann 2000).

There are many classification schemes used to identify fiber heterogeneity. No one scheme adequately describes the nature of muscle fibers, so consideration must be extended to various descriptions. Thick myofilaments (mostly myosin) possess various specific heavy chains that confer contractile speed. as well as enzymatic properties, that allows different rates of hydrolysis of adenosine triphosphate (ATP) by adenosine triphosphatase (ATPase) (Engel and Franzini-Armstrong 1994). Structural properties of myosin can be used to differentiate fiber types. Two identical myosin heavy chains (MHC) and two pairs of light chains form each thick filament. Different forms of MHC (termed isoforms) are expressed in individual muscle fibers.

Contractile Fiber Types: The speed of contraction of individual muscle fibers differs depending on the type of myosin and the activity of the myosin ATPase. Contractile properties of muscle fibers can be differentiated using a histochemical staining technique that is based on the sensitivity of myosin ATPase activity to acid and alkaline pre-incubation. Slow twitch type I fibers stain darkly after acid and lightly after alkaline pre-incubation. In contrast, fast twitch type II myofibers stain lightly with acid and darkly with alkaline pre-incubation (Brooke and Kaiser 1970). Some fibers do not reverse their staining properties in acid and alkaline media and are classified as type IIC or

intermediate myofibers. This likely corresponds to fibers containing either slow and fast twitch myosin or embryonic myosin. Further, type IIA and type IIB myofibers may be differentiated if muscle sections are pre-incubated at pH 4.6 prior to ATPase staining.

Immunohistochemical techniques recently have been developed to identify fiber types based on antibodies directed against MHC isoforms (Gorza 1990). Embryonic and slow twitch myosin isoforms as well type 2a, type 2b and 2x (called 2d) in skeletal muscle and type 2m fibers in the jaw muscles can be distinguished by this technique. Some hybrid fibers contain a mixture of these isoforms (i.e., type 2a/x). Fibers with type 2x myosin contract faster than fibers with type 2a myosin, which in turn contract faster than type 1 myosin.

Unfortunately, type IIB fibers distinguished by myosin ATPase activity do not correspond to type 2b fibers distinguished by Immunohistochemical staining for MHC. Rather, type IIB fibers correspond more closely with type 2x fibers and type 2b fibers correspond to a very rapidly contracting fiber type found in rodents and camelids (Gorza 1990; Linnane et al. 1999). In the following sections information derived from studies using histochemistry, Roman numerals are used for fiber types (I, IIA, IIB), whereas studies using immunohistochemistry use types 1, 2a and 2x.

Metabolic Fiber Types: Type I fibers generally have higher concentrations of triglycerides and myoglobin and are better suited to derive their energy by oxidative phosphorylation via the electron transport system following the oxidation of fatty acids

and glucose via the Krebs cycle. The oxidative capacity of type 1 fibers can be demonstrated by dark histochemical staining for the activity of oxidative enzymes such as succinate dehydrogenase (SDH) and reduced nicotinamide adenine dinucleotide - tetrazolium reductase (NADH- TR).

In general, type II fibers are suited to derive energy for contraction by anaerobic glyco(geno)lysis. Fast twitch fibers, particularly type IIx and IIb fibers, tend to have higher concentrations of glycogen as well as higher activities of enzymes associated with glycogenolysis and glycolysis such as phosphofructokinase (PFK) and lactate dehydrogenase (LDH) activity. The oxidative staining of type II fibers can be variable with some fibers containing relatively higher oxidative staining. Generally, type 2a fibers have higher oxidative staining than type 2b fibers; however, this is not always the case and may vary with age and training (Valberg et al. 1988). A further means to subtype fibers is by both their ATPase and oxidative staining for SDH or NADH. This fiber typing distinguishes slow twitch, fast twitch oxidative and fast twitch glycolytic fibers (or non-oxidative).

Comparative physiology shows us that horses have a highly developed musculature compared to other mammals (30 – 40% of bodyweight vs. 42% in non-athletic horse breeds) (Hinchcliff 1994). Skeletal muscle comprises approximately 50% of the horse body's mass (Cardinet and Holliday 1979), with variations between breeds, notably Thoroughbreds, which have a high proportion (~55%) of muscle in total body mass (Gunn 1987). The equine anatomy features large muscles used for movement placed

proximally on the skeleton, creating energy efficient movement of limbs. The arrangement of fibers within the muscle also maximizes efficiency and power. Pennate arrangement of fibers, in which fibers join tendinous insertions at an angle to the direction of force maximizes the cross-sectional area and power of the muscle in relation to the limited space available on the upper limb (Snow and Valberg 1994). Strap muscles maximize range of movement by using a parallel organization of muscle fibers along the direction of force. Slower contracting fiber types commonly used for postural support are frequently located deeper in the muscle and the faster-contracting fibers used for higher speeds are located more superficially (Snow and Valberg 1994).

Water and protein: Lean muscle tissue in humans (and horses) is composed of almost 72% - 80% water and 20% protein (Wortmann 2000).

Carbohydrate: The resting glycogen content of equine muscle varies from 1 - 1.5% tissue weight (Snow and Guy 1980). In humans, muscle glycogen content is 0.7% - 1% of tissue weight (Engel and Franzini-Armstrong 1994; Murray et al. 2006).

Fat: Lipids are stored within the muscle as well as in deposits in adipose tissue and around organs. In equine muscle tissue, intramuscular triglycerides at rest range from 26 to 40 mmol/kg, with large individual variation (Essen-Gustavsson and Lindholm 1985; Valberg et al. 1985). In human muscle tissue, about 5% is lipid (Engel and Franzini-Armstrong 1994) These lipids are about 33% neutral lipids (80% triglycerides, major

constituents are palmitic, oleic and linoleic acids) and 66% phospholipids (phosphatidylcholine mostly) (Engel and Franzini-Armstrong 1994).

Measures of Metabolic Capacity: Aerobic pathways such as the citric acid cycle, beta oxidation of free fatty acids and the electron transport chain are located within mitochondria and provide the bulk of ATP for the cell as long as oxygen is plentiful. The activity of key enzymes in the pathways can be used as an indicator of oxidative capacity in muscle tissue. Citric acid cycle enzymes such as citrate synthase (CS) and SDH are often measured in snap frozen muscle biopsies as oxidative markers. 3 Hydroxy-acyl-CoA dehydrogenase (HAD) is often used as a marker for the capacity for beta oxidation of free fatty acids. Anaerobic pathways such as glycolysis, creatine phosphate shuttle, and the purine nucleotide cycle are found within the cell cytoplasm. Markers for the capacity for anaerobic glycolysis that are commonly measured in frozen muscle tissue include PFK and LDH enzyme activities (Murray et al. 2006).

In horses, increased activity of CS and a slight increase in activity of HAD were observed in muscle after 10 weeks of eating a fat supplemented diet (Essen-Gustvasson 1989). It has been speculated that increased citrate production could inhibit PFK (rate-limiting step in glycolysis) with secondary accumulation of glucose-6-phosphate (G-6-P). The G-6-P subsequently inhibits glycolysis and glycogenolysis (Kronfeld et al. 1994).

The main fuels for aerobic muscular contraction are fatty acids and glucose, found in intramuscular (lipid droplets, β glycogen particles) and extramuscular (liver and adipose

tissue) depots, which are accessed during exercise. The rate of FFA release from adipose tissues appears to be the limiting factor in the supply of plasma free fatty acid (FFA) to muscle (Bennard et al. 2005). Glucose uptake by the myofibers under the influence of insulin appears to be the rate-limiting factor in the extramuscular supply of glucose to working muscle. Muscle triglyceride levels can be estimated by Oil red O staining in muscle. Glycogen stores in muscle can be measured biochemically in snap frozen samples and estimated from periodic acid Schiff's stains of frozen sections.

Variation in Muscle Fiber Type Composition: The speed and force developed by a muscle during contraction differs qualitatively and quantitatively depending on its fast and slow twitch fiber type composition. Most muscles in horses contain a mixture of types 1, 2a, and 2x fibers (or I, IIA, and IIB, depending on the technique used for fiber typing). Locomotor muscles have a higher proportion of type 2a and 2x fibers relative to type 1 fibers than postural muscles. Conversely, postural muscles have a higher proportion of type 1 fibers than most locomotor muscles. The proportion of muscle fiber types within a given muscle will also vary along its length and depth.

Generally, deeper muscles or portions of muscles have a higher percentage of type 1 muscle fibers. Due to this variation, when comparing the fiber type composition of different individuals, a standardized depth and site of collection within a muscle is advised. Fiber type composition of muscles on the left and right side of the body will be identical in normal horses if samples are taken from the same site and depth. The gluteus muscle is often chosen for study in horses because it is a major propulsive muscle active

in locomotion, is easily accessible, and shows adaptations to growth and training. Some studies have also evaluated the semimembranosus or semitendinosus muscle.

When standardized techniques are used to assess muscle fiber composition, individual horses have been shown to have a wide variation in muscle fiber type composition. This phenomenon has been attributed to effects of genetic background, breed, sex, age, and state of training (Lopez-Rivero et al. 1992). Heritability is believed to have an estimated 13% influence on MHC isoforms in muscle and (Barrey et al. 1999; Rivero et al. 1993). Quarter Horses and Thoroughbreds have the highest percentage of type II fibers in the gluteus medius (~80-90%), while Standardbreds and Andalusians have a lower percentage (~75%) and donkeys have the lowest percentage of type II fibers in locomotor muscles (Hodgson and Rose 1994). There are however, wide variations in some breeds between individuals (Essen et al. 1980; Lopez-Rivero et al. 1992; Roneus et al. 1992).

Stallions have a higher proportion of type IIA and lower proportion of type IIB in their locomotor muscles than mares (Rivero et al. 1993). No difference in type I fibers or oxidative enzyme activities have been identified between age matched Standardbred mares and stallions (Roneus 1993). Age matched Andalusian mares have a higher percentage of type I fibers than stallions (Rivero et al. 1993, Roneus et al 1993), a finding which has been inconsistently reported in Thoroughbreds (Roneus et al. 1991; Snow and Guy 1980). Training and growth results in a change in the length and cross sectional area of fibers and a change in the proportion of fiber types, rather than an increase in the number of fibers (Snow and Valberg 1994).

Varying metabolic properties: Type I fibers appear red due to the presence of the oxygen binding protein myoglobin (Wortmann 2000). These fibers are suited for endurance and are slow to fatigue because they use oxidative metabolism to generate ATP. Type II fibers are white due to the absence of myoglobin and a reliance on glycolytic enzymes. These fibers are efficient for short bursts of speed and power and use both oxidative metabolism and anaerobic metabolism depending on the particular sub-type (Wortmann 2000). These fibers are quicker to fatigue (Table 1).

Variations in fat and glycogen content: The breed or type of horse also affects the amount of fat that can be used during exercise. Type I muscle fibers have the highest capacity for utilization of fat, while type II fibers are more adapted for use of carbohydrate (glycogen). Horses bred for sprinting activities (e.g., Thoroughbreds and Quarter Horses) have a greater proportion of type II fibers and are therefore more adapted toward utilization of carbohydrates. On the other hand, Arabians seem to be able to have a higher utilization of fat, with 17% type I muscle fibers in gluteal muscle tissue (D'Angelis 2005). Regardless of breed, exercise training results in a shift in metabolism such that there is a greater use of fat and a concomitant sparing of the more limited glycogen stores (Snow and Valberg 1994).

Recruitment Patterns of Fiber Types with Exercise: When a muscle contracts during exercise, it does with a predetermined recruitment of particular muscle fibers. The orderly recruitment of muscle fibers leads to smooth, coordinated movement. As exercise begins, a select number of motor units are recruited to provide the power to advance the limb. Motor units are recruited with respect to their contractile speed and

oxidative capacities (Burke et al. 1974; Valberg et al. 1988). At slow exercise intensities, type I and a small number of type II fatigue resistant muscle fibers are stimulated. The force produced by any muscle is proportional to the cross-sectional area that is active. As the speed or duration of exercise increases, more muscle fibers are recruited, and this occurs in the order of their contractile speed from type I to type IIA and type IIB (Lindholm and Piehl 1974; Valberg et al. 1988). With moderate intensity, type I and type IIA myofibers are preferentially recruited, whereas moderate intensity of long duration or maximal exercise intensity is required for recruitment of type IIB myofibers.

Studies suggest that muscle fiber recruitment may be regulated by the central nervous system, with an anticipated response governed by peripheral feedback mechanisms and predetermined patterns of recruitment acquired from training and modulated by conscious motivation (Noakes and St Clair Gibson 2004).

Oxidative metabolism

Energy Sources:

Energy necessary for muscle contraction is provided by the hydrolysis of adenosine triphosphate (ATP). Intercellular concentrations of ATP are normally maintained by the action of enzymes such as creatine kinase, and adenylate kinase. The energy needed to replenish ATP when it is consumed during muscle contraction is provided by the intermediary metabolism of carbohydrate and lipid by the pathways of glycolysis, the tricarboxylic acid cycle, beta oxidation, and oxidative phosphorylation (Engel and Franzini-Armstrong 1994).

In horses, a primary factor limiting muscular performance allowing for the type of exercise is the availability of substrates, mainly glycogen in skeletal muscle and the liver, and triglycerides in various fat reserves. The energy stores for a 500 kg horse with a muscle mass of 206kg, adipose mass of 25 kg and a liver mass of 6.5 kg were reported as ATP 9 kcal, creatine phosphate 45 kcal, glycogen 17,988 kcal, and fat 152,899 kcal (McMiken 1983). Amino acid degradation only contributes approximately 10 – 15% to energy production (Lewis 1995). For brief and intense exercise, ATP supplies anaerobic energy and phosphocreatine then provides sufficient fuel for about 30 seconds (Wortmann 2000).

Glucose and Glycogen: Glucose is the primary form of carbohydrate absorbed from the intestinal tract and presented to the tissues. A monosaccharide or simple sugar, glucose is one of the main products of photosynthesis. Starch and cellulose are polymers derived from the dehydration of glucose (Devlin 2002). Glycogen is the storage form of glucose, and is found as highly branched polymer granules in the cytosol of many cell types. While a less compact form of energy than lipids, glycogen can be quickly metabolized to supply energy.

Oxidative Metabolic Pathways: Aerobic energy is generated via the citric acid cycle (CAC) and oxidative phosphorylation. Glycolysis, or the Embden-Meyerhof pathway, is the anaerobic degradation of glucose to pyruvate and is the preparatory pathway for oxidative metabolism. More detail on this pathway is found later in this review. Different cells use the energy from glucose in different ways. For example, red blood cells have no mitochondria and use the pentose phosphate pathway to produce lactate,

thereby providing reducing agents for oxidative phosphorylation, while adipose cells convert glucose to lipids via lipogenesis (Devlin 2002).

Aerobic metabolism takes place in the mitochondria of cells and utilizes oxygen and the pyruvate derived from the anaerobic metabolism of glucose and glycogen within the cytoplasm. Fatty acids and glycerol and amino acids can also be metabolized to form the needed pyruvate molecules (Murray et al. 2006). Pyruvate is the starting molecule for oxidative phosphorylation via the CAC. Pyruvate dehydrogenase converts pyruvate to acetyl-CoA, which enters the mitochondria. The CAC reactions transfer all of the C-C and C-H bonds in pyruvate to oxygen. After pyruvate molecules are oxidized to acetyl coenzyme A, it can then join with the four-carbon oxaloacetate to generate a six carbon citrate. Carbons and hydrogen are gradually cleaved from this citrate until all that remains is the original four-carbon oxaloacetate (Devlin 2002). In the process, four NADHs, one FADH and one GTP are generated for each pyruvate molecule. The net production of energy from aerobic metabolism is approximately 30 ATPs per pyruvate molecule (Murray et al. 2006).

The initial step of the CAC is catalyzed by citrate synthase, an enzyme located in the mitochondrial matrix. The reaction combines the 4 carbon oxaloacetate and 2 carbon acetyl CoA and water to form citrate (Devlin 2002). The activity of this enzyme has been used as an indicator of oxidative capacity in a variety of studies in horses (Annandale et al. 2005; Essen-Gustavsson et al. 1997; Kinnunen et al. 2009; Ribeiro et al. 2004; Rietbroek et al. 2007a; Rietbroek et al. 2007b; Wijnberg et al. 2008).

Activity of the CAC is regulated by various factors. The supply of acetyl units from pyruvate (carbohydrate) or fatty acids is a critical factor in determining the rate of the cycle (Devlin 2002). Regulation of pyruvate dehydrogenase, and the transport of fatty acids into the mitochondia and beta oxidation of fatty acids affect cycle activity. Because dehydrogenases are dependent on the constant reduction of nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD), which are controlled by the respiratory chain, the reactions of oxidative phosphorylation and ATP generation also control the rate of the cycle. Finally, since the reactions of the cycle are reversible, concentrations of intermediates affect cycle activity. For example, high concentrations of succinyl CoA inhibit the first reaction (formation of citrate), while high concentrations of acetyl CoA inhibits the degradation of pyruvate, and high concentrations of ATP will inhibit the formation of α-ketoglutarate, another CAC intermediate. Ultimately, the CAC serves to generate a small amount of ATP and more importantly to replenish coenzymes for the electron transport chain, the oxidation – reduction reactions that use the mitochodrial membrane to produce ATP. In the presence of oxygen, this system converts reducing agents (complete oxidation of NADH and $FADH_2$) into utilizable energy by the process of oxidative phosphorylation (Devlin 2002).

Intracellular lipid and plasma free fatty acids: Lipids are used as an energy source via beta oxidation in the mitochondrial matrix. At rest, fat provides more than 50% of energy requirements (Kronfeld et al. 1994). As the duration of submaximal exercise increases, increased oxidation of free fatty acids provides the majority of the aerobic energy supply (Essen et al. 1980; Valberg et al. 1985). During exercise, the fatty acids stored in adipose

tissue and muscles become the primary source of fat energy. In general, providing that the exercise intensity is below 75% to 80% of maximum aerobic capacity (VO_2max), fat is an important energy source. Above this exercise intensity, carbohydrates provide most of the energy for muscle contraction. Diet and feeding strategy can influence the rate of fat utilization during exercise (Hyyppa et al. 1999; MacLeay et al. 2000; Pagan et al. 2002). Generally, increased fat use will decrease glycogen breakdown and enhance performance.

Horses undertaking endurance racing are working between 30% and 60% of VO^2_{max} during which fatty acids are an important energy source. . Corn oil added to the diet of exercising Thoroughbreds (8 km) protected against a decline in blood glucose during exercise compared to grain-only diets (Duren et al. 1999). On the other hand, fat does not supply significant energy during Thoroughbred or Standardbred racing that involves exercise at an intensity well above VO_{2max}. One of the reasons for this limitation in fat use during intense exercise is the time required to generate energy (as ATP) from the breakdown of fatty acids. The breakdown of muscle glycogen can produce ATP up to six times faster compared to the breakdown of an equivalent amount of fat (Devlin 2002). Because intense exercise requires very rapid generation of ATP, this energy must be provided by carbohydrate rather than fat.

Metabolic Pathways: Fatty acid oxidation metabolizes long chain fats in two carbon units of acetyl-CoA, which then enter the CAC. Fatty acids must be activated for degradation by conjugation with CoA in a reaction catalyzed by acyl-CoA ligases

(thiokinases). The enzymes are associated with the endoplasmic reticulum and outer mitochondrial membrane and require ATP. This activation step occurs in the cytosol, but they are oxidized inside the mitochondrion. Long chain fatty acyl-CoA must be transported across the impermeable inner mitochondrial membrane. First, the acyl group is transesterified to carnitine in a reaction catalyzed by carnitine palmitoyl acyl transferase I (CPTI), located on the external surface of inner mitochondrial membrane, or CPT II, on the inner surface of inner mitochondrial membrane. The protein responsible for transferring acyl carnitine transfers acyl carnitine inward as it transfers free carnitine outward. Once inside, the acyl carnitine is transferred to mitochondrial CoA and free carnitine is transported back outward in another iteration of this shuttle (Devlin 2002). Several metabolic deficits are associated with these processes (Roe et al. 2002; Roe et al. 2008) and have been successfully treated with provision of triheptanoin, a short-chain (7 carbon), odd-carbon fat.

Beta oxidation occurs inside the mitochondrion, with four primary reactions (Devlin 2002):

1) Acyl-CoA dehydrogenase removes two hydrogen from acetyl CoA between carbons 2 and 3 (from CoA attachment), forming a trans enoyl-CoA and $FADH_2$. Three variations of this enzyme are in the mitochondria, each specific for short, medium, or long chain acyl groups.
2) Water is added across the double bond by enoyl-CoA hydratase, forming 3-L-hydroxyacyl-CoA.

3) 3-L-hydroxyacyl-CoA dehydrogenase removes hydrogen, forming 3-ketoacyl CoA, and generating NADH.
4) The terminal acetyl-CoA group is cleaved in a thiolysis reaction with CoA catalyzed by beta-ketothiolase, forming a new acyl-CoA two carbons shorter than the previous one.

Odd-chain fatty acids: Two carbons are cleaved from acyl-CoA molecules in beta oxidation in each cycle in the presence of several enzymes known collectively as fatty acid oxidase. Fatty acids with an odd number of carbon atoms are oxidized by beta oxidation producing acetyl-CoA until a three carbon residue called propionyl-CoA remains. This compound is carboxylated to D-methylmalonyl-CoA by propionyl-CoA carboxylase (biotin co-factor) with ATP and bicarbonate. After a reconfiguration reaction, L- methylmalonyl-CoA is converted to succinyl-CoA, via methylmalonyl-CoA mutase and co-enzyme B12. Succinyl-CoA is a constituent of the citric acid cycle, making odd-chain fatty acids uniquely glycogenic (Murray et al. 2006).

Of interest to this review is the CAC intermediate, succinyl CoA (4C). Short chain fatty acids (some odd carbon moieties) can be metabolized to replenish this intermediate independent of pyruvate. (Roe et al. 2002; Roe and Mochel 2006; Roe et al. 2008; Roe et al. 2006). Reactions that replenish intermediates are termed anapleurotic and can utilize odd carbon fat (Devlin 2002). The glucogenic property of odd-chain fats could be used in the dietary management of horses with polysaccharide storage myopathy (PSSM). Dietary management of horses with PSSM recommends the provision of fat as an alternative energy substrate to carbohydrate and stabilization of blood insulin levels by

limiting the daily consumption of starch. This has been very successful in decreasing muscle stiffness and eliminating episodes of exertional rhabdomyolysis (ER) in 75% of horses when combined with gradually increasing daily exercise (Firshman et al. 2003; Ribeiro et al. 2004). This increases circulating free fatty acids, thereby enhancing their availability for muscle metabolism (Ribeiro et al. 2004). Quarter Horses have a low capacity for oxidative metabolism in skeletal muscle and low intramuscular lipid stores, so exercise is likely essential to increase muscle oxidative capacity and enhance the muscle's capacity to oxidize fat (Brojer et al. 2006; Firshman et al. 2005; Ribeiro et al. 2004).

Despite the success of this dietary management, 25% of horses still have intermittent episodes of muscle pain (Ribeiro et al 2004). Odd carbon fats could potentially be of benefit to horses with PSSM by providing intermediates to the CAC that could be depleted with muscle damage (McCue et al. 2009) during acute episodes of rhabdomyolysis.

Anaerobic metabolism

Glucose and glycogen: Carbohydrate in the form of muscle glycogen is the primary source for anaerobic glycolysis (and oxidative phosphorylation) during intense exercise. Despite increased reliance on lipid use, intramuscular glycogen is also an import fuel during prolonged exercise. Intramuscular glycogen and lactate contributed approximately 50% (0 – 30 min) and 20% (60 – 90 min) of the total energy expenditure during submaximal (35% of maximal oxygen uptake) exercise of

fasted horses (Geor et al. 2000). Plasma glucose accounts for only about 10% of oxidized carbohydrate (Geor et al. 1999; Lacombe et al. 2001).

Glycolysis: The goal of glycolysis is to produce energy as ATP from the catabolism of glucose to pyruvate. Glucose in the bloodstream is transported into the myoplasm via insulin-mediated glucose transporter 4 (GLUT 4) and is locked within the cytosol as G-6-P by phosphorylation via hexokinase (Devlin 2002). A glucose molecule is then rearranged slightly to fructose and phosphorylated again to fructose diphosphate via phosphofructokinase. These steps actually require energy, in the form of two adenosine triphosphates (ATP) per glucose. The fructose is then cleaved to yield two glyceraldehyde phosphates (GPs). In the next steps, energy is finally released, in the form of two ATPs and two nicotinamide adenine dinucleotides (NADH) as the GPs are oxidized to phosphoglycerates. One of the key enzymes in this process is glyceraldehyde phosphate dehydrogenase (GPDH), which transfers a hydrogen atom from the GP to NAD to yield the energetic NADH. Due to its key position in the glycolytic pathway, biochemical assays of GPDH are often used to estimate the glycolytic capacity of a muscle cell. Finally, two more ATPs are produced as the phosphoglycerates are oxidized to pyruvate (Devlin 2002). Anaerobic metabolism provides a limited amount of energy rapidly, but in an inefficient manner, as aerobic metabolism yields 13 times more ATP per glucose than anaerobic pathways (Eaton et al. 1999a).

Purine nucleotide and adenylate kinase or myokinase cycles: The purine nucleotide and myokinase cycles are of particular interest, as past studies suggest that despite the

apparent availability of excessive glycogen for energy metabolism, horses with PSSM have decreased oxygen consumption and an energy deficit during submaximal aerobic exercise as evidenced by an accumulation of IMP (Annandale et al. 2005; Valberg et al. 1999a). The amount of IMP in some muscle fibers of PSSM horses after 11 minutes of trotting is almost as high as that found in normal horses performing at twice the speed for almost five times as long (Annandale et al. 2005; Essen-Gustavsson et al. 1989; Essen-Gustavsson et al. 1997).

When muscle contraction occurs under conditions that lead to insufficient creatine phosphate levels to buffer adenosine triphosphate (ATP) concentrations adequately, ATP levels decline. Under strenuous exercise, the myokinase reaction utilizes two molecules of accumulated adenine diphosphate (ADP) to regenerate ATP and produce adenine monophosphate **(**AMP**).** The purine nucleotide cycle occurs when the accumulation of AMP activates AMP deaminase and leads to an increase in muscle IMP (Cutmore et al. 1986; Heller et al. 1986; Jansson et al. 2002). The enzyme myoadenylate deaminase converts AMP to muscle inosine monohosphate (IMP) with the release of ammonia. The metabolic flux through the cycle has been proposed to play a role in the regeneration of ATP by pulling the adenylate kinase reaction in the direction of formation of ATP. Ammonia stimulates phosphofructokinase, a rate-limiting glycolytic enzyme. IMP increases stoichometrically as ATP decreases. When oxidative conditions are restored, AMP is regenerated with the release of fumarate. Fumarate is converted to malate, an intermediate of the CAC. The increased levels of malate drive the cycle, increasing the generation of ATP through oxidative phosphorylation (Murray et al. 2006).

MECHANISMS OF FATIGUE

The immediate source of energy for skeletal muscle is found in compounds containing high energy phosphate such as ATP and creatine phosphate. Creatine kinase and its products, creatine and creatine phosphate also play a significant role in the transport of energy from the mitochondrial matrix to myofibrils in the sarcoplasm (creatine-creatine phosphate shuttle).

Lactate concentrations in equine muscle increase in a rate proportional to the percentage of type II fibers recruited at high speeds (Rivero et al. 2007) and disappear at a rate similar to humans (Harris et al. 1987). High lactic acid levels associated with fatigue following maximal exercise are known to interfere with normal cellular metabolism (Byrd et al. 1989a; Byrd et al. 1989b; Hyyppa et al. 1999; Wilson et al. 1998). Lactic acidosis is characterized by elevated blood lactate levels (normal <2mmol/l in the horse, anaerobic threshold 4 mmol/l), decreased blood pH, and decreased bicarbonate concentrations. The significant reduction in cytoplasmic pH is suggested to be the main cause of fatigue during maximal exercise (Rivero et al. 2007). Lactate is produced when there is an insufficient amount of oxygen available for complete oxidation of pyruvate. Post exercise, most lactate is degraded to CO_2 and H_2O or converted back to glucose via gluconeogenesis, both of which require oxygen (Devlin 2002), or is converted to glucose via the Cori cycle in the liver or kidney.

Muscle glycogen becomes depleted during both aerobic and anaerobic exercise in horses (Essen-Gustavsson et al. 1989; Hodgson and Rose 1987; Snow and Mackenzie 1977; Valberg et al. 1988; White and Snow 1987). Studies have reported glycogen depletion up to 30% in single bouts of exercise in Thoroughbreds (800 -2000M) and Standardbreds (1600M) (Harris and Snow 1986; Hodgson et al. 1984; Lindholm and Piehl 1974). Longer exercise bouts (50 to 100 km) can deplete up to 75% of muscle glycogen (Hodgson et al. 1983).

Maximal exercise: As a measure of maximal exercise in horses, VO_2 max occurs when the oxygen uptake does not increase despite an increase in workload. Studies of Thoroughbred horses during maximal exercise report values of 140 – 187 ml O_2 /kg/min (Eaton et al. 1999b; Rose et al. 1988; Williams et al. 2001b). For most studies, maximal exercise is > 85% VO_2 max, and the high demands of this level of exercise requires the recruitment of most motor units of locomotor muscle fibers. Type II B motor units are recruited at near-maximal intensity and/or extremely prolonged submaximal exercise (Rivero et al. 2007).

When VO_2 max is reached, oxygen consumption plateaus and energy production is drawn from anaerobic pathways, resulting in lactate production (Essen-Gustavsson et al. 1989; Essen-Gustavsson et al. 1997; Essen-Gustavsson et al. 1999; Harris et al. 1997; Schuback and Essen-Gustavsson 1998). This point (anaerobic threshold) depends on a range of factors, including muscle fiber type composition, rate of oxygen delivery to muscles, mitochondrial density, catecholoamine levels, intracellular enzyme concentrations, prior training, nutritional status, warm up, and the rate of increase in the workload (Hinchcliff

et al. 2004). As described above, muscle can use the limited muscular stores of ATP and phosphocreatine and the adenylate kinase pathway for energy for the first few minutes of exercise. High levels of ammonia, lactate, and IMP with low ATP levels were seen after intense exercise in Standardbreds and in type 2 fibers (Essen-Gustavsson and Lindholm 1985; Essen-Gustavsson et al. 1997; Gottlieb et al. 1989; Harris et al. 1987; Lindholm and Piehl 1974). However, relatively high concentrations of ATP is sometimes found in fatigued muscle, as fatigue may be an adaptation to prevent rigor that result when ATP concentrations become very low (Wortmann 2000).

After two 900m sprints glycogen depletion of middle gluteal tissue was approximately 40% and repletion was affected by diet in thoroughbreds, with a high carbohydrate diet restoring glycogen more rapidly than a low carbohydrate diet(Snow et al. 1982; Snow et al. 1985). Lacombe et al (2001) demonstrated that glycogen stores could alter fatigue during high speed exercise, with decreased anaerobic capacity in subsequent high speed exercise challenges (glycogen depleted state). Further, glucose infusion after glycogen depletion restored anaerobic capacity.

Endurance exercise: With submaximal exercise (<85% VO_2 max), muscle glycolysis and blood glucose are the main mechanisms for providing acetyl-CoA from pyruvate. As the duration of exercise increases and higher rates of pyruvate oxidation occur, free fatty acid oxidation becomes the major source of energy for exercising muscle tissue. As a result, glycogenolysis in muscle declines and beta oxidation of fatty acids increases with

the duration of submaximal exercise. Fatigue with endurance exercise is related to the depletion of intramuscular glycogen stores (Rivero et al. 2007).

Because glycogen is a limiting fuel at low-to-moderate exercise, the capacity to maximize muscle glycogen replenishment after exercise is an important factor for optimizing performance; however complete resynthesis of glycogen pools in muscle require 48-72h post-exercise (Brojer et al. 2006; Jensen-Waern et al. 1999; Lacombe et al. 2003b; Lacombe et al. 2003b; Lacombe et al. 2004; White and Snow 1987).

II. EFFECT OF TRAINING ON SKELETAL MUSCLE

CONVENTIONAL TRAINING:

With growth and training, there is a change in the length and breadth of all fibers, and a change in the proportion of fiber types rather than an increase in the number of muscle fibers (Snow and Valberg 1994). Training or conditioning programs often strive to improve different attributes of athleticism which are realized through changes in oxidative capacity (endurance), fiber type and cross sectional area (strength), and glycolytic capacity (speed), among others. Different techniques are employed to maximize training impact. Training effects differ with age, sex, and state of training (Rivero et al. 2007).

The metabolic and contractile adaptations in skeletal muscle that are present at birth provide the means by which young foals stand within minutes of being born and by which the quick burst of speed and rapid glycolytic metabolism are used to evade predators. Equine muscle contains high muscle glycogen content and glycolytic capacity

from birth and mare's milk provides a rich source of sugar for energy metabolism. During the first years of a horse's life, there is a shift in fiber type proportions in favor of type 2a fibers at the expense of type 2b fibers (Dingboom et al 1999). Depending on the breed, there may be a gradual increase in the oxidative capacity of type 2 fibers in the first year of life, which progressively evolves over the next 2 years providing enhanced staying power and a slightly slower speed of muscle contraction (decreased type 2a:2x ratio). Initiating training at less than a year of age does not appear to hasten the changes that occur naturally with growth and its impact appears to be less in young growing horses than is seen when training is begun at 18 months to 3 years of age (Henckel 1983, Eto et al 2003).

Effect of duration: Endurance training incorporating submaximal exercise over long periods has been shown to generate hypertrophy (enlargement) in fibers of type I and IIa and increased the number of capillaries surrounding type I fibers (Rivero et al. 1993) (Rivero et al. 1995a; Rivero et al. 1995b). One study specifically designed to compare the effects of duration and intensity in Thoroughbred horses, suggests that both intensity and duration are critical factors in determining the nature and magnitude of short term (3 wk) training adaption in skeletal muscle (Hodgson 1985).

Duration was shown to have more impact than intensity on the degree of training adaption in muscle oxidative capacity and capillarization. Fiber type composition changed most with exercises of short duration (25 min), with increases in the percentage of type IIA fibers (Rivero et al. 2007). Increased oxidative capacity and capillarization in

a training/detraining study of Standardbred trotters, and a reduction in the area of type IIa fibers (Essen-Gustavsson et al. 1989). Even 10 days of low intensity work over long distances (55% of VO_2 max, 60 min)can increase aerobic capacity (Geor et al 1999).

Effect of intensity: Low intensity training has been shown to produce increases in $VO_{2\ max}$ (Bayly et al. 1983; Rose et al. 1983; Rose et al. 1988; Thornton 1985). Transformation of fiber types has also been recorded (type 2B to type 2A to type 1) in rodents and humans in response to low intensity training (Green et al. 1984). CK levels can be mildly elevated after low intensity exercise in unfit horses, even in the absence of rhabdomyolysis (Valberg 1999).

High intensity training, such as sprints, can increase anaerobic capacity as measured by increases in activities of markers of glycolytic enzymes such as phosphofructokinase. In addition, increased oxidative capacity gained with high intensity training results in an increased rate of glycogen utilization, increased run time to fatigue, and decreased concentration of lactate in muscle (Rivero 2007).

Alterations in muscle fiber types: It appears that there is generally a transformation from type IIB to IIA fibers with conditioning (Foreman et al. 1990a; Foreman et al. 1990b, Rivero 2007). Comparisons of trained and untrained Standardbreds showed no difference in type 1 fibers. However, there was an increase in IIA compared with IIB fibers, with the best performers (58% IIA, 15% IIB) having more IIA than moderate performers (49% % IIA, 26% IIB) and untrained (41% IIA, 35% IIB) horses (Essen-

Gustvasson and Lindholm 1985). Thoroughbreds in high intensity work (100% VO_2 max, 3day/wk, 3 wks) developed an increase in IIA:IIX ratio (Miyata et al. 1999; Tyler et al. 1998; Yamano et al. 2005), while Standardbreds showed an increase in type IIA fibers and IIA:I fiber ratio (35%) (Essen-Gustavsson and Lindholm 1985; Lindholm and Piehl 1974). Draft load interval-training resulted in an increase of type IIA and decrease in IIB fibers in gluteal muscle (Gottlieb et al. 1989). Submaximal exercise in Thoroughbreds on a sloped treadmill at 80% $VO_{2\ max}$ and in Arabians on a flat treadmill resulted in an increase of 11A:IIB ratio (Sinha et al 1993, D'Angelis 2005). Dressage training resulted in a 6.5 fold increase in IIA:IIX fiber ratio (Rivero et al 2001). Thoroughbreds in jumping training also showed an increase in IIA:IIX fiber ratio (Rietbroek et al 2007). Regular training in Standardbreds over a several years resulted in significant changes in muscle composition with a shift among type II fibers towards IIA (fast oxidative) fibers from IIB fibers (Roneus et al 1992).

Snow and Valberg (1994) reported increases in type 1 fibers with training, something not previously detected. They interpreted this finding as suggested the increase of type 1 fibers in response to training could be the result of fiber splitting or gradual transformation of type IIa to type I fibers, or even loss of type II fibers with age. A comparison of Dutch Warmbloods that were either stall-rested, turned out on pasture or chased in the paddock for repeated sprints from birth to 5 months of age showed little effect of exercise on contractile fiber types (Dingboom et al. 1999; Dingboom et al. 2002, Essen-Gustavsson et al 2002). However, low intensity training in young Andalusians produced an increase in IIA:IIX fiber ratios and I:II fiber ratios (Serrano and Rivero

2000). In one study, Thoroughbreds were trained on a land treadmill for 6 wks (40% and 80% VO_2 max) and then detrained for 6wks. These horses showed no change in proportions of fiber type in gluteal or bicep muscle, but an increase of 54% in capillary density in bicep muscle in the 80% VO_2 max group (Sinha et al 1993). This increase was reversed with detraining. The study suggested that a more intense exercise regime would be needed for more detectable alterations in muscle fibers.

Alterations in fiber areas: The tendency for fiber type to shift from low oxidative fibers to high oxidative fibers with training suggests that a decrease in fiber area would be expected, since high oxidative fibers generally have a smaller area than low oxidative fibers at the same depth in tissue (Snow and Valberg 1994). Smaller fiber area appears to be advantageous, as the best performing racing Standardbreds demonstrated a smaller mean area size (IIA 3075 μm^2 , IIB 3378 μm^2) of II fibers compared with moderately successful (IIA 3185 μm^2, IIB 4252 μm^2) and untrained (IIA 3714 μm^2 , IIB 5935 μm^2) horses (Essen-Gustavsson and Lindholm 1985; Lindholm and Piehl 1974). Draft loading in Standardbreds demonstrated no change in type I and IIA fiber area in gluteus muscle during training, but a significant increase in IIA fiber area (2103 μm^2 to 2632 μm^2) (Gottlieb et al 1989).

However, as different breeds have a varying muscle group proportions and different rates of maturation, and all muscles demonstrate variability in fiber type, it is difficult to generalize about increasing fiber area with maturation and onset of training. A study of young (4 year old) Andalusians ridden for dressage showed an increase in the diameter of

type I (15%) and type IIA fibers (10%). Thoroughbreds just starting jumping training also showed a 23%increase in type IIA fiber area (Gottlieb et al. 1989) (Rivero 2007).

Alterations in capillarization: Endurance training can induce growth of new blood vessels (angiogenesis), but the effect is dependent on the type and intensity of training (Jensen 2004), Near maximal training in humans (70-80% VO_2max) has been shown to increase capillarization (Denis et al. 1986). Low intensity training in humans (45% VO_2max) had little effect on capillarization(Schantz et al. 1983). In horses, five weeks of intense (up to 54 min per bout, 6 days per wk) training in Standardbred showed increased oxidative capacity and capillary density (Essen-Gustvasson 1989) .

The capillary supply to type I and II muscle fibers has been observed to increase equally in response to exercise at moderate intensities, during which mainly type I and IIA fibers are recruited. However, as exercise at higher intensities causes marked activation of type II fibers, it is reasonable to expect that training at higher intensities will also increase capillarization supplying those fibers. In Thoroughbreds trained at different intensities (as measured by blood lactate levels, 2.5 and 4 mmol/l respectively), there was an increase in capillarization in gluteus medius muscle with all fiber types except type IIX (Rivero et al 2007). The comparison of data is for fiber type I pre 11.5%, post 12.3%; type IIA pre 41.3%, post 43.5%; type IIAX pre 12.6%, post15.6 %; type IIX pre 34.4 %, post 28.6%. The highest intensity exercise (4 mmol/l, 25 min) generated a significant increase in type I (pre 11.3%, post 13.1%) and type IIAX (pre 11.5%, post 18.9%), and indeed created the greatest increase s in capillarization of all muscle fiber types. Exercise intensity affects capillarization of type IIA fibers, with increases of 6.4% between the 2.5

mmol/l and the 4 mmol/l intensities. Effect of duration was seen only in type I fibers, with an increase of 8.2% from 5 to 25 min and 12.2% in 5 to 25 min when intensities were combined.

The mechanisms that regulate exercise-induced capillarization are not well understood in human medicine, but a study demonstrated that although 5' – AMP- activated kinase (AMPK) regulates basal skeletal muscle capillarization and gene expression, it is not necessary for the angiogenic response to exercise(Zwetsloot et al. 2008).

Alterations in metabolic properties of fiber types: Training studies of 2-4 year old Standardbred, Andalusian and Thoroughbred horses show an increase in the type IIA:IIB or 2a:2x ratio and an increase in oxidative capacity (CS; 31% increase Andalusians, 50% increase Standardbreds, SDH; 37% increase Thoroughbreds (Roneus et al. 1992; Serrano et al. 2000; Yamano et al. 2005). Enhanced oxidative capacity of type 2X and 2B fibers may occur with training but this change is not permanent, as studies have shown that after 3 months of detraining oxidative enzyme activity will revert to pre-training levels (Serrano et al. 2000).

RESISTANCE TRAINING:

Resistance training in particular increases the workload on the muscle without increasing exercise speed and may in so doing provide a means to quickly enhance muscle oxidative capacity (Gottlieb et al. 1989). Further, resistance training was shown to increase the percentage of type I fibers and increase electromyographic activity in the

Brachiocephalicus muscle (Tokuriki et al. 1999). Gottlieb et al (1996) showed that a small increase (20 kilogram-force) in load resistance could increase plasma lactate (10.8 mmol/l) and heart rate (214 bpm) in Standardbreds(Gottlieb-Vedi et al. 1996a; Gottlieb-Vedi et al. 1996b). When young (18-20 months) grade horses (n=17) carrying a load of 45 kg over a period of 78 days with an increasing submaximal walk/trot regime of 70 min was compared with horses carrying no weight with the same exercise regime, no conclusive results were found (O'Connor et al 2002).

WATER TREADMILL TRAININ:

Exercising horses using water treadmills is one method of resistance training. However, while some studies explored biomechanics and heart rate there are no studies of muscle metabolic properties to indicate this modality's effectiveness. Heart rate and blood lactate levels remain unchanged by water treadmill training, and any training effect on specific muscle groups or cardiocirculatory effects remains unclear (Lindner et al 2003, Tokuriki et al 1999; Nankervis and Williams 2006; Voss et al. 2002; Reimer 2003).

Human studies suggest that it is difficult to detect the effect of resistance training provided by water treadmill use. Walking backward in a water treadmill increased heart rate and muscle activation when compared to walking forward, but there was no comparison the same exercise on a land treadmill (Masumoto et al. 2007). Hall et al (2004) compared land and water treadmill exercise in rheumatoid arthritis patient and found heart rate and perceived exertion was lower when walking on the water treadmill as compared to the land treadmill. Another study with obese humans showed no

difference in land or water treadmill training in measured VO_{2max} or lean body mass (Greene et al. 2009).

III. EQUINE NUTRITION AND IMPACT ON SKELETAL MUSCLE

DIETARY CARBOHYDRATES

The horse is a hindgut fermenting herbivore, and can digest a range of hydrolysable and fermentable carbohydrates (Hoffman 2003). The equine diet relies primarily on forage carbohydrates as an energy source, with supplementation usually consisting of grain concentrates for high performance animals. The carbohydrates found in grasses and dried forage (hay) includes simple and complex sugars, and digestible and non-digestible carbohydrates (Figure 1).

Types of carbohydrates: Simple sugars and some starches consist of chains of glucose molecules linked by α – 1,4 bonds (Cunningham 2002). Disaccharides, some oligosaccharides, and starch comprise most hydrolyzable fractions of dietary carbohydrates. Simple nonstructural carbohydrates are rapidly digested in the small intestine via hydrolysis (Hoffman 2003). Complex carbohydrates are linked using β - 1, 4 bonds, and are resistant to enzymatic action and are fermented by microbes in the hind gut producing volatile fatty acids (Cunningham 2002). Starches resistant to enzymatic action, hemicelluloses, cellulose, and lignocelluloses, soluble fibers, some oligosaccharides (fructans) make up the fermentable carbohydrate fraction (Hoffman 2001).

Sources of dietary carbohydrates for horses: Cereal grains, such as corn and oats, contain abundant starch. Pasture grass and dried grass (hay) structural and nonstructural carbohydrate content varies with season and weather (Hoffman et al. 2001) as well as other factors such as cutting and drying time (MacKay 2003). Some grasses and hays have been found to contain high concentrations (>12%) of water soluble carbohydrates such as sucrose, fructose, glucose and fructans (Watts personal communication). Consumption of forages usually creates small but measureable increases in blood glucose and insulin due to enzymatic digestion and absorption in the small intestine (Stull and Rodiek 1988).

Starch- grains: Starch, a carbohydrate, is the primary component of cereal grains (oats, corn, and barley). Oats are about 50% starch, while corn and barley have more than 60% starch. Oats contain more fiber and less starch than corn, but oat starch has been reported to be more readily digested than corn starch in the small intestine (Radicke et al 1991). The source of starch in the diet makes a difference in digestibility. For example, if oats are used as the main source of starch in a high starch diet, the oats may result in less overflow of starch into the cecum compared to corn.

Starch is highly digestible, and the rate and site of digestion is influenced by many factors other than the grain variety source. These factors include rate of intake, morphology of the foodstuff, processing of starch, size of ration, forage intake, rate of passage through the small intestine, level of amylase and individual variation of the horse (Jose-Cunilleras et al. 2006; Kienzle et al. 1997; Kienzle et al. 2002; Meyer et al. 1995; Vervuert et al. 2009).

Different processing techniques include popping, steaming, crushing, grinding, and cracking. Grinding reduces the particle size which affects the rate of passage (Jullian et al 2006). Heat and steam processing, and to a lesser degree, popping, is thought to disrupt the starch molecule and changes digestibility (Kienzle et al. 1997; Meyer et al. 1995). It is apparent that processing grains can influence the relative amounts of starch digested in the small intestine, and differences in animals responses should be expected (NRC).

Sugars- hay and grass: Plants contain simple sugars (glucose, fructose, sucrose). Many environmental factors affect the amount of sugar in plant tissue, as well as the method that the plant employs for photosynthesis. Many types of grasses used for forage use either C3 or C4 photosynthesis. C4 plants are warm weather plants, and the first product of photosynthesis is a 4 carbon acid. C3 plants are cool weather plants and produce a 3 carbon intermediary product. Examples of C3 grasses are brome, timothy, orchard, fescue, and examples of C4 grass are Bermuda, bluestem, Pangola, and many native prairie grasses. When the rate of photosynthesis exceeds respiration, sugars accumulated in plant cells. Respiration is reduced during dry or other stressful conditions (NRC). While some sugar is depleted during the curing process, hays made from stressed grasses will contain elevated sugar compared to the same species in an unstressed condition. Curing conditions and drying time can also affect sugar content in hay (Longland and Byrd 2006, Longland 2001, 2009a, 2009b).

Fructans: Fructan is produced by many C3 cool-season grasses and certain broadleaf plants. Fructan is a polymer of fructose, referred to as oligosaccharide in shorter chains. Plants accumulate fructan as a storage form of fructose during periods of stress (Watts personal communication). Fructans are polysaccharides comprised primarily of fructose and glucose, which vary in linkage and structure. In grasses, fructans are levans or phleins, and have 2-6 linkages, while fructan from broad leaved plants is stored as inulin (2-1 linkages) (Watts personal communication).

Fiber: Fiber is a variable mixture of indigestible or slowly fermenting feed components containing cellulose, hemicelluloses, some pectins, and lignin, plus indigestible protein and lipids like waxes (Mertens 1992). Van Soest (1991) has described the advantages and limitation of the different analytical procedures for fiber, most of which have been developed for ruminants or humans, and do not always translate well for horses (hind gut fermentation). Further, there is variation in how different methods determine fiber content, leading to confusion in interpretation of analysis. In general, fiber is determined as crude fiber (CF), total dietary fiber (TDF), neutral detergent fiber (NDF) and acid detergent fiber (ADF). Lewis (1995) considers NDF to include nearly all cellulose and over 50% of hemicellulose, but it also erroneously includes a high amount of amylase digestible starch.

TDF consists of the digestive resistant plant cell components, polysaccharides, lignin, and other resistant components. TDF becomes relevant to hindgut fermentation because of

the inclusion of cellulose, hemicellulose, non –α –glucose oligosaccharides, lignins, pectins, mucilages, gums, and waxes among others (Cottrell E. et al. 2005).

Measurement of dietary carbohydrates: There are two main analytical methods used to quantify fractions contained in forage, the Proximate Analysis method (Henneberg and Stohman 1860), which divides all feed into six fractions. These are water, ether extract, crude protein (CP), ash, crude fiber, and nitrogen-free extract. The weakness of this method is that although it does analyze feed for these six fractions, it does not determine how digestible the substances are within the fractions. The Proximate Analysis calculates NSC by subtraction and includes both hydrolyzable and non-hydrolyzable (fermentable) carbohydrates in the formula: NSC = 100- water-CP-fat-ash-NDF. One investigation assessing the proportion of hydrolysable carbohydrate in NSC as calculated by Proximate Analysis found 97% hydrolysable carbohydrate in concentrated feed and 33% in pasture and hay (Hoffman et al 1991).

The second method was developed by Van Soest (1991) that divides all matter into two categories – cell wall or cell content. Cell content includes sugars, starches, soluble carbohydrates, pectin, protein, nonprotein nitrogen, lipids, and water soluble minerals and vitamins. These contents are 98% digestible. Cell walls consist of cellulose, hemicelluloses, lignin, silica, keratin, waxes, cutin, insoluble minerals, lignified nitrogen compounds, and lignocelluloses. In the horse, the cellulose and hemicelluloses are partially digestible by fermentation in the hindgut.

In the Van Soest process, the hay sample is dried, then mixed with a neutral detergent. This produces two fractions - the cell contents (and pectins) and the cell walls. The cell wall fraction is called the neutral detergent fiber or NDF. The NDF is then mixed with an acid detergent and two fractions result - the digestible fraction or acid detergent soluble and indigestible or acid detergent fiber (ADF). NDF values differ from ADF in that it includes hemicelluloses. NDF analysis is considered more useful for predicting intake - the higher the NDF, the lower the intake. ADF is the plant fiber that remains (cellulose and lignin) after an acidic detergent removes more digestible cell components. As ADF increases, the digestibility of hay deceases. The lower the ADF and NDF values, the higher the quality of the forage. Higher values indicate over- or under-maturity, or heat damage from fermentation resulting from improper curing. For example, alfalfa hay with ADF < 27% and NDF < 34% are considered "supreme", while ADF > 35% and NDF > 44% is considered "utility" (Van Soest et al. 1991).

CP is the estimation of both protein and nonprotein nitrogen, or total nitrogen content of hay. Total digestible nutrients (TDN) is calculated from ADF and used to estimate the energy value of forage. It is the sum of all digestible organic nutrients (proteins, fiber, fat, nitrogen-fee extract). TDN is the most extensively used forage quality value for hay-marketing purposes. Digestible dry matter (DDM) is similar to TDN and is calculated from ADF as an estimate of the energy available in forages and is used for formulate rations. Non-structural carbohydrate (NSC) is the sum of carbohydrates, specifically water soluble carbohydrates (WSC) and starch, and some fructans. NSC is measured by

hydrolytic method and is not interchangeable with the term no fiber carbohydrate (NFC) (Smith 1981).

Water soluble carbohydrate include compounds soluble in cold water or in gastrointestinal contents such as monosaccharides (glucose, fructose, galactose), disaccharides (sucrose and melibiose), oligosaccharides (raffinose, stachyose) and some polysaccharides (Van Soest 1994). Fructans are included with the WSC fraction. WSC can be assessed by high-performance liquid chromatography (HPLC) analysis, which was determined to be less accurate in fresh cut ryegrass than for ryegrass hay (Longland and Harris 2009a).

Ethanol soluble carbohydrate (ESC, sometimes ESS) is the fraction of glucose, fructose, and sucrose soluble in 80% ethanol (NRC). Fructans are oligo- and polyfructosyl sucrose molecules, and some are included in NSC fractions. Fructan is the remainder of WSC-ESC, and can be assayed by enzymatic technique (MegaZyme), which may underestimate fructan content in timothy hay (Longland and Harris 2009b). Controversy exists on the best methodology for measuring fructan content.

Carbohydrate digestion of Starches and Simple sugars: In general terms, simple sugars and some starches are digested and absorbed in the small intestine through the action of enzymes and gastric acids acting on by $\alpha - 1,4$ bond resulting in the production of glucose (Cunningham 2002) . Interestingly, equine saliva contains little α-amylase, and limited hydrolysis occurs during mastication. Action of the pancreatic α-amylase and

amylopectinase in the small intestine produces disaccharides and oligosaccharides. The final digestion and absorption of these substances occurs in the villi, which line the inner surface of the small intestine. Digesta may pass though the small intestine in as little as 30 to 60 minutes (Dyer et al. 2002). The villi increase the surface area of the small intestine to many times and the apical surface of the epithelial cells of each villus is covered with microvilli (brush border). Incorporated in the plasma membrane of the microvilli are a number of enzymes that complete digestion, including sucrase, lactase and maltase, complete hydrolysis to yield free sugars, glucose, galactose, and fructose (Cunningham 2002). Fructose simply diffuses into the villi, but both glucose and galactose are absorbed by active transport. Transport of glucose through the apical membrane of intestinal epithelial cells depends on the presence of secondary active Na+/glucose symporters, SGLT-1, which concentrate glucose inside the cells, using the energy provided by cotransport of Na+ ions down their electrochemical gradient (Cunningham 2002).

Complex carbohydrates: Complex carbohydrate digestion occurs predominately in the hindgut or large intestine via fermentation performed by anaerobic bacteria; some in turn produce the cellulase that cleaves the β-1,4 linkages in hemicellulose and cellulose. The cecum and large colon provide the retention time, and suitable pH for bacteria to flourish. Complex carbohydrates are fermented to produce volatile fatty acids, mainly acetate, propionate, and butyrate. The relative proportions of volatile fatty acids produced are dependent on the amount of forage and concentrates fed (Cunningham 2002). Volatile fatty acids are absorbed via passive diffusion down the pH gradient of the large intestinal wall in the form of free acids. Molecular weight affects absorption, with acetate the

most quickly absorbed, and lactate the least quickly (Hintz et al. 1971). Absorbed volatile fatty acids pass into hepatic portal blood to circulate as neutral anions at blood pH (Argenzio et al. 1974).

Overloading the amount of hydrolysable carbohydrates (i.e. concentrates or cereal grains) can increase the proportion of propionate as well as lactate and reduce the proportion of acetate due to rapid fermentation (Cunningham 2002). This can in turn alter the equine microbial ecosystem, creating increased lactate levels, lowered pH, colic, or laminitic episodes (de Fombelle et al. 2004; Hudson 2001)).

Glucose uptake in liver: Once glucose is transported out of the intestine and into the bloodstream, it is taken up by many tissues; primarily by liver and skeletal muscle. Blood glucose is delivered to the liver via the hepatic portal vein, and galactose and fructose are readily converted to glucose in the liver. Uptake of blood glucose into the liver is by the high capacity, low affinity passive glucose transporter 2 (GLUT 2) in a non-insulin dependent manner.

Glucagon is released from the pancreas in response to low blood glucose and the action of glucagon is confined to the liver. Epinephrine is released in response to a threat or stress and acts on both liver and muscle glycogen. Both hormones act upon enzymes to stimulate glycogen phosphorylase to begin glycogenolysis and inhibit glycogen synthetase (to stop glycogenesis).

The reverse process, or glycogenolysis, degrades glycogen to glucose, when blood glucose levels are low. In glycogenolysis, glycogen stored in the liver and muscles is converted first to glucose-1- phosphate and then into glucose-6-phosphate. Two hormones which control glycogenolysis are glucagon from the pancreas and epinephrine from the adrenal glands. First individual glucose molecules are hydrolyzed from the chain, followed by the addition of a phosphate group at C-1. In the next step, the phosphate is moved to the C-6 position, producing G-6-P, the first step of the glycolysis pathway if glycogen is the carbohydrate source and further energy is needed. If energy is not immediately needed, the G-6-P in the liver is converted to glucose for distribution in the blood to various cells. Muscle lacks glucose-6-phosphatase so glucose is not released from muscle cells with glycogen breakdown (Devlin 2002).

Glucagon stimulates hepatic gluconeogenesis. Gluconeogenesis results in the generation of glucose from carbon substrates such as lactate, glycerol and glucogenic amino acids. In animals, gluconeogenesis takes place mainly in the liver and, to a lesser extent the kidneys. Lactate is transported back to the liver where it is converted into pyruvate using the enzyme lactate dehydrogenase via a process known as the Cori cycle. Pyruvate, the first designated substrate of the gluconeogenic pathway, can then be used to generate glucose. All Krebs's cycle intermediates, through conversion to oxaloacetate, some amino acids, or glycerol can also function as substrates for gluconeogenesis. Transanimation or deanimation of amino acids facilitates entering of their carbon skeleton into the cycle directly (as pyruvate or oxaloacetate), or indirectly via the citric acid cycle (Devlin 2002).

Role of VFA in glucogenesis:

In horses, much of the energy from forage is derived from volatile (VFA) or short-chain fatty acids that are produced by microbial fermentation in the large intestine and do not need to undergo beta-oxidation (Hoffman 2003). The release of VFAs in response to a grain meal occurs in a delayed fashion due to the transport time of the digesta through the horse's GI tract. It can remain in the small intestine for 30 -60 minutes and then in the cecum for up to 7 hours. An increase in blood glucose concentrations (and insulin levels) due to VFA production in the cecum would be delayed in relationship to the time it takes for the undigested starch to reach the cecum and the time it takes for microbial fermentation and glucogenesis (Lewis 1995).

Eades et al (2007) created a large bolus of undigested starch in the cecum by overloading carbohydrates in a meal fed to 20 adult horses. Results from this study show a peak serum insulin (46.8 ± 6.3 μU/mL) at 5 hours post carbohydrate feeding (17.6g starch/kg BW) and plasma glucose peaked (124 ± 4 mg/dl) at 4 hours post feeding, suggesting that VFAs may cause a secondary elevation in blood glucose and insulin concentrations resulting from the same meal.

The three most common VFA are acetate, propionate, and butyrate. Acetate can be absorbed directly into the bloodstream and used as energy. During exercise the absorption from the muscle tissue of VFA increases (NRC). Propionate and butyrate can also be used as energy, but must first be converted to other products and eventually to glucose.

The amount of energy derived from starch via VFA varies with amount fed and transit time through the small intestine. The digestible energy (DE) derived from VFA also varies with the particular system used to calculate energy obtained from feeds. Presently, the French system is the most fully developed for horses and the expected net energy (NE) gained from VFAs is 63-68% (NRC). The VFA produced in the cecum may be sufficient to meet up to 30% of the a horse's energy needs at maintenance (NRC). Additional VFAs are produced in the colon by bacterial activity. Horses eating primarily hay may get as much as 80% of their energy needs met from VFAs (Vermorel et al 1997a), and they can be metabolized to long-chain fatty acids or glucose (Glinsky et al. 1976). Simmons and Ford (1991) used forage fed ponies to show that up to 50-60% of circulating glucose is derived from propionate.

GLUCOSE UPTAKE IN SKELETAL MUSCLE:

Uptake of glucose into muscle and adipose tissue is mediated by insulin induced translocation of GLUT4 transporters from intracellular endosomes or by exercise induced activation of GLUT4 translocation (Devlin 2001). The effect of exercise on glucose uptake may be initiated by calcium release from the sarcoplasmic reticulum leading to the activation of other signaling intermediaries (Hayashi et al 1997).

In the absence of insulin, GLUT-4 exists in membrane vesicles located within the cytosol of cells where it cannot facilitate glucose transport. Binding of insulin to its receptor on its receptor on the plasma membrane initiates a signaling cascade that promotes translocation and fusion of GLUT-4 containing vesicles with the plasma membrane, where it can facilitate glucose transport (Murray and Knovel 2003). Insulin binding triggers autophosphorylation of the receptor, which creates a recognition motif for the binding domain of insulin receptor substrates (IRSs). These proteins are tyrosine phosphorylated upon binding to the insulin receptor leading to a cascade to activation of protein phosphoinositide 3-kinase (PI3K), which then catalyzes the formation of phosphoinsitol lipids and subsequently activates 3-phosphoinositide-dependent protein kinase (PDK1). This kinase then phosphorylates and activates the other kinases that mediate the translocation of GLUT – 4 to the cell membrane (Corcoran 2006).

GLYCOGENESIS*:*

Under conditions of adequate ATP, the liver will attempt to convert glucose into glycogen via a process known as glycogenesis. Found mainly in muscle and liver tissue, glycogen is the principal storage form of carbohydrate in animals. Insulin promotes the glucose conversion into glycogen. In the synthesis of glycogen, one ATP is required per glucose incorporated into the polymeric branched structure of glycogen. Glucose-6-phosphate (G-6-P) is a key molecule in this pathway. Glucokinase in the catalyst for the creation of glucose-6-phosphate (G-6-P) from glucose and ATP in hepatic tissue, and hexokinase is the catalyst in peripheral tissues (Devlin 2002). G-6-P is then converted to glucose-1-phosphate, which in turn forms UDP-glucose and PP_i with the catalyist action

of glucose–1- uridylyltransferase. UDP-glucose is an activated molecule, and energy for glycogen synthesis comes from UTP (Devlin 2002).

Glycogen is synthesized from monomers of UDP-glucose by the enzyme glycogen synthase, which progressively lengthens the glycogen chain with (α1→4) bonded glucose. As glycogen synthase can lengthen only an existing chain, the protein glycogenin is needed to initiate the synthesis of glycogen (Figure 2). The glycogen-branching enzyme, amylo (α1→4) to (α1→6) transglycosylase, catalyzes the transfer of a terminal fragment of 6-7 glucose residues from a nonreducing end to the C-6 hydroxyl group of a glucose residue deeper into the interior of the glycogen molecule. The branching enzyme can act upon only a branch having at least 11 residues, and the enzyme may transfer to the same glucose chain or adjacent glucose chains. It transfers a block of 7 glycosyl residues from a growing chain and transfers it to another chain to produce an α—1,6 linkage (Devlin 2002).

A mutation in the *GYS1* glycogen synthase gene has been identified as the cause for type 1 PSSM in horses (McCue et al 2008b). This mutation results in a gain of function of glycogen synthase, and as this is a highly regulated pathway results in detrimental myopathies, since glycogen synthase must be inactive for glycogen degradation to occur.

Regulation of glycogen formation and breakdown is complex, and ideally prevents futile cycles of formation and breakdown. Glycogen phosphorylase and glycogen synthase are the principal enzymes controlling glycogen metabolism and are subject to allosteric activitation by AMP and allosteric inhibition by glucose and ATP. This is to regulate the reversible phosphorylation and dephosphorylation of enzymes in response to hormone

action (Murray et al. 2006). Cyclic adenosine 5' monophosphate (AMP, cAMP) acts as a second messenger in response to hormones such as epinephrine and glucagon. Control of phosphorylase differs between liver and muscle tissue, as the role of glycogen in liver is to provide free glucose to maintain blood glucose levels, while that in muscle is to provide G-1-P for glycolysis.

In liver and muscle tissues, phosphorylase is activated by phosphorylation catalyzed by phosphorylase kinase (phosphorylase a) and inactivated by dephosphorylation catalyzed by phosphoprotein phosphatase (phosphorylase b) in response to hormonal and other signals (Figure 3). Active phosphorylase a in liver and muscle tissues is inhibited by ATP and G-6-P allosterically. In muscle, inactive phosphorylase (dephosphorylated) is activated by 5' AMP. Phosphorylase kinase is activated in response to cAMP. Increasing concentrations of cAMP activates cAMP-dependent protein kinase, which catalyzes the phosphorylation by ATP of inactive phosphorylase kinase b to active phosphorylase kinase b, which then phosphorylates phosphorylase b to phosphorylase a (Murray et al. 2006). In the liver (but not in muscle) cAMP is formed in response to glucagon, norephinephrine signals increased cAMP in muscle. Insulin can inhibit activation of phosphorylase b indirectly by increasing uptake of glucose, leading to increased formation of G-6-P, which inhibits phosphorylase kinase.

The effect of phosphorylation is reversed in the regulation of glycogen synthase. Active glycogen synthase a is dephosphorylated and inactive glycogen synthase b is phosphorylated. Six different kinases act on glycogen synthase, and insulin promotes glycogenesis in muscle while inhibiting glycogenolysis by raising G-6-P concentrations,

which stimulates dephosphorylation and activation of glycogen synthase. Dephosphorylation of glycogen synthase b is carried out by protein phosphatase-1, which is under the control of cAMP-dependent protein kinase (Murray et al. 2006).

Inhibition of glycogenolysis enhances net glycoegenesis, and inhibition of glycogenesis enhances glycogenolysis. Also, the dephosphorylation of phosphorylase a, phosphorylase kinase, and glycogen synthase b is catalyzed by a single enzyme, protein phosphatase-1, which is inhibited by cAMP-dependent protein kinase via inhibitor-1. Thus glycogenolysis can be terminated and glycogenesis can be stimulated in a coordinated fashion (Murray et al. 2006) (Figure 3).

ASSESSING INSULIN SENSITIVITY AND GLUCOSE DISPERSAL IN HORSES:

Insulin sensitivity can be assessed in horse by a variety of methods. Below is a summary of some tests as presented by McAuley et al (2001).

Hyperinsulinemic-euglycemic clamp. The gold standard for evaluating insulin sensitivity, the hyperinsulinemic-euglycemic clamp technique requires a steady IV infusion of insulin to be administered in one vein. The serum glucose level is "clamped" at a normal concentration by administering a variable IV glucose infusion in another vein. Numerous blood samples are then taken to monitor serum glucose so that a steady level can be maintained. The degree of insulin resistance should be inversely proportional to the glucose uptake by target tissues during the procedure. The IV insulin infusion should completely suppress hepatic glucose production and not interfere with the test's ability to determine how sensitive target tissues are to the hormone, theoretically. A variation of

this technique, the hyperinsulinemic-hyperglycemic clamp provides a better measurement of pancreatic beta cell function but is less physiologic than the euglycemic technique.

Insulin tolerance test (ITT). ITT measures the decline in serum glucose after an IV bolus of regular insulin (0.1–0.5 U/kg) is administered. Insulin and glucose levels are sampled over the following 15 minutes. The ITT primarily measures insulin-stimulated uptake of glucose into skeletal muscle. Because this test is so brief, there is very little danger of counter-regulatory hormones interfering with its results. (McAuley et al. 2001).

Oral Glucose Tolerance Test. This assesses small intestinal absorption of glucose, hepatic glucose uptake and the endocrine function of the pancreas. It has been found to be a valuable tool for assessing small intestinal malabsorption in horses (Mair et al 1991). This test requires an overnight fast. The oral glucose tolerance test uses IV catheters to facilitate collection of samples after a 10% glucose solution (1 g of glucose/kg, via a nasogastric tube) is administered to feed horses during a 10 minute period. A peak in blood glucose levels occurs 90-120 minutes of administration of the glucose and blood glucose levels should return to normal after 4-6 Blood samples are collected before and 2,4,8,16,30,60,90,120,150,180,and 240 minutes after glucose administration (de la Corte 1999). Oral glucose challenge results may vary with age, diet, and previous fasting and the effect of incretions may produce differences between oral and IV challenges.

Frequently sampled glucose insulin tolerance test. One practical means to assess insulin sensitivity that is in current use is the frequently sampled glucose insulin tolerance test (FSGIT) combined with Minimal Model Analysis The FSGIT is commences with a 300 mg/kg bwt IV glucose bolus, administered to each horse over a period of approximately

2 min. Venous samples are then collected via the catheter at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min. At 20 min after the glucose dosing, an IV insulin bolus of 30 mU/kg bwt is administered. Blood samples are subsequently collected via the catheter at 22, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, and 180 min after glucose administration (De la Corte 1999, Hoffman 2003). Whole blood glucose is measured immediately with a handheld glucometer.

Minimal model analysis is a mathematical construct that partitions glucose disposal. This approach has been used in human medicine, and has been proven effective in the horse (Bergman et al. 1979, Toth 2009). Glucose effectiveness (Sg) (minutes) is the capacity of glucose to mediate its own dispersal independent of a change in plasma insulin. Insulin sensitivity (Si) ($L*mU^{-1}*min^{-1}$) is the capacity of insulin to promote glucose disposal. Acute insulin response to glucose (AIRg) ($mU*min* L^{-1}$) quantifies endogenous insulin secretion in response to the glucose dose. The disposition index (DI) is an index (AIRg x Si), which describes pancreatic β-cell responsiveness and accounts for the influence of both endogenous insulin secretion and insulin sensitivity (Toth 2009).

DIETARY FAT:

Fats (lipids) in the diet have chain lengths that vary from 4 to 24 carbons, usually in even numbers, and are classified as saturated (no double bonds) or unsaturated (containing double bonds), and are not water soluble. Simple fats, called triglycerides, are composed of three fatty acids attached to a glycerol backbone. Fats can provide a large amount of caloric energy in a concentrated form, and horses are capable of efficient digestion and

utilization of dietary fat (Harris et al. 1999; Pagan et al. 2002). Once adapted, horses will digest over 90% of the vegetable oil in a ration, even when fed at levels as high as 500 – 600 ml/day (Pagan 2010). Fats are digested by lipases secreted by the pancreas.

Sources of dietary fat for horses: Fats or oils are generally used in equine diets to increase energy density and/or substitute for hydrolysable or rapidly fermentable carbohydrates in the form of cereal grains. The benefits of a high fat diet include increase energy efficiency (Geelen et al. 1999; Kronfeld et al. 1994), tractability (Holland et al. 1996), metabolic adaptations that increase fat oxidation during exercise((Dunnett et al. 2002) and increased body condition. Fat-soluble vitamins and essential fatty acids (linoleic acid and α-linolenic acid) can also be increased with fat supplementation. Kronfeld et al (2004) reported that many different types of feeds with added fats (corn oil) demonstrated a true digestibility approaching 100% but that an accommodation period was required for substrate-adaptive lipolytic enzyme changes, and that low fat diets resulted in low fat digestability. Rapid introduction of fat-supplemented diets produced some adverse effects (Kronfeld et al. 2004). The upper limit of soy oil digestibility may be 7% DM, however (Zeyner 2002), as soybean oil was shown to reduce digestibility of fiber at 1g/kg BW/d, but not at 0.7g/kg BW/d. When approaching 22 to 32% DM a significant depression of gut microbial fermentative activity and decreased fiber digestibility was detected as well (Zeyner et al. 2002). Added soybean oil to the diet changed plasma lipid concentrations and increased fatty acid flux (specifically lipoprotein lipase) suggesting increased oxidative capacity compared to a low fat diet (Geelen et al. 1999).

Animal fats: Both animal and vegetable fats and oils have been fed to horses, as well as some byproducts with relatively high fat content (e.g., stabilized rice bran, wheat germ). Two studies (McCann et al 1987; Kronfeld et al 2004) failed to detect an effect of chain length or saturation of fatty acids using vegetable oils, tallow, and vegetable-tallow blends.

Vegetable fats: Holland et al (1998) demonstrated that corn oil was most palatable to horses, and vegetable fats or oils preferred to animal fats. These include soy, canola, flax, sunflower, safflower, coconut, and peanut oils, which are all long chain fatty acids with even number carbons.

Long chain vs short chain, odd vs even: Fatty acids are simple in structure and even with their derivatives can be subdivided into well-defined families. Among straight-chain fatty acids, the simplest are referred to as saturated fatty acids. When double bonds are present, fatty acids are said to be unsaturated, monounsaturated (MUFA) if only one double bond is present, and polyenoic (or polyunsaturated fatty acids, PUFA) if they have two or more double bonds. These bonds are generally separated by a single methylene group (methylene-interrupted unsaturation). The last class is used only for fatty acids with three to six double bonds, such as those found in fish oil or in brain tissue. Fatty acids may be more complex with an odd number of carbon atoms, branched chains or contain a variety of other functional groups, including acetylenic bonds, epoxy-, hydroxy- or keto groups and even ring structures or a coenzyme A moiety (acyl CoA). These are more common in bacteria and plants fats (Devlin 2002).

Except fatty acyl-CoA, classification of fatty acids is based first on the type of carbon chain: either straight (or normal), or branched, or containing a carbon ring. In each category, subdivisions are created according to the functional groups substituted on the carbon chain. To describe the unsaturated fatty acids, two ways are offered. Chemists use the number of carbon atoms counted from the carboxyl group which put the emphasis on the double bond closest to this group (D-notations). As an example linoleic acid is 18:2 D9,12 or cis-9, cis-12-octadecadienoic acid. Biochemists or physiologists use a numbering system for the unsaturation of fatty acids, the "omega nomenclature". The double bonds are counted from the methyl group determining the metabolic family, noted by wx (w for the terminal carbon) or better n-x (n for the total number of carbon, x being the position of the distal double bond). The other double bonds are deduced from the first one by adding 3 (this is the most frequent structure, non-conjugated fatty acids, but sometimes by adding 2, these double bonds are said to be conjugated). Thus, linoleic acid would be cis-9, cis-12-octadecadienoic acid, also named in the shorthand nomenclature 18:2 (n-6). This compound has 18 carbon atoms, 2 double bonds and 6 carbon atoms from the last double bond to the terminal methyl group (Devlin 2002).

Chain length is also used for nomenclature purposes. Up to 6 carbon atoms, organic acids are considered "short-chain organic acids" and they have substantial solubility in water (Table 2). Short chain fats do not behave physiologically like other fatty acids since they are more rapidly digested and absorbed in the intestinal tract and have unique properties in regulating sodium and water absorption through the mucosal epithelium. Biochemically, short chain fats are more closely related to carbohydrates than to longer

chain fats. From 8 to 10 carbon atoms, fatty acids are said to have a medium chain Fatty acids which have 14 (or 12) and more carbon atoms are considered long-chain fatty acids (Devlin 2002).

DIGESTION OF FATS

FAT ABSORPTION BY GI TRACT:

Bile salts assist in emulsifying and reducing the size of fat globules in the small intestine, which are then hydrolyzed by pancreatic lipases before absorption into the bloodstream can occur. The lipase successively split off 2 carbons at a time, and the resultant monoglyceride are absorbed into the intestinal mucosa, where triglycerides are resynthesized. The resynthesized triglycerides, along with other lipids and some free fatty acids are combined with protein to form chylomicrons. Chylomicrons leave the mucosal cells, enter the lymph system and finally enter circulation. After being absorbed, the triglycerols are transported by lipoprotein carriers (Murray et al. 2006).

There are four classes of circulating lipids based on density; chylomicrons (in lymphatic system draining the small intestine), very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). VLDL is of hepatic origin and the vehicle of triglyceride transport to extrahepatic tissue (Murray et al. 2006).

MECHANISM AND REGULATION OF RELEASE OF FREE FATTY ACIDS:

Triglycerol stores in adipose tissue are continually undergoing lipolysis and reesterification. These two processes are entirely different pathways involving different

reactants and enzymes, allowing for separate regulation by many nutritional, metabolic, and hormonal factors. The result of these processes determines the level of free fatty acids circulating in the bloodstream. Hormone sensitive lipase (HSL) hydrolyzes triaglycerol to form free fatty acids and glycerol and is activated by glucagon and inhibited by insulin. The free fatty acids formed by lipolysis can be reconverted in adipose tissue to form triacylglycerol in a continuous cycle. Glycerol in adipose tissue is produced by glycolysis due to the lack of glycerol kinase in the tissue (Devlin 2002).

F*AT UPTAKE INTO ADIPOSE AND MUSCLE CELLS:*

Triaglycerol is transported from the intestine in chylomicrons and from the liver in very low density lipoproteins (VLDL). The clearance of chylomicrons from the blood is rapid, and larger particles are catabolized more quickly than smaller ones. Fatty acids originating from chylomicron triacylglycerides are delivered mainly to adipose tissue, heart, and muscle (80%), while 20% goes to the liver (Murray et al. 2006).

Plasma lipids consist of triacylglycerols, phospholipids, cholesterol, and cholesteryl esters, and a much smaller fraction as unesterified long-chain fatty acids (NEFA), which are the most metabolically active of the plasma lipids and must be bound to albumin in plasma or a binding protein in the cell. Free fatty acid (FFA) arises in the plasma from lipolysis of triacylglycerols in adipose tissue or as the result of the action of lipoprotein lipase during uptake of plasma triaglycerols into tissues. Some shorter chain fatty acids are more water soluble and can exist as a fatty acid anion (Murray et al. 2006).

The FFA uptake by tissues is related directly to the plasma FFA concentration, which in turn is determined by the rate of lipolysis in adipose tissue. After dissociation of the fatty acid-albumin complex, at the plasma membrane, fatty acids bind to a membrane fatty acid transport protein that acts as a transmembrane cotransporter with Na^+. Upon entering the cytosol, FFA are bound by intracellular fatty acid binding proteins(Murray et al. 2006).

EFFECT OF FAT SUPPLEMENTATION:

Fat supplementation has been shown to either increase activity of oxidative capacity enzymes(Orme et al. 1997b) or have no effect in different skeletal muscle (Geelen et al. 2000) and in different breeds ((Geelen et al 2001). In Thoroughbred horses, feeding fat or oils in concentrated feeds that replaced starches resulted in decreased blood glycemic and insulinemic responses to meal feeding (Williams et al. 2001a; Zeyner et al 2005).

Enhanced expression and activity of enzymes associated with the transport of FFA into muscle and beta-oxidation can result from high fat diets. In the horse, there is evidence that some increase in lipoprotein lipase activity (LPL), decrease in plasma triglyceride (TAG) concentrations and increase in plasma cholesterol and phospholipid concentrations occurs with fat-supplemented diets (Geelen et al. 1999; Orme et al. 1997a). As LPL is involved with the removal of FFA and glyercol from the lipoprotein particles in skeletal muscle and adipose tissues, the increase in LPL activity with fat-supplementation suggests increased availability of FFA in these tissue beds (NRC). One study suggests in fasted ponies, the fat-induced decrease in plasma TAG concentrations

may be due to a decreased de novo fatty acid synthesis in response to decreased acetyl-CoA carboxylase and fatty acid synthase activities (Geelen 2001b).

IV. EQUINE GLYCOGEN STORAGE DISORDERS

Several different glycogenoses exist in humans and in animals due to glycogenolytic or glycolytic enzyme deficiencies resulting in increased muscle glycogen concentrations (DiMauro and Lamperti 2001). Such enzyme deficiencies result in abnormal substrate utilization, interruption of the flux through the glycolytic pathway and decreased or absent lactate accumulation during exercise. These glygenoses are associated with acute, recurrent muscle dysfunction with exercise intolerance, myalgia, cramps and myoglobinuria (DiMauro and Lamperti 2001).

GLYCOGEN BRANCHING ENZYME DEFICIENCY (GBED)

GBED is a fatal disorder caused by an autosomal recessive genetic mutation of *glycogen branching enzyme 1* gene (*GBE1*) and is purported to cause at least 3% of all abortions and stillbirths in Quarter Horses (Valberg et al 2001). In a study of 7 dead Quarter Horse foals, an accumulation of an unbranched polysaccharide in tissues was found and glycogen branching enzyme activity was virtually absent in cardiac and skeletal muscle, as well as in liver and peripheral blood cells (Valberg et al. 2001). Carriers express about half the normal activity of glycogen branching enzyme and can appear clinically normal in most instances (Wagner et al. 2006). *GBE1* is responsible for the formation of α-1,6 branch points in all tissues, whereas glycogen synthase produces the straight-chain α-1,4

linkages. The numerous branch points in animal glycogen provide abundant terminal glucose residues for rapid mobilization of glucose by glycogen phosphorylase. The lack of highly branched glycogen results in a clinical systemic deficiency of glucose for energy metabolism (Valberg et al. 2001). The prevalence in Quarter Horses is estimated at 11 % for GBED (Tryon et al. 2009).

.*POLYSACCHARIDE STORAGE MYOPATHY (PSSM)*

Definition: PSSM is a one variant of episodic exertional myopathies that is found in several equine breeds (Firshman et al. 2003; Firshman et al. 2006; Valberg et al. 1992; Valentine et al. 2000; Valentine et al. 2001; Valentine and Cooper 2005). PSSM is characterized as a glycogen storage disorder because skeletal muscle has 1.5 -4 times normal glycogen concentrations and because amylase-resistant polysaccharide accumulates in the type 2A and 2B skeletal muscle fibers (Valberg et al. 1992, Valentine 2000, 2001, 2005, Firshman 2003, 2006). Two forms of PSSM have now been identified, and are denoted as type 1 and type 2. An autosomal dominant mutation in the gene encoding the skeletal muscle glycogen synthase enzyme (*GYS1*) characterizes type 1 PSSM (McCue et al 2008b). Type 1 PSSM affects at least 20 breeds including Quarter Horse-related breeds, and European breeds and their derivatives (McCue et al. 2006, 2008a). Additional cases of PSSM have been identified which do not have the *GYS1* mutation but have muscle biopsies characterized by abnormal granular polysaccharide accumulation in skeletal myofibers (McCue 2009). Allelic heterogeneity of the disease gene, environmental factors, and modifying genes may all contribute to variable phenotypic expression of PSSM horses.

Early studies of PSSM in which horses were diagnosed based on muscle biopsy findings estimated the prevalence of PSSM at 6% - 12% in Quarter Horses (McCue and Valberg 2007). Using the same diagnostic criteria, PSSM is estimated to have 34% prevalence in Belgians (Firshman et al. 2008). More recent studies of prevalence have utilized the *GYS1* mutation as the gold standard for diagnosis. In these studies, the prevalence of type 1 PSSM in 11 breeds ranges from 0.5% to 62.4% (McCue in press). Further, a study by Tryon et al (2009) looked at the prevalence of type 1 PSSM in Quarter Horse performance types. This study found the prevalence of 11.3 % for type 1 PSSM. Subgroups of the breed had different prevalence percentages (Tryon et al. 2009). Other genes that modify clinical expression of PSSM include an *RYR1* mutation, responsible for malignant hyperthermia (MH). A treadmill trial and controlled exercise trial demonstrated that Quarter Horse types with both the *GYS1* and *RYR1* mutation had a more severe clinical phenotype and higher serum CK activity than horses with the GYS1 mutation alone (McCue et al. 2009).

Environmental factors affecting PSSM include the amount and timing of exercise, number of episodes, season of the year, stall rest, and diet. It has been demonstrated that 60% of PSSM horses had episodes during exercise and that submaximal exercise causes elevated CK activity in PSSM horses (Annandale et al. 2005) . Many horses had more than one episode (85%), and 29% of episodes were associated with season. Stall rest is another factor, with 35% on stall rest prior to episode (Firshman et al. 2003). Training after stall rest, or the onset of training in young horses, can result in elevated serum CK

activity in PSSM horses (Valberg et al. 1997). Ribeiro et al (2004) demonstrated that a high starch diet results in muscle stiffness and exercise intolerance in PSSM horses.

Clinical signs of PSSM are usually associated with a bout of light exercise, and occur either during or immediately after a session. The severity of the episode varies from slight stiffness and gait abnormality to the inability for movement and recumbency. Muscles groups affected are usually those of the back and hindquarters, and usually are affected bilaterally. During an episode, muscles become firm, swollen and painful, and the animal may have profuse sweating, elevated pulse, respiration and temperature. Some horses may become colicky, and discolored urine due to myoglobin excreted via the kidneys from damaged muscle cells (Valberg et al. 1992).

The extent of muscle damage can be determined by measurement of the activity of specific muscle enzymes that have leaked into the bloodstream. Specifically, CK and aspartate aminotransferase (AST) enzyme activities can be determined. An episode of rhabdomyolysis can generate a peak in serum CK activity 4 to 6 hours later, occasionally in excess of 100,000 U/L, which returns to normal in the absence of further damage within days (Valberg et al. 1992).

Diagnosis: Currently there are two diagnostic tools available for type 1 PSSM, histochemical staining and genetic testing. Diagnosis of PSSM using tissue samples is the presence of amylase-resistant periodic acid Schiff's positive (PAS) inclusions in type 2A and type 2B muscle fibers (De La Corte et al. 1999b; Valberg et al. 1992). This is

however, not specific for genotype. Particularly in young horses, amylase resistant polysaccharide may not yet be present leading to a false negative diagnosis using muscle biopsy for diagnosis (De La Corte et al. 1999a; De La Corte et al. 1999b). The presence of aggregates of amylase sensitive polysaccharide as a diagnostic criteria for type 1 PSSM may lead to false positive diagnoses, as these criteria more commonly describe type 2 PSSM (McCue 2009). There is no record of histopathologic abnormalities or abnormal glycogen accumulation in the liver of PSSM horses, however one horse homozygous for the *GYS1* mutation had abnormal polysaccharide in cardiac muscle (Annandale et al. 2005).

Other muscle pathology which may be present in biopsies of horses with type 1 PSSM include macrophages in necrotic fibers, regenerating myofibers, anguloid atrophy, centrally located nuclei and rimmed vacuoles within fibers with abnormal polysaccharide (Colgan et al. 2006; Firshman et al. 2006; McCue et al. 2009; Valberg et al. 1992; Valberg et al. 1999b).

The *GYS1* mutation on ECA 10 is a G-to-A base substitution in exon 6, which results in and arginine to histidine amino acid substitution. A restriction fragment length polymorphism assay is used to genotype samples to identify those with the *Arg309His* allele of the *GYS1* gene. A 230-base pair segment of DNA contacting *GYS1* exon 6 and the flanking intronic sequence is amplified by polymerase chain reaction (PCR). In horses without the G-to-A, polymorphism a single restriction site for the enzyme *Hpy*CH4V is present in this fragment; in horses with the G-to-A polymorphism, a second

restriction site is created. Restriction fragments are resolved using 3% agarose gel electrophoresis(McCue 2009).

The mechanism by which a dominant genetic mutation in the *GYS1* gene causes exertional rhabdomyolysis is not known. The Arg309His substitution occurs in a critical region of glycogen synthase (GS), which appears to result in the enzyme being in a continuously active state. It appears that unregulated GS activity may be responsible for the accumulation of excess glycogen and abnormal, less branched polysaccharide in type 1 PSSM. However, how this impacts energy generation in muscle has yet to be explained (McCue et al. 2008b).

Pathophysiology: Initial studies of PSSM did not recognize that more than one form of PSSM existed and focused on glycogenolytic and glycolytic enzymes because of high resting muscle glycogen concentrations (Valberg et al. 1998). Polysaccharide extracted from PSSM muscle had a less highly branched structure (Valberg et al. 1992; Valentine et al. 2000; Valentine et al. 2001; Valentine and Cooper 2005). Mean activities of phosphorylase and phosphorylase B kinase enzymes were normal in type 1 PSSM horse muscle tissue (Firshman et al. 2003). Similarly, in PSSM muscle tissue, enzyme activities of the glycolytic enzymes phosphofructokinase, aldolase, triosphosphate isomerase, glyceraldehydes 3 phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase and lactate dehydrogenase were not different from healthy horses (Valberg et al. 1992; Valberg et al. 1998; Valberg et al. 1999a). The effect of allosteric activators and inhibitors on phosphofructokinase was not

different between type 1 PSSM and normal horses ((Valberg et al. 1998) . Thus, although glycogenolytic and glycolytic defects were the most common cause of glycogenoses in humans, type 1 PSSM horses had normal glycogenolytic and glycolytic metabolism (Brojer et al. 2006; Dranchak et al. 2007).

During maximal exercise tests horses with PSSM depleted 26% of the resting glycogen concentration and accumulate twice as much muscle lactate as normal horses, but had similar glucose-6-phosphate and pyruvate concentrations (De La Corte et al. 1999a; De La Corte et al. 1999b; Valberg et al. 1999a). This suggested that horses with PSSM could utilize glycogen as a substrate and glycolysis was functioning during maximal exertion, In contrast, PSSM horses showed exercise intolerance and muscle pain during sub-maximal exercise (Annandale et al. 2005; Valberg et al. 1998; Valberg et al. 1999b). Light exercise has been shown to augment uptake of glucose in skeletal muscle in PSSM (Brojer et al. 2006).

Muscle metabolic flux is regulated by the cytoplasmic ATP/ADP ratio. A decrease in this ratio stimulates glycolytic and oxidative metabolism during times of high energy demand. Submaximal exercise in PSSM horses does not produce differences in lactate, ATP, ADP and AMP concentrations compared to normal horses; however, IMP concentrations are significantly elevated in myofibers of horses with PSSM after light exercise (Annandale 2005). This may be attributed to energy deficits and abnormal flux of metabolic substrates in horses with type 1 PSSM.

An increased rate of blood glucose clearance has been demonstrated in affected horses after a carbohydrate meal or intravenous or oral glucose tolerance tests (De La Corte et al. 1999a; De La Corte et al. 1999b). In addition, an increased rate of whole body glucose disposal in Quarter Horses with PSSM has been shown using a euglycemic hyperinsulinemic clamp technique (Firshman 2005). During the euglycemic hyperinsulinemic clamp, Quarter Horses with PSSM require 1.4 to 2.6 fold higher rate of glucose infusion to maintain euglycemia than did healthy horses. This likely reflects an increased uptake of glucose into skeletal muscle in response to insulin, which is the site of 75 – 80% of insulin-dependant glucose disposal. Further studies showed that, during basal resting conditions, myocyte free glucose concentrations are not different in PSSM and control horses, however, after light exercise myocyte free glucose concentrations are significantly higher in PSSM horses. This suggests an increase in skeletal muscle glucose uptake in response to exercise. Despite enhanced insulin sensitivity in Quarter Horses with PSSM, GLUT 4 content and cellular distribution of GLUT 4 in the basal metabolic state does not differ from normal horses (Annandale et al. 2004; Firshman et al. 2005).

Treatment: Immediate treatment depends on the severity of the episode of rhabdomyolysis, but is focused at alleviating pain, muscle cramping and dehydration. Cessation of exercise immediately when sign appear is critical to reduce the muscle damage.

Management of PSSM: Although type 1 PSSM is due to a genetic defect, phenotypic expression can be dramatically affected by controlling environmental factors such as diet and exercise. Past studies and clinical outcomes have demonstrated the benefit of regular

exercise in conjunction with dietary management of PSSM horses, especially as it relates to reduced number of episodes of rhabdomyolysis (Brojer et al. 2006; De La Corte et al. 1999a; De La Corte et al. 1999b; Valberg et al. 1999a). The continuous production of muscle glycogen is offset by glycogenolysis associated with regular exercise. Continuous turn out or large stabling is also of benefit in this manner. Inconsistent exercise regimes, however, have been shown to exacerbate rhabdomylodic episodes and therefore should be avoided (Annandale et al. 2005; Valberg et al. 1998; Valberg et al. 1999a). Exercise has been shown to reduce insulin resistance (Carter et al. 2009; Carter et al. 2010; Pratt et al. 2006; Pratt et al. 2007), increase cardiocirculatory efficiency (Munoz et al. 1999a; Munoz et al. 1999b; Munoz et al. 1999c; Valberg et al. 1985), alter muscle fiber make up (D'Angelis et al. 2005) and metabolic capacity of muscle fibers in horses (Geor 1999; Roneus et al. 1992 ;Serrano et al. 2000; Yamano et al. 2005).

The work contained in the following dissertation examines the impact of nutrition and exercise on metabolism in healthy horses and PSSM horses. The broad approach to this research necessitates an understanding of different metabolic pathways. The impact of training on muscle metabolism, the impact of carbohydrate concentrations on equine digestion, and the impact of fat supplementation in the equine diet are explored in this research.

Table 1. Fiber type characteristics of equine skeletal muscle

Fiber Type	***Type I fibers***	***Type II a fibers***	***Type II b fibers***
Contraction time	Slow	Moderately Fast	Very fast
Size of motor neuron	Small	Medium	Very large
Resistance to fatigue	High	Fairly high	Low
Activity Used for	Aerobic	Both aerobic and anaerobic	Short-term anaerobic
Onset of fatigue	Hours	<30 minutes	<1 minute
Power produced	Low	Medium	Very high
Mitochondrial density	High	High	Low
Capillary density	High	Intermediate	Low
Oxidative capacity	High	High	Low
Glycolytic capacity	Low	High	High
Major storage fuel	Triglycerides glycogen	Creatine phosphate, glycogen, triglycerides	Creatine phosphate, glycogen
Fiber size(can vary within muscle and between muscle)	smallest	Moderate	Largest

Table 2. Some common fatty acids:

Systematic name	**Trivial name**	**Shorthand designation**	**Source**	Notes
Acetic, proprionic	Acetate, proprionate	2:0 3.0		Hindgut bacterial fermentation VFA
Butanoic	Butyric	4:0	milk fats	Butyrate, hindgut bacterial fermentation VFA
Pentanoic	Valeric	5:0	Petroleum, fementation	
Hexanoic	Caproic	6:0	Milk fats	
Octanoic	Caprylic	8:0	animal and vegetable fats	Ghrelin
Nonanoic	Pelargonic	9:0	sebaceous glands	first natural odd-numbered carbon fatty acid
Decanoic	Capric	10:0		
Dodecanoic	Lauric	12:0	coconut oil, palm oil	widely distributed saturated fatty acid
Tetradecanoic	Myristic	14:0	Coconut, milk fats,	excess induces a rise in plasma cholesterol
Hexadecanoic	Palmitic	16:0	common saturated fatty acid	peanut, soybean, corn, coconut, lard, tallow, cocoa butter palm oil
9-hexadecanoic	Palmitoleic	16.1		Animal fats
octadecanoic	Stearic	18.0		Animal fats
9-octadecenoic	oleic	18.1		Olive oil
9,12-octadecadienoic	linoleic	18.2		Grape seed oil
9,12,15-octadecatrienoic	Alpha-linolenic	18.3		Flaxseed oil
Eicosanoic	arachidic	20.1		Peanut, fish oil

FIGURE 1. Carbohydrate fractions of plant cells

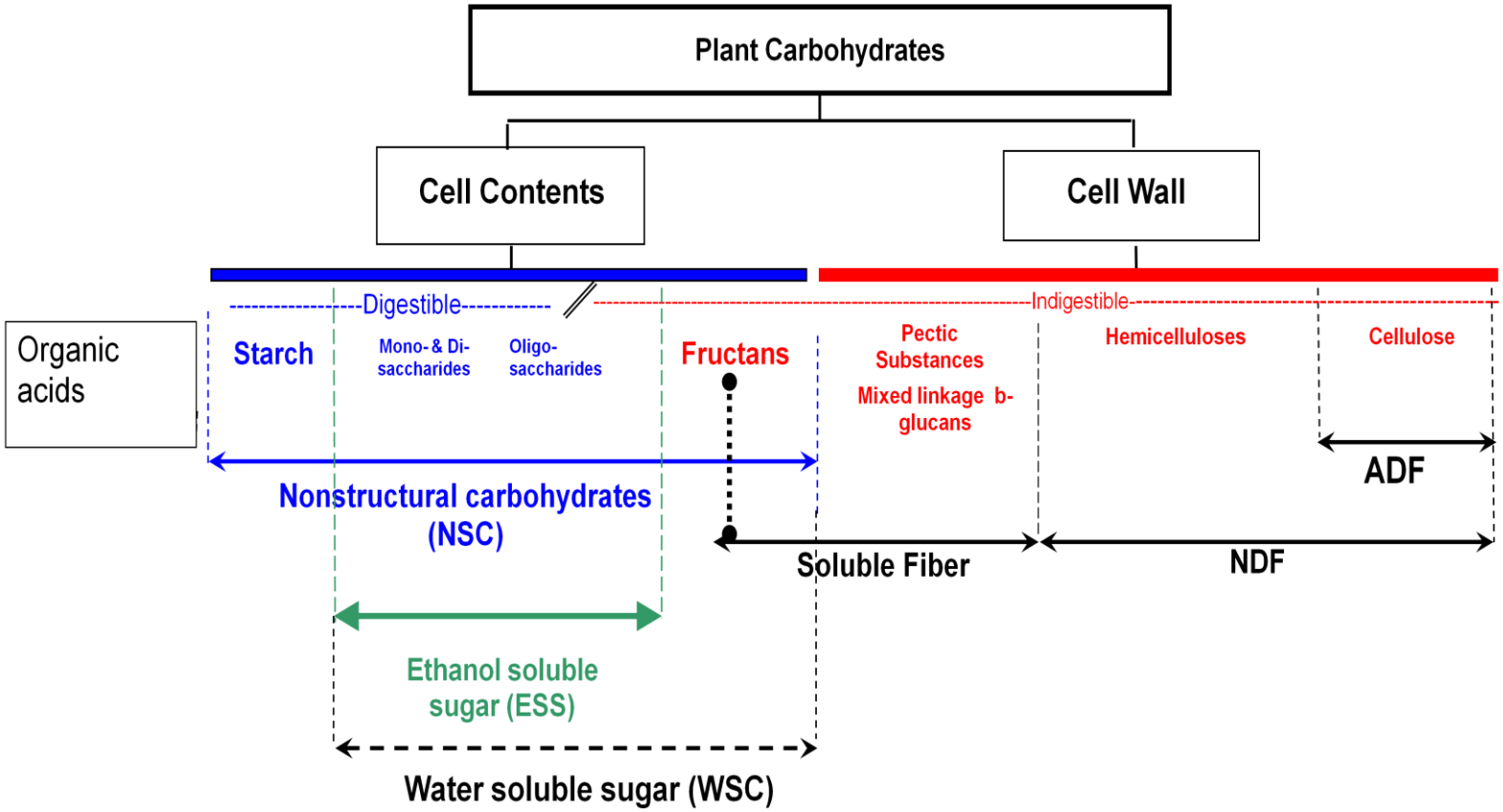


Diagram provided by Dr. Mary Beth Hall, US Dairy Research Center, Madison, WI

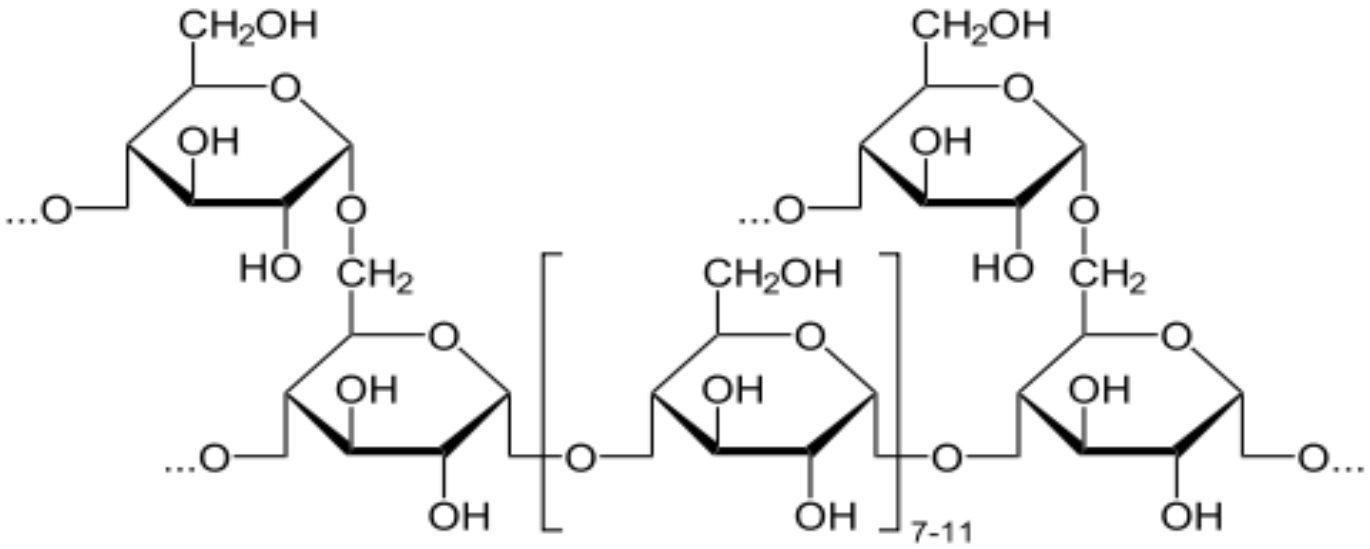
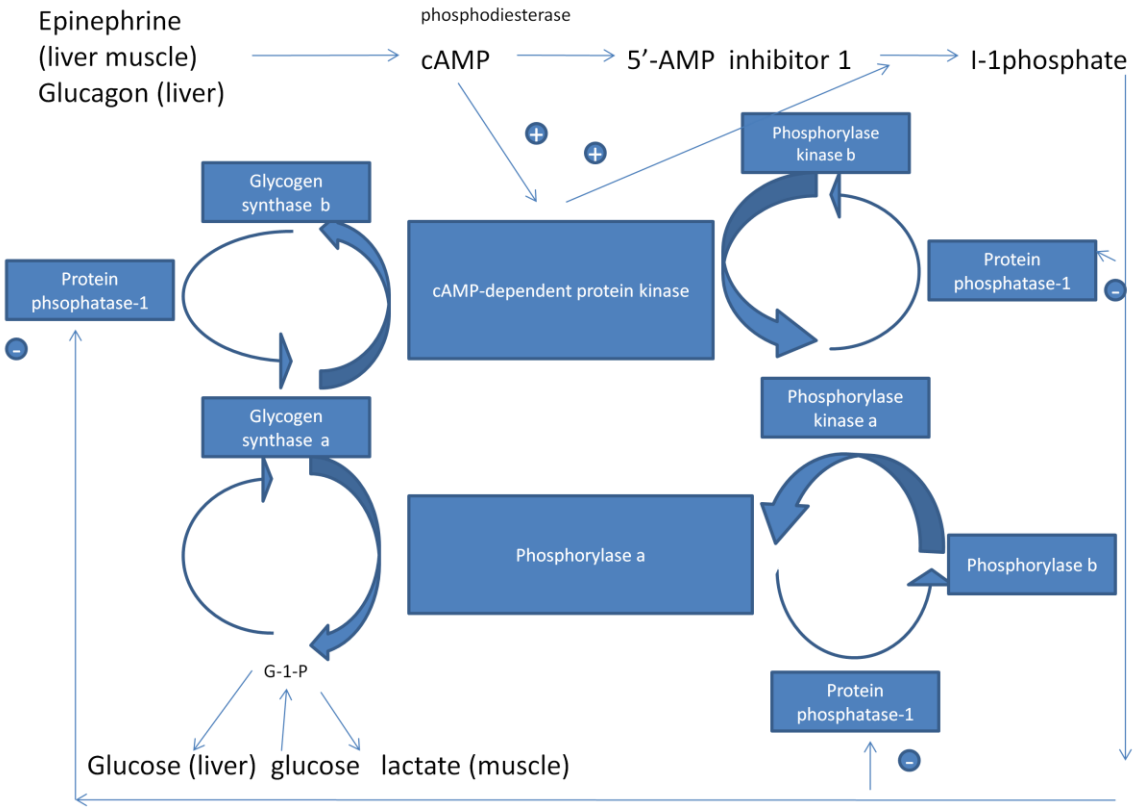


Figure 2. Glycogen molecule showing α1→4 bonds between molecules and α—1,6 branching linkage.

Figure 3. Representation of phosphorylation/dephosphorylation interaction on glycogen synthase.



CHAPTER 2

EFFECTS OF UNDERWATER TREADMILL TRAINING ON CARDIOCIRCULATORY PARAMETERS AND METABOLIC PROPERTIES OF EQUINE GLUTEUS MEDIUS AND SUPERFICIAL DIGITAL FLEXOR MUSCLES

Effects of Underwater Treadmill Training on Cardiocirculatory Parameters and Metabolic Properties of Equine Gluteus Medius and Superficial Digital Flexor Muscles

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Keywords: Oxidative Capacity, Aquatreadmill, Heart rate, Fiber Type, Exercise

Running title: Underwater Treadmill Training

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Rationale: Flexor tendon injury may be due to flexor muscle fatigue, contributing to fetlock joint hyperextension and tendon damage. A water treadmill provides resistance training on flexor tendon muscles, which might reduce the risk of tendon injury.

Objectives: Determine effect of water treadmill training on gluteal and superficial digital flexor (SDF) muscles properties and on cardiocirculatory response to a standardized exercise test (SET).

Methods: Five healthy unfit horses were trained on a water treadmill for 5 days/week for 4 weeks, starting with 5 min/day increasing to 20 min/day. Before and after the water treadmill training, an incremental SET was performed on a land treadmill to determine velocity at a heart rate 200 bpm (V_{200}) and resting gluteal and SDF muscle biopsies were obtained for biochemical analyses.

Results: There was no measurable difference in resting concentrations of gluteal or SDF muscle glycogen, lactate, ATP or glucose-6-phosphate, or activities of citrate synthase (CS), 3-hydroxyacyl CoA dehydrogenase (HAD) and lactate dehydrogenase (LDH) after training and no change in $V_{200.}$ Lactate, glycogen, G-6-P and ATP concentrations were 50% lower and type 1 fibers 30% higher in SDF compared to gluteal muscles. CS and HAD activities were similar between SDF and gluteal, while LDH was lower in the SDF muscle.

Conclusions: A more strenuous water treadmill conditioning protocol may be needed to induce a training effect in gluteal and SDF muscle and heart rate response. The low substrate concentrations and oxidative capacity of SDF may predispose this muscle to catastrophic fatigue during maximal exercise.

Introduction:

Injury to the SDF tendon generates substantial loss within the horse racing industry, (Jeffcott et al. 1982; Rossdale et al. 1985; Wilsher et al. 2006) and as many as 10% of Thoroughbred racehorses, as well as other sport horses, suffer such injury (Rossdale et al. 1985; Palmer et al. 1994; Goodship et al. 1994). Williams (2001) reported that 46% of limb injuries per 1000 British race starts over three years involved flexor and suspensory tendons. Biomechanical modeling suggests that the SDF tendon is exposed to considerable mechanical stress as it acts to stabilize fetlock and pastern joints during the stance phase, potentially flex the limb during the swing phase, and store and utilize elastic strain energy during running (Anderson and Pandy 1993; Brown et al. 2003; Zarucco et al. 2004). This stress and the relatively small cross-sectional area of the superficial digital flexor tendon in the metacarpal region (Kerr 1988) combine to create a higher frequency of tendon breakdown than what is observed in the deep digital flexor tendon (Hermanson and Cobb 1992).

The ability of the SDF muscles to maintain a forceful contraction over a range of muscle fiber lengthening and shortening, and properties of the collagen fibrils within the tendon, may influence the degree of strain placed on the SDF muscle and tendon during the stance phase (Brown et al. 2003; Swanstrom et al. 2004; Zajac 1989). Furthermore, the ability of the flexor muscles to resist fatigue while shortening may play a key role in injury to the flexor tendon following maximal exercise (Butcher 2007).

The combined effects of low oxidative capacity, lactic acidosis, and depletion of myofiber ATP and deamination with subsequent production of IMP contribute to

fatigue in equine skeletal muscle following maximal exertion (Essen-Gustavsson et al. 1997; Harris et al. 1997; Valberg et al. 1988). However, there are no studies of the metabolic properties of the SDF muscle at present, whereas there are numerous studies that describe the morphological and biomechanical properties of the SDF muscle tendon complex (Biewener 1998; Brown et al. 2003; Dowling and Dart 2005; Firth et al. 2004; Firth 2006; Hermanson and Cobb 1992; Kasashima et al. 2002; Lin et al. 2005a; Lin et al. 2005b; Swanstrom et al. 2004; Swanstrom et al. 2005; Zarucco et al. 2003; Zarucco et al. 2004).

It is well understood that the metabolic properties of skeletal muscle can be modulated with as little as10 days of training, thereby delaying the onset of fatigue (Geor et al. 1999). Resistance training in particular increases the workload on the muscle without increasing exercise speed and may in so doing provide a means to quickly enhance muscle oxidative capacity (Gottlieb et al. 1989). Further, resistance training was shown to increase the percentage of type I fibers (Gottlieb et al. 1989), and increase electromyographic activity in *brachiocephalicus* muscle (Tokuriki et al. 1999). One method that uses resistance training is exercising horses using water treadmills. These treadmills are currently popular and are being used to train horses; however, while some studies explored biomechanics and heart rate (Lindner et al 2003, Tokuriki et al 1999) there are no studies of muscle metabolic properties to indicate this modality's effectiveness (Nankervis and Williams 2006; Voss et al. 2002). One study suggested that heart rate and blood lactate levels remain unchanged by water treadmill training (Lindner et al. 2003) but any training effect on specific muscle groups or cardiocirculatory effects remains unclear.

The purpose of this study was to determine if exercising horses using an water treadmill and the manufacturer recommended protocol altered the velocity at which heart rate during maximal exercise reached 200 bpm, or altered SDF and gluteal muscle oxidative and glycolytic capacity, or the metabolite and substrate concentrations of those muscles. In addition, this study compared markers of muscle oxidative and glycolytic capacity, as well as substrate and metabolite concentrations, between the SDF and gluteal muscles.

Methods:

Horses: Five healthy horses, 1 Arabian stallion, 1 Quarter Horse Arabian cross gelding, 3 mares (one Paint, one Thoroughbred, and one Quarter Horse Arabian cross) owned by the University of Minnesota were used. Horses were unfit and had no forced exercise for at least 12 months prior the start of the study. Mean age was 6 ± 3.8 yrs. Horses were weighed and examined by a clinician prior to the commencement of any acclimation or training. During the study, all horses were cared for in accordance with principles outlined by the University of Minnesota Animal Use and Care Committee and housed in accredited facility.

Acclimation: All horses were acclimated to the high-speed (land) treadmill [a] and water treadmill [b] for two days.

Standardized Exercise Test: Prior to starting the water treadmill training regime, and at the completion of training, horses performed a SET on the high-speed treadmill at 0% slope preceded by 24 hours of rest. The SET consisted of a 4 minute walk at 1.9 m/s, followed by 2 minute intervals of trot (3.0 -3.8 m/s), canter (8.5- 9 m/s) and gallop (10m/s and 11 m/s). The horses performed until a heart rate of 200 bpm was attained. Heart rate was recorded by a Polar [c] equine heart monitor for the last 15 sec

at each speed and plotted against treadmill speed. The treadmill velocity at a heart rate of 200 bpm was considered the V_{200}. A catheter was placed in the jugular vein prior to the SET and heparinized venous blood samples from each speed were spun down in a micro centrifuge and packed cell volume (PCV) measured as a percent. Whole blood lactates were measured with a hand held lactate meter, however values proved to be inaccurate and were not included in analysis. Horses repeated the identical SET protocol after their training period.

Water Treadmill Training: The training regime recommended for bowed tendon rehabilitation by the water treadmill (Aquapacer) manufacturer was used. The water treadmill training protocol, performed daily Monday through Friday, started with 5 minutes during week 1, progressed to 10 minutes during week 2 and 15 minutes during week 3, and finished with 20 minutes during week 4. The water treadmill was filled over a period of 5 minutes until the water reached the level of the ventral abdomen while the horse walked continuously. Once the proper water level was attained, timing of the training interval commenced. After the proscribed training interval, the water level was lowered over a period of 5 minutes, while the horse walked continuously. The treadmill pace was 2.0 m/s, with slight variation for each horse's stride and ability to maintain pace. No horse exhibited discomfort or lameness; nor was any session terminated early in this study. Venous blood samples were collected 4-hr post-exercise for measurement of plasma CK by use of an automated chemistry analyzer, once per week [d].

Muscle Biopsy: Biopsy specimens were obtained at rest 24 hours after the SET from gluteal and SDF muscle in the same approximate site, but in the opposite limb, for the

first and second SET. Biopsy specimens were obtained with a 6 mm diameter Bergstrom biopsy needle by use of SC local anesthetic and incision over the right or left gluteal or SDF muscle at a depth of 60 mm for the gluteal and 10 mm for the SDF. Gluteal specimens were obtained 17 cm along a line running from the most dorsal part of tuber coxae to the head of the tail. SDF specimens were sampled caudally on the forearm, at a third of the distance distal from the olecranon between olecranon and carpus. Biopsy specimens were immediately frozen in liquid nitrogen and stored at -80 degrees C until biochemical analysis was performed. A portion of muscle was rolled in talc and frozen until histochemical analysis was performed.

Muscle Fiber Typing: Pre-training frozen muscle specimens were mounted in cross section in optimal cutting temperature (OCT) media and sectioned 10 µm thick. Muscle fiber types were determined by staining for myosin adenosine triphosphatase activity after pre-incubation at pH 4.6 (Brooke and Kaiser 1970; Pestronk et al. 1992). A minimum of 250 muscle fibers were typed per biopsy to calculate fiber type proportions.

Muscle Biochemistry: Frozen muscle specimens were lyophilized; dissected free of blood, fat, and connective tissue; and then weighed. Glycogen was assayed fluorometrically in muscle biopsy specimens as glucose residues remaining after portions (1-2 mg) of muscle tissue were boiled for 2 hours in 1 M HCl (Lowry 1972). A separate portion (4 mg) of muscle was homogenized by crushing with a glass rod in 1.5M perchloric acid and then was cold centrifuged for 10 minutes at 9300g. The supernatant was neutralized with KHCO3, centrifuged again and the remaining supernatant used for analysis of lactate, glucose 6 phosphate (G6P) and ATP

concentrations via fluorometric techniques (Lowry 1972). CS, LDH and HAD activities were determined from muscle tissue homogenized in phosphate buffer using fluorometric techniques (Essen-Gustavsson and Lindholm 1985).

Statistical analysis:

Muscle lactate, glycogen and ATP concentrations and CS, HAD and LDH enzymatic activities were compared before and after training using two-way ANOVA blocked for muscle and training state. Muscle lactate, glycogen, G-6-P, and ATP concentrations and CS, HAD and LDH enzymatic activities were compared between muscle groups using two-way ANOVA. Fiber type proportions were compared using two-way ANOVA blocked for muscle group and fiber type. The V_{200} responses to the SET were compared before and after training using paired t-tests. Significance was set at $p < 0.05$. Results were presented as mean ± SD.

Results:

Plasma CK activities were found to be within the normal range for all horses during the entire training session (272.4 ± 52.8 U/L). Horses' weight did not change (mean weight 438.2 ± 67.3 kg before training, 444.5 ± 55.0 kg after training) during the water treadmill training period.

Cardiocirculatory effects: There was no significant difference in PCV (maximum 44.4 ± 4.1% first SET and 42.9 ± 4.7% second SET) and V_{200} between the first and the second SET (Table 1). . The mean pre-training V_{200} was 9.0 ± 1.0 m/s and post-training V_{200} was 9.3 ± 2.4 m/s. Four horses attained V_{200} at a slightly higher velocity (2 at 10 m/s, 2 at 11 m/s), while one attained V_{200} at a slower velocity (9 m/s).

Training effect on skeletal muscle: The concentrations of glycogen, ATP, G-6-P, and lactate as well as activities of CS and HAD enzymatic activity, were not significantly different before and after training in either gluteal or SDF muscle biopsy specimens (Fig. 1).

Characteristics of SDF and Gluteal Muscle: The SDF (Fig. 2 A) muscle tissue had a higher proportion of type 1 muscle fibers and lower proportion of type 2B muscle fibers than the gluteal muscle (Fig. 2B). The mean percentage of type 1 fibers found in the SDF biopsy specimens was 45.2 ± 10.5% compared to 14.8 ± 7.7% in gluteal specimens. The mean of type 2A fibers in the SDF was not different (52.7 ± 12.4%) compared to the percent found in gluteal biopsy specimens (30.8 ± 12.9%) and the mean of type 2B fibers in the SDF was 2.1 ± 4.2% compared to 54.4 ± 17.8% in the gluteal muscle.

The SDF muscle had significantly lower concentrations of glycogen, ATP, G6P and lactate than gluteal muscle biopsy specimens (Table 2). CS and HAD activity were not different between SDF and gluteal muscle specimens; whereas LDH activity was significantly lower in the SDF muscle as compared with the gluteal biopsy specimens (Fig. 1).

Discussion:

The results of this study show that no demonstrable cardiocirculatory or skeletal muscle training effect occurred with 4 weeks of water treadmill exercise using the protocol recommended by the consultant for the water treadmill manufacturer. SET

V_{200} is often used as used as a measure of cardiocirculatory fitness (Persson 1997) and has been shown to increase with training in horses (Evans et al 2006). No significant change in V_{200} occurred in the present study after water treadmill training in agreement with results seen by Lindner et al (2003) in their study of water treadmill conditioning. This may not be surprising, since heart rates of approximately 78 bpm have been previously reported during water treadmill exercise at a walk (Nankervis and Williams 2006; Voss et al. 2002) and walking produces little change in blood lactate concentrations (Weber et al 1987).

Furthermore, no change in oxidative capacity occurred in either the SDF or gluteus medius muscle in the present study. Using weights for resistance training, Standardbreds performing 3 to 5 intermittent bouts of 2 min trot (7m/s) on a treadmill while pulling weights 3 times per week showed no change in V_{200} during a SET after 4 weeks of training; however, there was an increase in gluteal muscle CS activity after 2 weeks of exercise (Gottlieb-Vedi et al. 1996). Based on the lack of change in SET V_{200} and muscle oxidative enzyme activities, it would appear that in order to induce a training effect with a water treadmill, a protocol involving more prolonged exercise sessions, greater water resistance and/or velocity of exercise will be necessary.

The muscle fiber type composition of the biopsy specimens of SDF muscle tissue in this study was similar to those identified in a previous study of Standardbreds, Thoroughbreds and Quarter Horses (Hermanson and Cobb 1992). In the previous study, fiber type composition of the midbelly of the SDF muscle was type 1 54 ± 6 %, type 2A 45 ± 5 %, and type 2B 1 ± 1%. Past research has shown that fiber type composition of gluteal muscle can vary by breed (Rivero and Diz 1992, Snow and

Guy, 1980), and by sample depth (Rivero et al.1993). Less is known about variability in fiber type distribution in SDF muscle by breed. It is possible that variability in the fiber type composition in the present study is due to the study horses being of different breeds.

Based on previous findings of fiber type composition in SDF and gluteus medius muscle specimens, the expectation was that SDF muscle would have a higher oxidative and lower glycolytic capacity than gluteal muscle, which has a high proportion of type 2B fibers. It is well known that type 1 fibers have high oxidative and low glycolytic capacity relative to type 2B fibers (Valberg et al. 1988). While glycolytic capacity was lower in the SDF than was found in gluteal biopsy specimens, surprisingly no difference was detected in the activities of oxidative enzymes CS and HAD between the two, despite a 3-fold higher percentage of type 1 fibers found in the SDF specimens. This confirms previous reports, which determined that contractile fiber types in horses do not always correspond to their expected metabolic properties (Karlstrom et al. 1994; Valberg et al. 1988).

The apparently low oxidative capacity of the SDF muscle specimens might suggest a greater reliance on anaerobic glycolysis during muscle contraction. However, the SDF does not appear highly suited for anaerobic metabolism, as indicated by low LDH activity and low resting concentrations of glycogen and ATP found in the specimens. The resting ATP concentrations in SDF muscle tissue samples were 50% lower than resting gluteal muscle levels, and only slightly higher than concentrations measured in fatiguing gluteal muscle after maximal exercise (Valberg 1987; Schuback and Essen-Gustavsson 1998). Rat soleus muscle, which is predominantly slow twitch fibers like

the SDF, has a resting ATP concentration of 18 mmol/kg dry weight (Meyer and Terjung 1979). These past findings make the 12-14 mmol/kg measured in the equine SDF muscle in the present study surprising. It is possible that the small number of biopsies collected were not a comprehensive representation of the mean type 1 fibers, as one study showed that SDF muscle has a unique and uneven distribution of type 1 fibers (Hermanson and Cobb 1992). While SDF muscle, by its slow contractile nature, requires less ATP, the combination of low oxidative and glycolytic capacities and low substrate concentrations in the SDF muscle could potentially predispose this muscle to early onset of fatigue during maximal exercise.

ATP and adenine nucleotide depletion are believed to be strong contributing factors to fatigue at maximal exercise intensity (Essen-Gustavsson et al. 1999; Essen-Gustavsson and Jensen-Waern 2002; Schuback and Essen-Gustavsson 1998). With the very low resting ATP concentrations found in the SDF muscle specimens in this study, any further decline in ATP concentrations with maximal anaerobic exercise could contribute to the inability to prevent hyperextension of the fetlock, with subsequent damage to tendon collagen fibrils.

Another potential interpretation of why the metabolic capacity found in SDF muscle varies from what is expected based on fiber composition relates to the function of muscle-tendon complex. Previous studies demonstrate that the SDF muscle functions in a passive manner as a support to the fetlock joint and does not actively flex the forelimb (Swanstrom et al. 2005). The muscle-tendon complex stiffens as the fetlock hyper-extends (Hermanson and Cobb 1992), and dampens high-frequency oscillations during loading (Wilson et al. 2001). Swanstrom et al (2004) demonstrated that the

SDF muscle-tendon complex has a relatively small active force component, suggesting a primary function in storing energy during loading. In a related study (Zarucco et al. 2004), it was determined that the SDF contribute to predominately tendinous support with little muscle fascicular shortening during stance at rest and in locomotion. In comparison, the gluteal muscle is well characterized as actively generating propulsion and locomotion (Essen et al. 1980). It is therefore possible that the metabolic properties of the SDF tissue relative to gluteal tissue reflect a more passive role during locomotion.

In conclusion, the water treadmill protocol used in this study did not produce an increase in fitness as measured by V_{200} and by muscle oxidative enzyme activities. If increased fitness was the purpose for water treadmill use, then a more strenuous protocol would be needed to provide intended results. Furthermore, the finding of approximately 50% lower oxidative, glycolytic and substrate concentrations in the SDF than gluteal muscle at rest suggest this muscle is either passive during locomotion or could be precariously predisposed to fatigue during maximal exercise.

Footnotes:

[a] SÄTO AB Knivsta Sweden
[b] FernoVeterinary Systems, Wilmington, Ohio
[c] HRM USA Inc. Warminster PA
[d] Marshfield Laboratories, Marshfield WI

Table 1. Mean heart rates ± SD during a standardized exercise test (SET) before and after a 4 week period of underwater treadmill training for 5 horses recorded during the last 15 seconds of 2 minute intervals at different speeds on a land treadmill. One horse reached V_{200} at 9 m/s, two reached V_{200} at 10 m/s and two reached V_{200} at 11 m/s.

Speed (m/s)	**Heart rate (bpm) Before Training**	**Heart Rate (bpm) After Training**
0	44 ± 8	41 ± 6
1.9	98 ± 13	101 ± 12
3.4	131 ± 14	129 ± 11
9	195 ± 8	192 ± 9
10	204 ± 6	203 ± 8
11	209 ± 3	208 ± 1
5 minutes after exercise	111 ± 6	108 ± 7

Table 2. Concentration of substrates and metabolites at rest in the gluteus medius and superficial digital flexor muscle (SDF).

Substrate or Metabolite (mmol/kg)	Gluteus medius		SDF	
	Before	After	Before	After
Glycogen	517 ± 92 §	582 ± 130 §	254 ±48	271 ±29
ATP	23 ± 6 §	24 ± 4 §	12 ± 4	14 ± 2
Lactate	20 ± 9	17 ± 7	8 ± 4	8 ± 3
G-6-P	2.3 ±.6 §	2.4 ± .4 §	1.2 ± .4	1.4 ± .2

§ =Significant difference between gluteus medius and superficial digital flexor muscle biopsy specimens for corresponding time point No difference was detected between values for biopsy specimens obtained before and after training within the same muscle.

Figure 1. Citrate synthase (CS), 3-hydroxyacyl CoA dehydrogenase (HAD) and lactate dehydrogenase (LDH) ± sd from gluteal (white) and superficial digital flexor (black) muscle biopsies taken from 5 horses before and after water treadmill training. LDH activity was significantly lower in SDF muscle than gluteal muscle. CS, HAD, and LDH activities did not change with training.

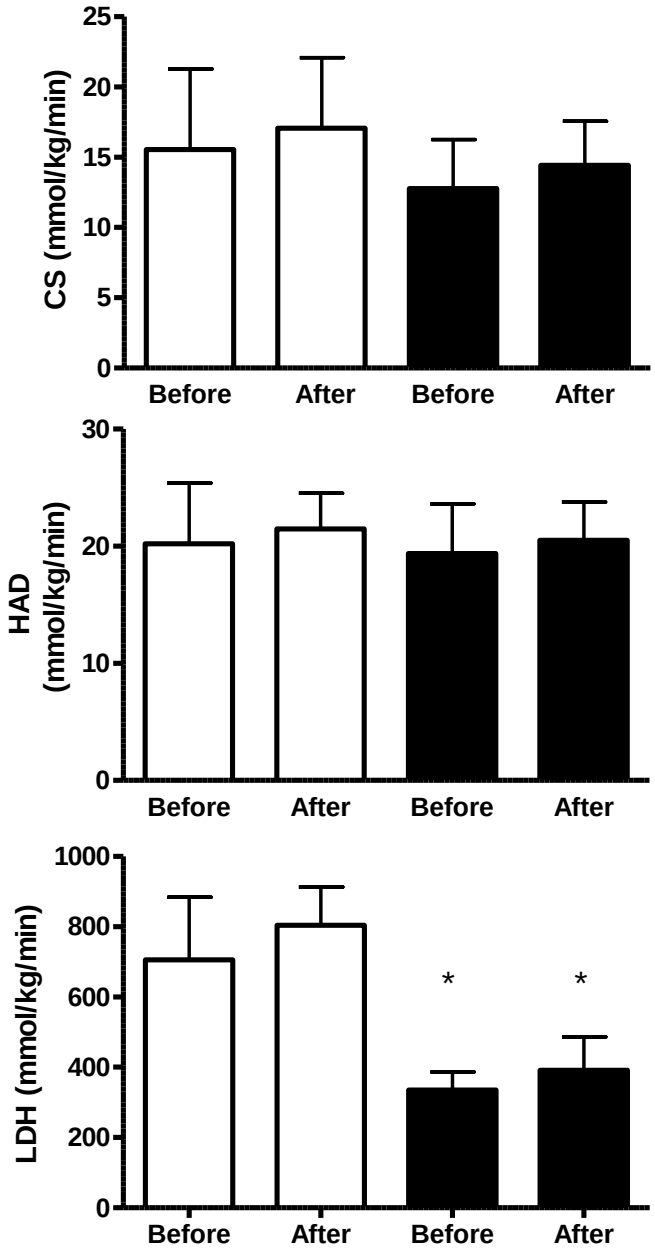
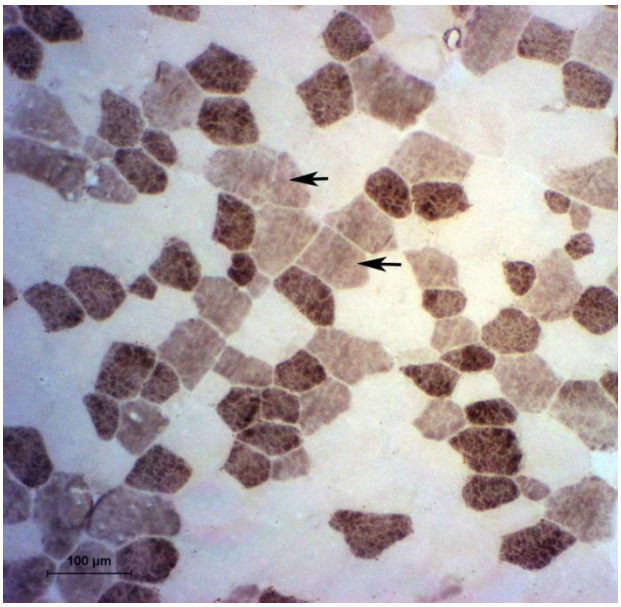


Figure 2. Muscle fiber type composition of the SDF (A) and gluteus medius muscle (B). Note the proportion of the type 1 muscle fibers (black) than type 2B muscle fibers (arrow) in the SDF compared to gluteus medius muscle

A

B



CHAPTER 3

GLYCEMIC AND INSULINEMIC RESPONSE TO FEEDING HAY WITH DIFFERENT WATER SOLUBLE CARBOHYDRATE CONTENT IN HEALTHY AND POLYSACCHARIDE STORAGE MYOPATHY-AFFECTED HORSES

TITLE: Glycemic and insulinemic response to feeding hay with different water soluble carbohydrate content in healthy and polysaccharide storage myopathy-affected horses

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Objective: To determine if the glycemic/insulinemic responses to hay with nonstructural carbohydrate (NSC, soluble carbohydrate) of 17% (HC), 10% (MC) or

4% (LC) differs in control horses and if these responses differ between control and horses with polysaccharide storage myopathy (PSSM).

Horses: 5 clinically normal control horses and 7 PSSM horses, all unfit and of Quarter Horse breeding (age 9.4 ± 3.4 yrs, BCS range 4.5 – 6).

Methods: A crossover design compared the HC and LC hay, with horses randomly assigned to hay type for 5 days, and all horses fed the MC hay during washout, after which the diets were switched. Horses were fed 1.5% BW (as fed) divided into 2 feeding per day; no grain. On morning of the 5th day of each block (7th day for washout) horses were given 0.5% BW in hay, blood was drawn before and every 30 min for 5 hours after feeding, and rate of intake was measured. Whole blood glucose and plasma insulin were measured.

Results: Intake rate was significantly higher for HC. In control horses, the insulin AUC (6891.7 ± 3524.2 HC vs.1185.4 ± 530.2 LC) was significantly higher. PSSM horses had significantly higher glycemic and insulinemic responses to HC vs. LC however; the magnitude of insulin response was lower and glucose response higher in PSSM vs. control horses.

Relevance and conclusions: Results suggest that insulin responses can differ significantly with the NSC content of hay. Feeding hay with 17% NSC produces elevations in insulin that could be detrimental for PSSM horses.

Introduction:

Altering dietary components has become a key strategy for managing muscle disorders in horses (Valberg et al. 1997, Valentine et al. 1998). One such disorder is Polysaccharide Storage Myopathy (PSSM), which is characterized by clinical signs of muscle pain and cramping with exercise (Firshman et al. 2003, Valberg et al. 1997). Muscle damage in horses with PSSM is associated with a deficit in energy generation and adenine nucleotide degradation during short-term aerobic exercise (Annandale et al. 2005). Several studies show that feeding sweet feed or other grain-based concentrated feeds that contain a large amount of starch to PSSM horses (Firshman et al. 2003, Ribeiro et al. 2004, Borgia et al. 2010) exacerbates exercise intolerance and muscle pain in PSSM. PSSM horses appear to have limited substrate availability for skeletal muscle oxidative metabolism, which is impaired by the starch and sugar (nonstructural carbohydrate (NSC)) content of the diet (Borgia, et al. 2010). PSSM horses have enhanced sensitivity to insulin compared to control horses since they secrete relatively less insulin in response to the same glucose load (De La Corte et al. 1999a).

One potential mechanism for the impairment in energy generation in PSSM horses may be that high NSC concentrates increase serum insulin, which has a stimulatory effect on glycogen synthase. Increased insulin concentrations could further perturb the already enhanced glycogen synthase activity present in PSSM horses as a result of a dominant gain of function mutation in the *glycogen synthase 1 (GYS1)* gene (McCue et al. 2008). Disruption and dysregulation of oxidative substrate flux and energy generation may then occur. The precise reason for the limitation in oxidative flux of

glycogen to pyruvate to acetyl CoA in PSSM muscle has yet to be elucidated (Borgia et al 2010).

Feeding a diet low in starch (<15% of DE as NSC) and high in fat and fiber in conjunction with a regular exercise program dramatically reduces signs of muscle pain in PSSM horses (Firshman et al. 2003, Ribeiro et al. 2004). This feeding regime produces a low glycemic and insulinemic response, while providing an alternate energy source in the form of plasma free fatty acids and volatile fatty acids (VFA) (Ribeiro et al. 2004). Although hay is usually the primary source of energy for PSSM horses, no studies have at this time evaluated whether the NSC content of hay, provided primarily by water-soluble carbohydrates (WSC), affects the glycemic or insulinemic response to a meal.

Sucrose, fructose, and glucose are components of WSC, as well as some poly- and oligosaccharide fructans, although not all oligosaccharides are fructans. The presence of fructans in the estimated NSC content, as well as the prolonged time needed for hay consumption and digestion, may mitigate a measurable glycemic response to forages (Cottrell E. et al. 2005, Hoffman et al. 2001). Whereas simple sugars produce a glycemic response via enzymatic digestion, fructans are typically digested by hind gut microbial fermentation and therefore may not have a major impact on the glycemic or insulinemic response to fed hay (Longland 2001). However, feeding inulin (one form of fructan) added to hay (1500g/500kg BW) to ponies has produced an increase in insulin levels when compared to feeding hay alone (Bailey et al 2007).

The objectives of the present study were to determine if the glycemic and insulinemic response to a meal of hay is impacted by the NSC content of hay and to determine if the postprandial glycemic and insulinemic response to hay with high or low NSC content differs between PSSM horses and control horses.

Materials and Methods:

Horses: Five healthy unfit control horses (QH or Paint breeding; all housed in Minnesota), mean age 9.4 ± 2.7 yr, weight 498.1 ± 22.1 kg and 7 unfit PSSM horses (2 Appaloosa, 2 QH, 3 QH-cross; 2 housed in MN and 5 housed in Kentucky), mean age 9.4 ± 4.5 yr, weight 531.2 ± 46.3 kg were used. Horses had not been exercised for at least 12 months prior to the study. All horses were housed in stalls at night and turned out on drylot (MN) or wore muzzles that did not allow them to graze but allowed them to drink while turned out on fall pasture (KY) during the day. All horses were fed mixed grass hay (at 1.5% BW as fed), a vitamin/mineral supplement, free choice salt, and no grain for at least 1 month prior to and during the study. The body condition score for all horses ranged from 4.5 – 6 out of 9 (Henneke) for the at least the previous 4 months, and all horses received annual dental examinations. Frequently sampled IV glucose tolerance testing (FSIGT) was performed prior to the present study (Hoffman et al 2003) and all horses were within two standard deviations (4.28 ± 2.56 L/min/mU) of the mean insulin sensitivity (Si) reported for control Quarter Horses by Toth et al (2010). Horses were cared for in accordance with principles outlined by the University of Minnesota Animal Use and Care Committee.

Dietary NSC: High NSC (HC) (Italian Rye, *Lolium multiflorum*, 17.1% NSC), low NSC (LC) (Blue Grama, *Boutelous gracilis*, 4.4% NSC), and medium NSC (MC)

(mixed grass, MN 10.7% NSC; KY 10.6% NSC) hays were identified by estimating NSC (WSC plus starch) and fructan (WSC- ethanol soluble carbohydrate) (Table 1)[a]. This method has previously been used to provide a rough estimate of fructan content for fresh ryegrass, but less accurate when compared to HPLC analysis for ryegrass hay (Longland and Harris 2009a). Fructan and starch contents were subsequently assayed by enzymatic technique (MegaZyme)[b], which may underestimate fructan content in timothy hay (Longland and Harris 2009b). While controversy exists on the best methology for measuring fructan content and may not be considered as validated for horse feeds

(Longland and Harris 2009b), these two methods were used to determine if fructan content was similar across hay types. As such, the fructan content is provided for completeness only. The sugar profile of HC and LC hay was analyzed using high-performance liquid chromatography (HPLC)[c]. The HC and LC hay were both harvested from single monoculture fields, and duplicate samples were submitted for hay analysis. The gross energy (GE) of each hay type was estimated using equations from Pagan (1998) assuming GE of NSC and NDF of 4.15 kcal/g. The digestible energy (DE) calculated from equations using Fonnesbeck (1981) assuming coefficients of 90% for NSC and NDF.

Study design: A crossover design was used to compare the HC and LC hay with horses randomly assigned to consume one of the hay diets for 5 days. Three PSSM and 3 control horses were fed the HC hay and 4 PSSM and 2 controls were fed the LC hay in the first block. This was followed by a washout period when all horses were fed MC hay for 7 days, after which the hay diets were reversed. Horses were fed 1.5% BW (as fed) of hay, divided into 2 equal feedings per day (7:00 and 15:00 h).

[a] Equi-Analytical Laboratories, Ithaca, NY 14859
[b] NP Analytical Laboratories St Louis, MO 63164
[c] NP Analytical Laboratories St Louis, MO 63164

Identical HC and LC hay and locally purchased MC hay of mixed grass composition (primarily orchard grass mixed with other grasses) and similar NSC (MN 10.7%; KY 10.6%) were fed to all horses. While the study design focused on comparison of HC and LC hay, we also examined the effect of feeding MC hay by conducting the same sample collection on the last day of the washout period as performed for the HC and LC hays.

Sample collection: On the fifth day of each block (seventh day of the washout period), after a 12-hr fast, a jugular catheter was placed 1 hour before time-zero blood samples were drawn from each horse. Horses were then fed 0.5% BW (as fed, equivalent to 33% daily DE) in hay divided into 1 kg portions (for ease of monitoring intake) every 30 min, (or upon complete consumption of 1 kg portion if this occurred before 30 min). Horses consumed the hay continuously under this protocol, and the time to consumption of hay or the residual hay left was recorded. Blood samples were taken prior to the portions being fed and every 30 min for 5 hr thereafter. Whole blood glucose was measured immediately with a handheld glucometer[d] previously validated in the horse (Wess and Reusch et al. 2000a). Blood samples were centrifuged at 2000 rpm x g within 30 min, and the plasma removed and frozen at -20 degrees C, and plasma insulin was measured by radioimmunoassay (Reimers et al. 1982). This assay provides accurate results in the horse for concentrations not requiring dilution (Tinworth et al. 2009).

Statistical analysis: Mean time to consumption for all horses was analyzed by GLM ANOVA blocked for hay type and disease using NCSS[e]. The glycemic and insulinemic response was defined as all of the values measured between time zero and

[d] AccuCheck Handheld Glucometer
[e] NCSS Kaysville, Utah 84037

300 min after feeding and was analyzed by repeated measures ANOVA, blocked for diet and disease. Analysis included MC hay with the qualification that it was not fed in a block design. Area under the curve (AUC) for blood glucose and plasma insulin from a baseline of the individual value at time zero was calculated by trapezoidal method of numerical integration using NCSS[f] and was compared by GLM ANOVA, blocked for hay type and disease, using NCSS[g]. Baseline and peak glucose and insulin values as well as time to peak glucose and insulin were compared by two-way ANOVA, blocked for diet and disease. This study did not use an ideal randomized design for assessing MC hay and therefore, primary comparisons focused on HC vs. LC hay. Tukey-Kramer multiple comparison test was used to determine differences in means. Linear regression analysis was performed in GraphPad Prism[h] to determine a potential correlation between time to consumption and peak glucose, peak insulin concentrations, and glucose and insulin AUC. To control for individual variation in insulin and glucose responses a paired t-test was also performed on baseline glucose and insulin, peak glucose and insulin as well as AUC for glucose and insulin for the HC and LC hay. Further, the peak insulin concentration on HC was divided by peak insulin on LC and the relative difference in peak insulin concentration determined. Results are expressed as mean ± sd with significance set at $p<0.05$.

Results:

Dietary Energy and NSC: The total estimated GE was similar amongst the hay types; however, total estimated DE was higher in the HC compared to the LC hay (Table 1). WSC content of the HC hay was nearly 4 times greater than the LC hay and the DE

[f] NCSS Kaysville, Utah 84037
[g] NCSS Kaysville, Utah 84037
[h] GraphPad Prism 5 San Diego, CA USA 92121

provided by estimated NSC was at least 3 fold higher for HC than LC hay (Table 1). The WSC content and DE from NSC of MC hay were nearly 2 times greater than the LC hay (Table 1 and 2). The HC hay contained more sucrose, glucose and fructose than LC hay (Table 2). The NDF content and the DE from NDF was lowest in the HC and highest in the LC hay, and the HC had higher protein concentration than the LC or MC hays although the DE from protein was only moderately higher for HC than LC hay (Table 1 and 2). The estimated fructan content of the LC and HC hays were both < 3.5% (MegaZyme) (Table 2).

Time to consumption: When data for control and PSSM horses were combined, the HC hay was consumed faster than the LC hay (Table 3). However, when PSSM and control horses were analyzed separately, there was no difference between the rate of consumption for the HC and LC hay (Table 3). Control horses had a significantly faster rate of intake than the PSSM horses for all hay types. The rate of intake increased similarly by 1.4 times for both PSSM and control horses eating HC compared to MC hay (Table 3). There was no significant correlation detected between time to consumption of HC hay and peak glucose concentration (r^2 =0.029). A weak negative correlation was detected between time to consumption and peak insulin concentration for all horses combined (r^2 = - 0.251). Linear regressions comparing the rate of intake (kg/min) to glucose and insulin AUC and peak values were not significant except for a significant negative correlation found with peak insulin in PSSM horses on HC hay (r^2 = -0.6).

Baseline glucose and insulin: There was no significant difference in baseline glucose and insulin amongst hay types (Table 4) or between PSSM and control horses (Table 4).

Glycemic and insulinemic response of control horses: There was no significant difference in postprandial glycemic response assessed by repeated measures ANOVA (Figure 1), peak glucose, time to peak or AUC between hay types for the control horses (Table 4). There was a large variation in AUC between individual horses. Insulinemic response assessed by repeated measures ANOVA (Figure 1), peak insulin, and AUC were significantly higher on the HC hay compared to the LC and MC hay, with the exception that the AUC was not different between HC and MC hay (Table 4). Control horses had similar peak values on the LC or MC, but higher peak values when fed HC (range peak insulin HC 40.9 - 77.6 µU/ml, MC 13.7 - 24.2 µU/ml, LC 11.2 - 24.7 µU/ml). Glycemic and insulinemic responses (Figure 1), peaks, and AUC when MC and LC hay was fed were not different (Table 4). There was no difference in time to peak insulin concentrations for any of the hay types (Table 4). Similar statistical results were obtained when comparisons were made between HC and LC hay using paired t tests.

Glycemic and insulinemic response of PSSM horses: The postprandial glycemic response of the PSSM horses was significantly higher after eating HC hay compared to the LC hay based on a repeated measures ANOVA (Figure 1). PSSM horses on the HC hay had a significantly higher postprandial insulinemic response based on repeated measures ANOVA (Figure 1) but not peak or AUC when compared with responses to LC hay (Table 4). There was a large variation in AUC between horses. Glycemic and insulinemic responses (Figure 1), peaks, and AUC for MC and LC hay were not different (Table 4). There was no difference in time to peak glucose or insulin concentrations for any of the hay types (Table 4). Similar statistical results

were obtained when comparisons were made between HC and LC hay using paired t tests.

Glycemic and insulinemic response of PSSM vs. control horses: A significantly higher postprandial glycemic and insulinemic response (Figure 1) and blood glucose peak (Table 4) occurred in PSSM vs. control horses when hay types were combined in the analysis, whereas glucose AUC was not different (Figure 1) (Table 4). On the HC hay, there was a significantly higher glycemic response as well as significantly lower insulinemic response (Figure 1), peak insulin value and AUC insulin in PSSM compared to control horses (Table 4). On the LC and MC hay, no significant differences were noted between PSSM and controls (Table 4). There was no difference in time to peak glucose or to peak insulin between control and PSSM horses (Table 4). When the relative change in peak insulin concentration was examined by normalizing the HC peak to the LC peak PSSM horses appeared to have a lower peak when consuming HC than controls (Figure 2). There was very little relative difference in the peak glucose between PSSM and control horses (Figure 2).

Discussion:

The faster time to consumption for high NSC hay in the present study may have been due to higher palatability of HC hay (Ordakowski-Burk et al. 2006, Ralston and Baile 1983), which had a lower NDF (grade 2 vs. 5)[i] and higher NSC content, when compared with the LC hay. It is unclear why control horses consumed the HC hay faster than PSSM horses and further research with additional horses in the same facility would be required to determine if this is a repeatable finding. A faster rate of consumption of hay could result in a more rapid rise in blood glucose and insulin. In

[i] Hay Marketing Task Force of the American Forage and Grassland Council Rating System

the present study, however, there was not a significant correlation between the rate of hay consumption and peak blood glucose or insulin concentrations. This is in agreement with a study of 12 Quarter Horses by Zeyner et al (2006) that found that time to total consumption did not affect the time to reach peak glucose concentrations after feeding 3 different low starch high fat concentrates and one high starch concentrate. This same study reported a lower glycemic and insulinemic response when the horses were fed the low starch high fat diets compared to a high starch grain meal (Zeyner 2006).

Postprandial glycemic responses were not impacted by the NSC content of hay in control horses in the present study. The glycemic response of a feed relative to a standard such as oats is often used to assess the metabolic impact of feeds (Kronfeld et al.2004, Rodiek, and Stull 2007, Stull and Rodiek 1988). The glycemic index of feeds such as rice bran, beet pulp, soy hulls, and alfalfa are reported to have a similar glycemic impact when compared to each other using this method of measurement (Rodiek and Stull 2007). However, results from the present study suggest that the glycemic response may not be the best means to assess the metabolic impact of a feed, as although hay with 4% and 17% NSC produced a similar glycemic response in healthy horses, the insulinemic response was significantly higher when the 17% was fed, compared to the 4% NSC hay. Thus, use of glycemic response alone may not reflect the true insulinemic response to feeds. A low glycemic response could also occur if the starch contained in a meal of high-amylose cereal grain such as corn has avoided enzymatic digestion in the small intestine (Harris and Geor 2009). This undigested starch could produce undesired hind gut fermentation.

The apparent disjoint between glycemic and insulinemic response to feeds containing a high NSC component has been reported previously (Hoffman et al. 2003a, Hoffman et al. 2003b, Kronfeld et al. 2004, Rodiek and Stull 2007, Stull and Rodiek 1988, Vervuert et al. 2003, Vervuert et al. 2004, Vervuert et al. 2009, Williams et al. 2001). Similar mean postprandial blood glucose concentrations were reported for isocaloric diets of corn or a combination of alfalfa and corn; whereas mean postprandial insulin concentrations were significantly different (5.7 ± 0.4 µU/ml alfalfa vs. 22.9 ± 2.2 µU/ml alfalfa and corn) (Stull and Rodiek 1988). In addition, a recent study that evaluated the glycemic/insulinemic response to increasing starch intake also found that the insulinemic response is more informative in determining the effect of feeding different NSC loads than blood glucose response (Vervuert et al. 2009). The high degree of variation in glucose responses to grain-based and forage-based diets of different glycemic loads between studies and between individual horses also led Kronfeld (2004) to conclude in his review that the use of glycemic indices in equine dietetics is limited.

In the present study, there was a dramatic increase in insulin concentrations following consumption of high NSC hay by control horses. In fact, if a single blood sample for insulin was taken approximately 90 min after feeding HC hay, the control horses in the present study might erroneously have been considered insulin resistant (mean insulin at 90 min 34.9 ± 20.1 µU/ml). Mean peak insulin values for control horses consuming 33% of daily DE as a meal of HC hay were higher (60 µU/ml) than those reported for Quarter Horses consuming 25% of daily digestible energy as a meal of alfalfa (9 ± 3 µU/ml) or corn (29 ± 10 µU/ml) (Stull and Rodiek 1988). Similar values were reported for alfalfa combined with corn (50 ±14 µU/ml)(Stull and Rodiek

1988). However, these comparisons should be interpreted bearing in mind that methodologies may have varied in insulin assays between 1988 and present. Peak insulin concentrations in control horses were not as high as those reported after a meal of sweet feed fed at 40% DE (117 ± 37 µU/ml) (De La Corte et al. 1999b). The marked insulin response to high NSC content of hay in healthy horses in the present study suggest that HC hay could have a detrimental effect if fed to horses with insulin resistance, such as horses with laminitis, metabolic syndrome and Cushing's disease. Acknowledging that the present study includes a small sample of PSSM horses, it is interesting to note that peak insulin concentrations in PSSM horses consuming the HC hay were lower than in control horses. Lower insulin responses in PSSM versus controls were also reported when sweet feed, oral glucose and IV glucose were administered (De La Corte et al. 1999a, De La Corte et al. 1999b). This has been attributed to increased insulin sensitivity in PSSM horses (Annandale et al. 2004, De La Corte et al. 1999a, De La Corte et al. 1999b) but could also reflect differences in digestion of carbohydrate. Glucose concentrations were higher in PSSM vs. control horses fed HC hay, whereas lower glucose was reported in PSSM vs. control horses when sweet feed, oral glucose or IV glucose were administered (De La Corte et al. 1999a, De La Corte et al. 1999b). The higher glucose concentrations detected in PSSM horses on HC hay in the present study compared with previous studies may reflect a lack of fitness, age difference, or individual variability (Annandale et al. 2004, De La Corte et al. 1999a, De La Corte et al. 1999b, Firshman et al. 2003, Firshman et al. 2005, Ribeiro et al. 2004). A similar relative increase in glucose peaks for both control and PSSM horses was observed by normalizing the HC glucose peak to the LC glucose peak.

The present study sought by design to determine if hay with a high NSC content generated a significant glycemic or insulinemic response in horses with PSSM. As the present study measured the response to a single meal of hay, comparisons to other studies measuring the response to single meals were made. The daily starch and WSC load fed to a 500-kg horse in this study was 1,282 g for the HC hay and only 330 g when fed the LC hay and resulted in a higher insulinemic response for the HC vs. the LC hay. The magnitude of increase in insulin (7-fold) in PSSM horses fed 33% DE in HC hay was similar to what was detected when feeding 20% of DE in sweet feed (7-fold mean base insulin approximately 7, peak approximately 49 µU/ml) reported previously (Ribeiro et al. 2004). Thus, since feeding 17% NSC hay produces a similar insulinemic response as sweet feed in PSSM horses, and this feed enhances muscle pain, it seems advisable to feed hay with NSC content less than 17%. Although the randomized design in the present study compared HC vs. LC hay, analyses were performed on the MC (washout) hay data as well to determine if a pronounced glycemic and insulinemic response could be detected. The similar minimal glycemic and insulinemic response to MC and LC hay suggests that a recommendation to limit the NSC content of hay to 11% or less for PSSM horses would be appropriate, although the study design did not block for the order in which MC was fed to study horses.

In addition to providing a low glycemic load (NSC <15% daily DE), an important component of management of PSSM horse's diet is to provide fat as an alternate energy source (15% of daily DE) (McKenzie et al. 2003, Ribeiro et al. 2004). The total digestible energy and starch and sugar load, including nutrients in hay, should be considered when formulating a feeding strategy that provides enough energy while

maintaining proper body condition. Feeding lower NSC hay may be advantageous to provide room to supplement the diet with calorie dense fat without excessive weight gain. Many PSSM horses are relatively easy keepers. For example, a 500-kg horse on a routine of light exercise generally requires 18 MCal (75.3 MJ)/day. Fed at 1.5 % of body weight, the HC hay provides an estimated 17 MCal/day (71.2 MJ), the MC provides 15 MCal/day (62.8 MJ) whereas the LC hay provides 14.4 MCal/day (60.3MJ). Thus, the daily calories that could be provided by fat without weight gain would be 1 MCal (4.2 MJ) (120 ml of vegetable oil) for HC hay which would provide 5.6% of DE (estimated) as fat. For MC hay, 3 MCal/day (12.6 MJ) (360 ml of vegetable oil) could be provided by fat which represents 16.7% of DE. For LC hay, 3.6 MCal/day (15.1 MJ) (420 ml of vegetable oil) could be provided by fat which represents 20% of DE (Lewis 1995). The lower caloric density of LC or MC hay may be beneficial in allowing a greater amount of fat to be fed without detrimental weight gain compared to HC hay. These calculations do not consider the amount of fat contributed by the hay to DE (0.05(0.21) – 0.06(0.25) Mcal/kg(MJ) per analysis). Further, individual variation in the utilization of dietary fat may affect DE.

It is reasonable to conclude that the results of the present study suggest that feeding hay with an NSC of 17% or greater that provides 31% of DE as NSC may be a poor choice for PSSM horses, because of the glycemic and insulinemic response such hay induces. Feeds providing >15% DE as NSC have previously been shown to trigger rhabdomyolysis in PSSM horses (Ribeiro et al. 2004). In contrast, hay with an NSC content of <12 % does not appear to produce the same high postprandial glycemic or insulinemic responses in PSSM horses. The importance of limiting the NSC content of hay may depend upon the total daily caloric requirements and the necessity of

providing calories in the form of fat without producing excessive weight gain. Further, in healthy Quarter horses, the insulinemic rather than the glycemic response may provide the most accurate assessment of the metabolic impact of feeding forage.

Acknowledgements:

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Table 1. Nutrient analysis of hays used in this trial. Values represent single analyses performed at Equi-Analytical Laboratories.

Assay (% as fed)	**Italian Rye (HC)**	**Blue Grama (LC)**	**Mixed MN (MC)**	**Mixed KY (MC)**
Dry Matter (DM)	91.7	94.0	89.6	91.6
Protein	12.1	9.7	11.8	10.2
Starch	1.3	0.4	0.9	0.8
NDF	46.4	66.2	54.0	56.7
ADF	31.0	38.9	34.2	36.9
WSC	15.8	4.0	9.8	9.8
ESC	10.2	1.3	8.9	6.4
Sugar Profile				
Fructan *	3.3	2.1	n/a	n/a
Fructose	2.0	0.9	n/a	n/a
Glucose	2.4	1.0	n/a	n/a
Sucrose	7.2	<0.2	n/a	n/a
Maltose	0.2	<0.2	n/a	n/a

*Fructan measurements in HC hay were lower when assayed by Megazyme than when estimated by WSC-ESC and are included for completeness only. The fructan content represented here was determined by Megazyme assay technique, which is not presently validated for horse forage performed at NP Analytical Laboratories.

Table 2. Estimated digestible energy (DE) and estimated gross energy (GE) for nutrient components in the hay fed in this trial.

Mcal/kg (MJ) **as fed**	**Italian Rye (HC)**	**Blue Grama (LC)**	**Mixed MN (MC)**	**Mixed KY (MC)**
Total GE	3.5 (14.8)	3.7 (15.5)	3.6 (14.9)	3.6 (15.0)
Total DE *	2.3 (9.5)	1.9 (8.0)	2.1 (8.9)	2.0 (8.3)
DE protein	0.5 (2.3)	0.4 (1.7)	0.5 (2.2)	0.4 (1.7)
DE NDF	1.0 (4.0)	1.3 (5.4)	1.1 (4.6)	1.1 (4.6)
DE NSC	0.7 (3.0)	0.2 (0.7)	0.4 (1.8)	0.4 (1.7)
DE fat	0.06 (0.25)	0.05 (0.21)	0.06 (0.25)	0.05 (0.21)

*The DE was calculated assuming GE of NSC and NDF (http://www.ker.com/library/advances/107.pdf) of 4.15 kcal/g and digestibility coefficient of 90% for NSC and 45% for NDF (http://www.ker.com/library/advances/108.pdf).

Table 3: Time in minutes (mean ± SD) for complete consumption of 0.5% BW (as fed) of hay types by PSSM and control horses.

Time (min)	**Italian Rye (HC)**	**Blue Grama (LC)**	**Mixed (MC)**	All hay types combined
PSSM (n=7)	104 ± 28[a]	131 ± 31[a]	140 ± 46[a]	125 ± 17[†]
Control (n=5)	68 ± 25[a]	93 ± 24[a]	96 ± 16[a]	86 ± 15
All horses combined (n=12)	89 ± 31[§]	145 ± 33	122 ± 42	

HC is high, LC is low and MC is medium nonstructural carbohydrate hay.
† PSSM significantly different from controls for all hay types combined
§ HC significantly different from LC and MC when data from PSSM and controls are combined
[a] different letters indicate significant differences within rows

Table 4. Mean (± sd) area under curve for whole blood glucose (g) and plasma insulin (i) response and peak values for PSSM and control horses. Significance is set at $p<0.05$.

High hay (HC)	**PSSM**		**Controls**		
	Units				
AUC, g	4410.0 ± 4023.9	[1,a]	3012.0 ± 2620.6	[1,a]	
Peak, g	130.6 ± 19.1	[1,a]	115.4 ± 10.1	[1,a]	mg/dL
Time to peak g	94.3 ± 40.4	[1,a]	126 ± 44.5	[1,a]	min
Baseline g	98.7 ± 10.1	[1,a]	92.8 ± 11.5	[1,a]	mg/dL
AUC, i	4716.2 ± 4376.3	[1,a]	6891.7 ± 3524.2	[1,b]	
Peak, i	31.6 ± 23.1	[1, a]	59.9 ± 17.7	[1,b]	µU/ml
Time to peak i	90.0 ± 30.0	[1,a]	111.4 ± 22.7	[1,a]	min
Baseline i	3.9 ± 2.7	[1,a]	3.9 ± 0.8	[1,a]	µU/ml
Low Hay (LC)	**PSSM**		**Controls**		
AUC, g	2605.7 ± 2339.5	[1,a]	1581 ± 2300.8	[1,a]	
Peak, g	122.0 ± 7.0	[1,a]	113.0 ± 7.5	[1,a]	mg/dL
Time to peak g	102.0 ± 62.2	[1,a]	102.0 ± 62.2	[1,a]	min
Baseline g	97.1 ± 5.8	[1,a]	97.4 ± 7.8	[1,a]	mg/dL
AUC, i	1331.1 ± 2313.4	[1,a]	1185.4 ±530.2	[2,a]	
Peak, i	19.3± 13.7	[1,a]	17.7 ± 5.5	[2,a]	µU/ml
Time to peak i	158.6 ± 33.4	[1,a]	111.4 ± 61.8	[1,a]	min
Baseline i	5.0 ± 2.9	[1,a]	4.4 ± 1.3	[1,a]	µU/ml
Medium hay (MC)	**PSSM**		**Controls**		
AUC, g	4212.9 ± 2301.7	[1,a]	2421.0 ± 1340.6	[1,a]	
Peak, g	128.0 ± 4.6	[1,a]	119.8 ± 5.2	[1,a]	mg/dL
Time to peak g	114.0 ± 53.7	[1,a]	132.0 ± 45.5	[1,a]	min
Baseline g	97.1 ± 7.6	[1,a]	97.0 ± 6.2	[1,a]	mg/dL
AUC, i	2229.3 ± 1363.6	[1,a]	1980.2 ± 952.8	[2,a]	
Peak, i	19.0 ± 11.5	[1,a]	19.1 ± 5.3	[2,a]	µU/ml
Time to peak i	154.3 ± 20.7	[1,a]	120.0 ± 17.3	[1,a]	min
Baseline i	3.5 ± 2.1	[1,a]	3.2 ± 1.5	[1,a]	µU/ml
All hays combined	**PSSM**		**Controls**		
AUC, g	3742.9 ± 2962.2	[a]	2338.0 ± 2087.4	[a]	
Peak, g	126.9 ± 12.0	[a]	116.1 ± 7.8	[b]	mg/dL
Time to peak g	135.7 ± 43.1	[a]	114.0 ± 51.0	[a]	min
Mean Baseline g	99.5 ± 8.7	[a]	95.1 ± 9.6	[a]	mg/dL
AUC, i	2758.8 ± 3172	[a]	3352.4 ± 3272.9	[a]	
Peak, i	23.3 ± 17.1	[a]	32.2 ± 22.7	[a]	µU/ml
Time to peak i	114.3 ± 37.5	[a]	108.0 ± 47.8	[a]	min
Mean Baseline i	5.4 ± 4.6	[a]	4.4 ± 1.2	[a]	µU/ml

[1] Different numbers indicate significant differences for corresponding values within column.[a] Different letters indicate significant differences for corresponding values within row.

Figure 1. Mean ± sd postprandial glucose and insulin concentrations for polysaccharide storage myopathy-affected and control horses fed high (solid square), medium (open triangle) and low (solid circles) nonstructural carbohydrate hay. Insulinemic response for control horses was significantly higher on HC than either MC or LC. Glycemic and insulinemic responses for PSSM horses were significantly higher on HC than MC or LC. PSSM horses had a higher glycemic and lower insulinemic response than control horses on the HC but not the MC or LC hay. Repeated measures ANOVA, significance $p<0.05$.

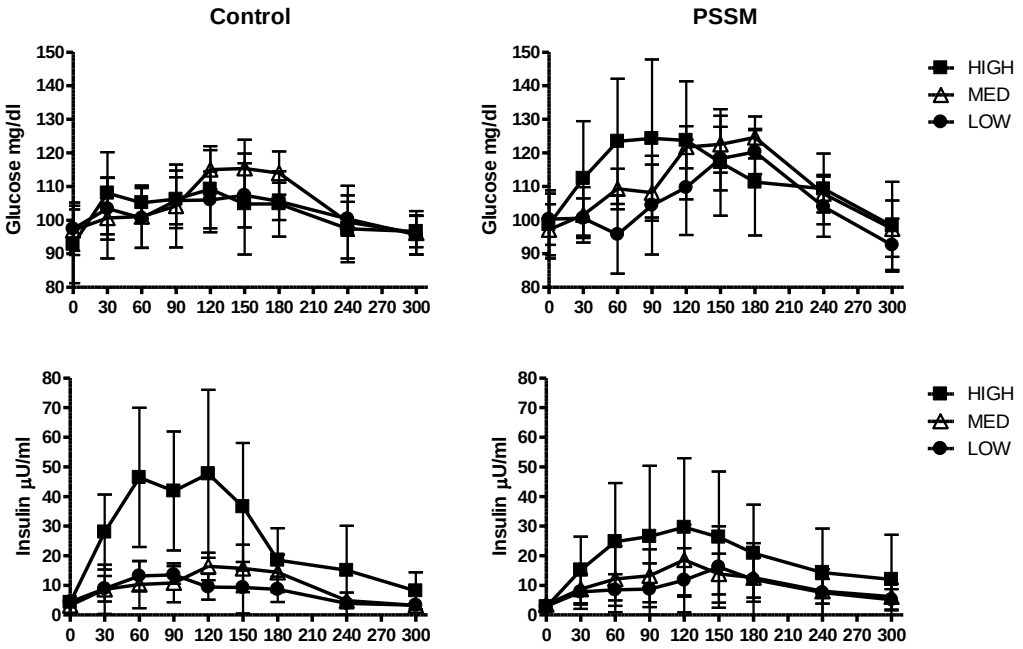
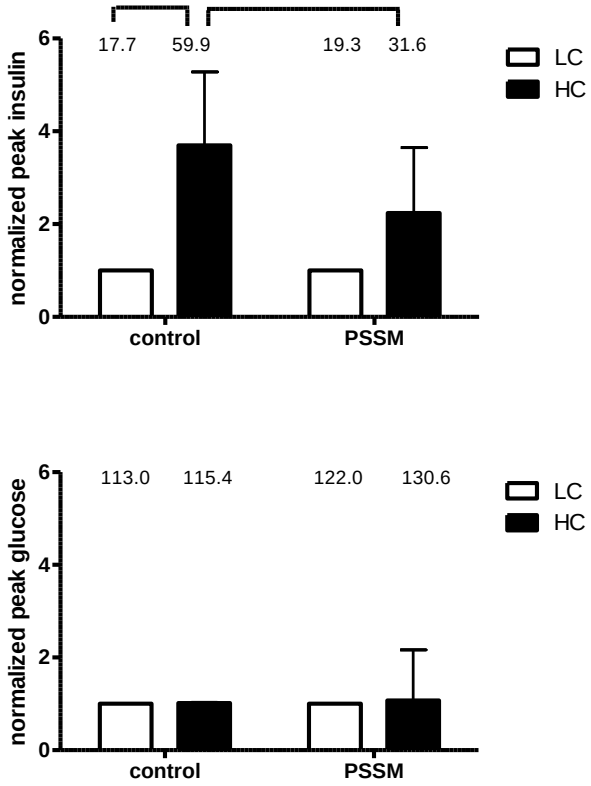


Figure 2. The relative change in peak insulin concentrations with the Italian Rye (HC) response normalized to the Blue Grama (LC) hay.



Significant differences ($p<0.05$) between peak concentrations are indicated by lines. Actual peak insulin response values represented

CHAPTER 4

GLYCEMIC AND INSULINEMIC RESPONSE TO FEEDING HAY WITH DIFFERENT WATER SOLUBLE CARBOHYDRATE CONTENT TO THOROUGHBRED AND QUARTER HORSE CROSSES

Glycemic and insulinemic response to feeding hay with different nonstructural carbohydrate content to Thoroughbred and Quarter Horse crosses

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Objectives: 1) To determine if glucose effectiveness and insulin sensitivity assessed by a frequently sampled glucose insulin tolerance test (FSGIT) differed between Thoroughbred (TB) and Quarter Horse (QH) breeds. 2) To determine if the glycemic/insulinemic response to hay with varying nonstructural carbohydrate (NSC) differed between TB and QH.

Horses: 5 QH-type mares and 5 TB geldings, all healthy and unfit. Mean ages and weights: QH 9.4 ± 2.7 yrs, 498.1 ± 22.1 kg; TB 12.6 ± 3.8 yrs, 565.0 kg ± 33.9 kg.

Methods: The FSGIT used an IV bolus of 300 mg/kg glucose followed 20 minutes later by 30mU/kg insulin. A crossover design compared 1.5% BW/day of HC (high nonstructural carbohydrate, 17.1% NSC), and LC (low carbohydrate, 4.4% NSC) hay, fed for 5 days each with the MC (medium carbohydrate, 10.7 - 10.6 % NSC) hay fed during a 7 day washout, after which the diets were switched. On the 5^{th} day of each block, horses were fed 0.5% BW in hay. Blood was sampled before and every 30 min for 5 hr after feeding and time to consumption was measured. Whole blood glucose and plasma insulin concentrations were measured.

Results: QH had higher insulin sensitivity (Si) and glucose mediated glucose disposal (Sg) than TBs in response to the FSGIT. The glycemic response, glucose area under the curve (AUC), and peak glucose did not differ between breeds or between hay types; however, insulin response and peak insulin was higher in QHs than TBs on the HC hay. Within breeds, QHs had a higher insulin response, insulin AUC, and peak insulin when fed the HC vs. LC hay, whereas these responses did not differ in TBs fed HC and LC hay. The time to complete consumption of hay was significantly less for QH than TB.

Conclusions: In response to an IV bolus of glucose and insulin, QH appear more insulin sensitive than TB. There appeared to be breed differences in response to HC

carbohydrate hay in that QH secrete more insulin than TB; however, since all QHs were mares and TBs were geldings, it is possible that differences identified reflect effects of gender as well as breed.

Introduction:

Management of equine muscle disorders such as exertional rhabdomyolysis and diseases such as laminitis and equine metabolic disorders includes strict dietary management of carbohydrates (Carter et al. 2009; Geor and Harris 2009; Harris and Geor 2009; Ralston 2007; Valberg et al. 1997; Valentine et al. 1998). Diets rich in starches have been associated with insulin resistance in several studies, suggesting that prolonged feeding starch-rich cereal grains many promote insulin resistance in horses (Frank et al. 2006; Garcia and Beech 1986; Kronfeld 2004; Kronfeld et al. 2005; Treiber et al. 2006c; Vervuert et al. 2003; Vervuert et al. 2004; Vervuert et al. 2009; Williams et al. 2001). Insulin resistance in horses has been associated with obesity, laminitis and pars pituitary intermedia dysfunction (Aleman 2008; Bailey et al. 2007; Firshman and Valberg 2007; Geor and Frank 2009; Geor and Harris 2009; Hoffman et al. 2003; Kronfeld D. 2005; Longland and Byrd 2006; Milinovich et al. 2006; Treiber et al. 2006b; Treiber et al. 2006d; van Eps and Pollitt 2006). Further, diets containing a high percentage of grain-based soluble carbohydrates are known to trigger exertional rhabdomyolysis in Thoroughbreds (MacLeay et al. 1999) and Quarter Horses with Polysaccharide storage myopathy (PSSM)(Firshman et al. 2003). PSSM is a glycogen storage disorder caused by a mutation in glycogen synthase 1(McCue et al. 2008; Valberg et al. 1992).

The nonstructural carbohydrate (NSC) content of hay consists of the sum of water-soluble carbohydrates (WSC) and starch. Sucrose, fructose, and glucose are components of WSC, as well as some poly- and oligosaccharides and fructans. The presence of fructans in the estimated NSC content, as well as the prolonged time needed for hay consumption and digestion, may mitigate a measurable glycemic

response to high NSC forages (Cottrell E. et al. 2005, Hoffman et al. 2001). Whereas simple sugars produce a glycemic response via enzymatic digestion in the small intestine, fructans are typically digested by hind gut microbial fermentation and therefore may not have a major impact on the glycemic or insulinemic response to fed hay (Longland and Byrd 2006). A recent study, however, showed that adding one form of fructan (inulin) to hay (1500g/500kg BW) produced an increase in insulin levels when fed to ponies, as compared to feeding hay alone (Bailey et al. 2007).

While much is known about the effect of NSC concentrates on clinical signs of exertional rhabdomoloysis and PSSM, there is limited scientific research documenting the glycemic/insulinemic response to fed hay containing different concentrations of NSC, and little research on the effect of feeding different concentrations of forage NSC in different breeds. One study exists with results suggesting that feeding hay with 12% "sugar" can produce a 20% increase in blood glucose from resting values (Cottrell E. et al. 2005). Therefore, the first objective was to determine if the glycemic and insulinemic response to a meal of hay is impacted by the NSC content and to determine if these responses differ between TB and QH.

Several equine studies have adapted a frequently sample glucose insulin IV tolerance test (FSGIT) to insulin sensitivity and glucose effectiveness (Bailey et al. 2007; Eiler et al. 2005; Frank et al. 2006; Haffner et al. 2008; Hoffman et al. 2003). Glucose dynamics are analyzed by minimal model analysis, which partitions glucose disposal into 1) glucose effectiveness (Sg), or the capacity of glucose to mediate its own disposal independent of plasma insulin and 2) insulin sensitivity (Si), or the capacity of insulin to promote glucose disposal (Bergman et al. 1979; Bergman 1997; Bergman

2005; Ward et al. 1991). This test has been used as a measure of insulin sensitivity in horses diagnosed with equine metabolic syndrome (Durham et al. 2009; Hoffman et al. 2003). A secondary objective of this study was to use the FSGIT and minimal model analysis to determine if Sg and Si differed between TB and QH breeds.

Materials and Methods

Horses: Five healthy unfit QH-type mares (Appaloosa, Paint, and QH cross) mean age 9.4 ± 2.7 yrs, weight 498.1 ± 22.1 kg, housed in MN. Five healthy unfit Thoroughbred geldings housed in KY, mean age 12.6 ± 3.8 weight 565 kg ± 33.9 kg. Horses had not been exercised for at least 12 months prior to the study. All horses were housed in stalls at night and turned out on drylot (MN) or wore muzzles that did not allow them to graze but allowed them to drink while turned out on fall pasture (KY) during the day. All horses were fed mixed grass hay (at 1.5% BW as fed), a vitamin/mineral supplement, free choice salt, and no grain for at least 1 month prior to and during the study. The Henneke body condition score for all horses ranged from 4.5 – 6 out of 9 for the at least the previous 4 months, and all horses received annual dental examinations. Horses were cared for in accordance with principles outlined by the University of Minnesota Animal Use and Care Committee.

Frequently sampled glucose insulin tolerance test (*FSIGT):* The horses were moved from pasture to stalls 15 to 18 hr before the onset of the FSIGT. Horses were housed in adjacent stalls and could see each other in order to avoid social dislocative stress and were allowed access to medium NSC content hay. To account for any diurnal variation for glucose tolerance, the modified FSIGT were initiated at approximately

the same time each morning (730) for all horses. On the morning of the FSIGT, each horse was weighed and a jugular catheter placed followed by an adaptation time of approximately 1 hr. Baseline blood samples were collected at -10 and 0 minutes. The FSGIT procedure of Hoffman was followed (Hoffman et al. 2003). A 300 mg/kg bwt IV glucose bolus, (dextrose solution 50% injection, Phoenix Pharmaceutical, Inc., St Joseph MO) was administered to each horse over a period of approximately 2 min, followed by infusion of 20 ml of saline solution containing heparin. This glucose dose was similar to other glucose doses used in horses (De La Corte et al. 1999; Hoffman et al. 2003). Venous samples were collected via the catheter at 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 19 min after the infusion of dextrose. At 20 min after the glucose dosing, an IV insulin bolus (Humulin R, Eli Lilly and Co, Indianapolis, IN) of 30 mU/kg bwt was administered followed by another infusion of 20 ml saline solution containing heparin. Blood samples were subsequently collected via the catheter at 22, 24, 25, 27, 30, 35, 40, 50, 60, 70, 80, 100, 120, 150, and 180 min after glucose administration. At each time point, 3 ml of blood was drawn and discarded. A blood sample was then collected. Whole blood glucose was measured immediately with a handheld glucometer previously validated in the horse (Wess and Reusch 2000)[j]. Remaining blood samples were immediately placed in heparinized sample tubes (Vacutainer, Fisher Health Care, Chicago IL) and kept in ice water until centrifuged within 30 min at 1000 RPM, and the plasma removed and frozen at -20 degrees C, and plasma insulin was measured by radioimmunoassay (Reimers et al. 1982) at a later date.

[3] AccuCheck Handheld Glucometer

A minimal model was used to interpret the modified FSIGT glycemic and insulinemic responses. Briefly, Sg (min^{-1}), Si (10^{-4})($L*mU^{-1}*min^{-2}$), acute insulin response to glucose (AIRg), and the disposition index (DI) were calculated using MinMod Millennium and WinSAAM and five equations (Bergman et al. 1979; Bergman 1997). AIRg ($mU*min^{-1}*L^{-1}$) quantifies endogenous insulin secretion in response to the glucose dose, while DI is a dimensionless index which describes pancreatic β-cell responsiveness and accounts for the influence of both endogenous insulin secretion and insulin sensitivity. The lowest glucose concentration below baseline, or nadir, was also calculated as Gmin.

Dietary NSC: High NSC (HC) (Italian Rye, *Lolium multiflorum*, 17.1% NSC), low NSC (LC) (Blue Grama, *Boutelous gracilis*, 4.4% NSC), and medium NSC (MC) (mixed grass, MN 10.7% NSC; KY 10.6% NSC) hays were identified initially by estimating NSC (WSC plus starch) and fructan (WSC- ethanol soluble carbohydrate[ESS]) (Table 1)and hay was analyzed by a commercial laboratory[k]. For a more accurate analysis, fructan was assayed by enzymatic technique (MegaZyme)[l]. The sugar profile of HC and LC hay was analyzed using high-performance liquid chromatography (HPLC)[c]. The HC and LC hay were both harvested from a single monoculture field and duplicate samples were submitted for hay analysis. The gross energy (GE) of each hay type was estimated using equations from Pagan (Pagan et al. 1998) assuming GE of NSC and NDF of 4.15 kcal/g (Table 2). The digestible energy (DE) calculated from equations assuming coefficients of 90% for NSC and NDF (Fonnesbeck 1981).

[k] Equi-Analytical Laboratories, Ithaca, NY 14859
[l] NP Analytical Laboratories St Louis, MO 63164

Study design: A crossover design was used to compare the HC and LC hay with horses randomly assigned to consume one of the hay diets for 5 days. This was followed by a washout period when all horses were fed MC hay for 7 days, after which the hay diets were reversed. Horses were fed 1.5% BW (as fed) of hay, divided into 2 equal feedings per day (7:00 and 15:00 h). Identical HC and LC hay and locally purchased MC hay of mixed grass composition (primarily orchard grass mixed with other grasses) and similar NSC (MN 10.7%; KY 10.6%) were fed to all horses. The study was conducted in the fall/winter, and no mares were in estrus. While the study design focused on comparison of HC and LC hay, we also examined the effect of feeding MC hay by conducting the same sample collection on the last day of the washout period as performed for the HC and LC hays.

Sample collection: On the fifth day of each block (seventh day of the washout period), after a 12-hr fast, a jugular catheter was placed 1 hour before time-zero blood samples were drawn from each horse. Horses were then fed 0.5% BW (as fed, equivalent to 33% daily DE) in hay divided into 1 kg portions (for ease of monitoring intake) every 30 min, (or upon complete consumption of 1 kg portion if this occurred before 30 min). Horses consumed the hay continuously under this protocol, and the time to consumption of hay or the residual hay left was recorded. Blood samples were taken prior to the portions being fed and every 30 min for 5 hr thereafter. Whole blood glucose was measured immediately with a handheld glucometer[m] previously validated in the horse (Wess and Reusch 2000). Blood samples were centrifuged at 2000 rpm x g within 30 min, and the plasma removed and frozen at -20 degrees C, and plasma insulin was measured by radioimmunoassay (Reimers et al. 1982). This assay

[m] AccuCheck Handheld Glucometer

provides accurate results in the horse for concentrations not requiring dilution (Tinworth et al. 2009).

Statistical analysis: Mean time to consumption for all horses was analyzed by GLM ANOVA blocked for hay type and breed using Statistical Analysis and Graphics (NCSS)[e]. The glycemic and insulinemic response was defined as all of the values measured between time zero and 300 min after feeding and was analyzed by repeated measures ANOVA, blocked for diet and breed. Analysis included MC hay with the qualification that it was not fed in a block design. Area under the curve (AUC) for blood glucose and plasma insulin from a baseline of the individual value at time zero was calculated by trapezoidal method of numerical integration using NCSS and was compared by GLM ANOVA, blocked for hay type and disease, using NCSS. Baseline and peak glucose and insulin values as well as time to peak glucose and insulin were compared by two-way ANOVA, blocked for diet and breed. Tukey-Kramer multiple comparison test was used to determine differences in means. Linear regression analysis was performed in GraphPad Prism[n] to determine a potential correlation between time to consumption and peak glucose, peak insulin concentrations, and glucose and insulin AUC. Results are expressed as mean ± SD with significance set at $p<0.05$. A two sample t test was used to compare Sg, Si, AIRg, and DI between breeds for the FSIGT analysis. Results are expressed as mean and standard error of the mean with significance set at $p<0.05$.

[e] NCSS Kaysville, Utah 84037
[f] GraphPad Prism 5 San Diego, CA USA 92121

Results

FSGIT: Insulin sensitivity (Si) and glucose mediated glucose disposal (Sg) were significantly higher in QHs than TBs (Table 3). Acute insulin response to glucose (AIRg) and the disposition index (DI) were similar between breeds (Table 3). No horses exhibited signs of hypoglycemia during the FSIGT. QH had a lower nadir of glucose (Gmin) during the FSGIT than TB, and QH glucose mean values failed to return to baseline levels at the end of the FSGIT (Figure 1).

Dietary Energy and NSC: The total estimated DE was higher in the HC compared to the LC hay (Table 2). WSC content of the HC hay was nearly 4 times greater than the LC hay and the DE provided by estimated NSC was at least 3 fold higher for HC than LC hay (Table 2). The WSC content and DE from NSC of MC hay were nearly 2 times greater than the LC hay (Table 1 and 2). The HC hay contained more sucrose, glucose and fructose than LC hay (Table 1). The NDF content and the DE from NDF was lowest in the HC and highest in the LC hay, and the HC had higher protein concentration than the LC or MC hays although the DE from protein was only moderately higher for HC than LC hay (Table 1 and 2). The estimated fructan content of the LC and HC hays were both < 3.5% (MegaZyme) (Table 1).

Rate of consumption: There was a large range in the rate which horses consumed hay (Table 4). The QHs consumed all hays significantly faster than TB horses; however, when broken down by hay type, there was no difference by breed within individual HC, MC or LC hay. A weak but significant negative correlation was detected when

all horses were grouped together (Figure 2) showing that consuming hay more quickly was associated with a higher peak insulin ($p<0.045$, $r^2 =0.136$).

Quarter Horse-related breed: There was no significant difference in postprandial blood glucose responses or peak values and AUC between hay types in QHs (Table 5). However, insulin responses and peak insulin [HC 60 ± 18, MC 19 ± 5, LC 18 ± 6 µU/ml] and AUC (Table 5) were significantly higher on the HC hay compared to the LC and MC hay in QHs (Fig. 3, Table 5).

Thoroughbreds: The postprandial blood glucose response to HC hay was higher than LC (Fig. 3) but glucose peak and AUC were similar among hay types in TBs (Table 5). There was no significant difference in postprandial insulin responses (Fig. 3) or peak values and AUC among hay types in TBs (Table 5).

Breed comparison: There was no difference between the glucose response, peak glucose or glucose AUC between the two breeds. When all hay types were combined, the insulin response (Fig. 3), insulin peak, and AUC were significantly higher in QHs compared to TBs (Table 5).

Discussion

Our results showing differences between QHs and TBs in the hay trial and FSGIT were an unexpected finding. There are no previous studies that have specifically examined differences between breeds in glucose and insulin responses. However, because our original study was not designed to investigate breed differences in insulin

responses to hay consumption we can not definitively conclude the identified differences are solely due to breed effects. Since all of our QHs were mares and all of our TBs were geldings our results could equally be attributed to gender effects. A better study design would have compared horses of similar genders across breeds; however, we did not have such horses available to us. To draw firm conclusions about breed differences in glucose and insulin responses to hay or IV glucose further studies are necessary. The following discussion of breed differences should therefore be interpreted with appropriate caution in light of our study design.

One of the difficulties in estimating the impact of NSC content of hays is the variation in consumption rates, digestion time, and how that may modulate large increases in blood glucose and insulin. QHs consumed hay more rapidly than TB, which may be a breed difference in their response to hay with variable NSC, ADF and NDF content, or may reflect differences in management prior to the study and individual preferences. The present study demonstrated a weak correlation between more rapid consumption and higher peak insulin concentrations in all horses, suggesting that a more rapid increase in glucose absorbed from hay may have produced a higher pancreatic insulin secretion. Thus, differences in the rate of consumption between breeds could have impacted our findings. Also, a secondary peak in insulin concentration and blood glucose may be detected due to the digestion of hay to volatile fatty acids (VFA) propionate and butyrate in blood samples 3 hours postprandial (Eades et al. 2007). These VFAs are fermented in the hind gut from forage, and can be gluconeogenic, and could affect timing of peak insulin if measurements are made over 3 hours (Table 4). The mean time to total consumption for TBs was over three hours, while QH was under 2 hours.

In the present study, we found a higher secretion of insulin in the QH group in response to the high NSC hay as compared to TBs. While TBs appeared to maintain similar insulin concentrations in response to HC, MC and LC hay, their glucose concentrations fluctuated. In contrast, QHs maintained similar glucose concentrations on all three hays but had significantly different insulin responses. Numerous equine diseases are impacted by the glycemic and insulinemic responses to equine feeds, including PSSM, (Ribeiro et al. 2004) recurrent exertional rhabdomyolysis (MacLeay et al. 2000), pituitary pars intermedia dysfunction(McCue 2002), equine metabolic syndrome (Geor and Frank 2009), osteochondrosis desicans (Treiber et al. 2005), and forms of laminitis (Geor and Harris 2009). Thus, the results of the present study would suggest that the NSC content of hay is particularly important to manage in horses of QH related breeds that are susceptible to the above mentioned conditions.

It is of note that the breed differences in insulin response to oral high NSC were not observed when glucose was given via jugular catheter (IV). One explanation for why QHs have the same insulin response to IV glucose as TBs, but a greater insulin response to oral NSC, may be a differential effect of incretins between breeds or consumption rate. Two primary incretins have been identified, glucagon-like-peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Drucker 2006). Incretins are secreted by intestinal mucosa of the proximal small intestine (GIP) in horses and stimulate glucose-dependent insulin synthesis and secretion resulting in amplified circulating insulin concentrations (Hampton et al. 1986). These peptides have been shown to lower postprandial and fasting glucose and suppress glucagon levels (Kim and Lee 2010). To date, only one study has measured GIP concentrations in horses and ponies, and found that GIP increased significantly only if glucose was

administered orally (not IV) and that plasma GIP levels were comparable between ponies and large horses (Duhlmeier et al. 2001). An alternative explanation for the breed differences noted here would be that QH may lack counterregulatory "anti-incretins" in response to a meal of oral starch and sugar when compared to TB (Rubino 2008).

There were several distinct differences between TBs and QHs identified during the FSGIT. QHs had a two fold greater capacity for glucose mediated glucose dispersal and 2-fold higher insulin sensitivity than TB. While there are no previous studies comparing breeds, our results for TBs are similar or slightly higher than previous studies with TBs using FSGIT (Hoffman et al. 2003; Treiber et al. 2005) and our QH Sg and Si results are higher than previous reports in other breeds (Bailey et al. 2007; Hoffman et al. 2003; Pratt et al. 2005; Tiley et al. 2007; Toth et al. 2009; Treiber et al. 2005; Treiber et al. 2006a). Another notable difference between QH and TB during the FSGIT was that following the IV bolus of insulin, blood glucose concentrations of QH were lower and dropped below baseline values compared to TBs. This suggests that in a delay may occur in QHs before counter regulatory hormones, such as glucagon, re-establishes normal blood glucose concentrations. The effects of obesity, diet and equine metabolic syndrome have been evaluated by FSGIT in Thoroughbreds, Standardbreds, Arabians, Quarter Horses, Tennessee Walking Horses, and ponies (Bailey et al. 2007; Eiler et al. 2005; Hoffman et al. 2003; Kronfeld et al. 2005; Pratt et al. 2005; Tiley et al. 2007; Toth et al. 2009; Toth et al. 2010b; Treiber et al. 2005; Treiber et al. 2006a). There are often differences in the dose of glucose and insulin used in these studies, suggesting caution when comparing exact values; however, results clearly show that the starch and fat content of the diet

(Hoffman et al. 2003; Toth et al. 2010a), age (Treiber et al. 2005), obesity (Hoffman et al. 2003), breed and conditioning (Treiber et al. 2006a), and disease predisposition (Bailey et al. 2007) all affect Sg and Si in horses, yet gender effects have not been studied.

Conclusions

In conclusion, this study provides preliminary evidence to suggest that Quarter Horses are more insulin sensitive and secrete more insulin in response to high NSC hay than Thoroughbreds. It is important to note however, that the QHs in this study were all female, the TBs were all geldings, and QHs consumed hay faster than TB. Thus, gender and rate of hay consumption may have confounded our results. More studies balanced for gender and rate of consumption are needed before any definitive conclusions can be made regarding breed differences.

Table 1. Nutrient analysis of hays used in this trial. Values represent single analyses performed at Equi-Analytical Laboratories. NDF (neutral detergent fiber), ADF (acid detergent fiber), WSC (water soluble carbohydrate), ESC (ethanol soluble carbohydrates).

Assay (% as fed)	**Italian Rye (HC)**	**Blue Grama (LC)**	**Mixed MN (MC)**	**Mixed KY (MC)**
Dry Matter (DM)	91.7	94.0	89.6	91.6
Protein	12.1	9.7	11.8	10.2
Starch	1.3	0.4	0.9	0.8
NDF	46.4	66.2	54.0	56.7
ADF	31.0	38.9	34.2	36.9
WSC	15.8	4.0	9.8	9.8
ESC	10.2	1.3	8.9	6.4
Sugar Profile				
Fructan *	3.3	2.1	n/a	n/a
Fructose	2.0	0.9	n/a	n/a
Glucose	2.4	1.0	n/a	n/a
Sucrose	7.2	<0.2	n/a	n/a
Maltose	0.2	<0.2	n/a	n/a

*The fructan content represented here was determined by Megazyme assay technique, which is not presently validated for horse forage performed at NP Analytical Laboratories.

Table 2. Estimated digestible energy (DE) and estimated gross energy (GE) for nutrient components in the hay fed in this trial.

Mcal/kg (MJ) **as fed**	**Italian Rye (HC)**	**Blue Grama (LC)**	**Mixed MN (MC)**	**Mixed KY (MC)**
Total GE	3.5 (14.8)	3.7 (15.5)	3.6 (14.9)	3.6 (15.0)
Total DE *	2.3 (9.5)	1.9 (8.0)	2.1 (8.9)	2.0 (8.3)
DE protein	0.5 (2.3)	0.4 (1.7)	0.5 (2.2)	0.4 (1.7)
DE NDF	1.0 (4.0)	1.3 (5.4)	1.1 (4.6)	1.1 (4.6)
DE NSC	0.7 (3.0)	0.2 (0.7)	0.4 (1.8)	0.4 (1.7)
DE fat	0.06 (0.25)	0.05 (0.21)	0.06 (0.25)	0.05 (0.21)

*The DE was calculated assuming GE of NSC and NDF (http://www.ker.com/library/advances/107.pdf) of 4.15 kcal/g and digestibility coefficient of 90% for NSC and 45% for NDF (http://www.ker.com/library/advances/108.pdf).

Table 3. Minimal Model analysis results from frequently sampled insulin glucose challenge test. Outputs are Si (insulin sensitivity, ($10^{-4)}$) L/mμ/min), Sg (glucose mediated glucose disposal, min^{-1}), AIRg (acute insulin response to glucose, a measure of pancreatic beta-cell responsiveness to exogenous glucose bolus, mU/min/L) and DI (disposition index, dimensionless). Baseline glucose and minimum glucose (mU/kg) concentrations during challenge test. Letters indicate significant differences, $p<0.05$.

Model parameters ± SD (significance $p<0.05$)						
Breed	Si	Sg	AIRg	DI	Baseline Glucose	Minimum Glucose
Quarter Horse	5.3 ± 1.5[a]	0.024 ± 0.008[a]	250 ± 100[a]	1415 ± 859[a]	100 ± 12	55.4 ± 11.8
Thorough bred	3.3 ± 1.4[b]	0.012 ± 0.008[b]	256 ± 97[a]	896 ± 587[a]	97.4 ± 8.4	86.2 ± 26

Table 4: Time in minutes (mean ± SD) for complete consumption of 0.5% BW (as fed) of hay types by Thoroughbred and Quarter Horses.

Time (min)	**Italian Rye (HC)**	**Blue Grama (LC)**	**Mixed (MC)**	All hay types combined
Thoroughbreds (n=5)	138 ± 27	234 ± 106	234 ± 106	202 ± 93†
Quarter Horses (n=5)	68 ± 25	93 ± 24	96 ± 16	86 ± 24
All horses combined (n=10)	103 ± 44	163 ± 104	165 ± 101	

HC is high (17% NSC), LC is low (4% NSC), and MC (11% NSC) is medium nonstructural carbohydrate hay.

† Thoroughbreds significantly different from Quarter Horses for all hay types combined.

Table 5. Mean (± sd) area under curve for whole blood glucose and plasma insulin response and peak values for glucose (g) and insulin (i). Significance is set at $p<0.05$.

High hay (HC)	**Thoroughbred**		**Quarter Horse**		**Units**
AUC, g	16588.2 ± 2263.9	[1,a]	16408.0 ± 1825.9	[1,a]	
Peak, g	126.6 ± 14.9	[1,a]	114.8 ± 9.9	[1,a]	mg/dL
Time to peak g	126.0 ± 44.5	[1,a]	126.0 ± 44.5	[1,a]	min
Baseline g	95.2 ± 8.8	[1,a]	92.8 ± 11.5	[1,a]	mg/dL
AUC, i	1491.0 ± 1275.9	[1,a]	3930.8 ± 1957.4	[1,a]	
Peak, i	20.9 ± 14.9	[1, a]	59.9 ± 17.7	[1,b]	µU/ml
Time to peak i	156.0 ± 57.7	[1,a]	111.4 ± 22.7	[1,a]	min
Baseline i	2.4 ± 1.2	[1,a]	3.9 ± 0.8	[1,a]	µU/ml
Low Hay (LC)	**Thoroughbred**		**Quarter Horse**		**Units**
AUC, g	16763.0 ± 875.5	[1,a]	16737.4 ± 869.0	[1,a]	
Peak, g	112.0 ± 7.2	[1,a]	119.8 ± 5.2	[1,a]	mg/dL
Time to peak g	174.0 ± 13.4	[1,a]	102.0 ± 62.2	[1,a]	min
Baseline g	93.2 ± 8.3	[1,a]	97.4 ± 7.8	[1,a]	mg/dL
AUC, i	432.5 ± 257.6	[1,a]	1089.1 ±335.7	[1,b]	
Peak, i	8.4 ± 4.0	[1,a]	17.7 ± 5.5	[1,b]	µU/ml
Time to peak i	144.0 ± 65.0	[1,a]	111.4 ± 61.8	[1,a]	min
Baseline I	2.7 ± 1.4	[1,a]	4.4 ± 1.3	[1,a]	µU/ml
Medium hay (MC)	**Thoroughbred**		**Quarter Horse**		**Units**
AUC, g	14754.6 ± 1147.7	[1,a]	15917.8 ± 968.0	[1,a]	
Peak, g	120.4 ± 3.4	[1,a]	113.3 ± 7.6	[1,a]	mg/dL
Time to peak g	126.0 ± 68.4	[1,a]	132.0 ± 45.5	[1,a]	min
Baseline g	97.4 ± 3.0	[1,a]	97.0 ± 6.2	[1,a]	mg/dL
AUC, i	613.6 ± 317.3	[1,a]	1338.3 ± 584.3	[1,b]	
Peak, i	12.7 ± 6.2	[1,a]	19.1 ± 5.3	[1,b]	µU/ml
Time to peak i	180.0 ± 36.7	[1,a]	120.0 ± 17.3	[1 a]	min
Baseline I	4.2 ± 4.0	[1,a]	3.2 ± 1.5	[1,a]	µU/ml
All hays combined	**Thoroughbred**		**Quarter Horse**		**Units**
AUC, g	16035.3 ± 1715.7	[a]	16546.4.0 ± 5604.4	[a]	
Peak, g	119.1 ± 11.2	[a]	115.9 ± 39.0	[a]	mg/dL
Time to peak g	142.0 ± 50.0	[a]	114.0 ± 51.0	[a]	min
Mean Baseline g	95.0 ± 6.9	[a]	95.1 ± 9.6	[a]	mg/dL
AUC, i	845.7.8 ± 861.3	[a]	2119.4 ± 1124.9	[b]	
Peak, i	14.0 ± 10.4	[a]	32.2 ± 17.2	[b]	µU/ml
Time to peak i	160.0 ± 52.8	[a]	108.0 ± 47.8	[a]	min
Mean Baseline I	3.1 ± 2.5	[a]	4.4 ± 1.2	[a]	µU/ml

[1] Different numbers indicate significant differences for corresponding values within column.
[a] Different letters indicate significant differences for corresponding values within row.

Figure 1. Frequently Sampled Glucose Insulin Tolerance test glucose and insulin responses ± SD. Glucose reported in mg/dl, insulin in µU/ml, time samples collected from time zero to 180 minutes. Solid black triangles ▲ Quarter Horses, green squares, ■ Thoroughbreds. Glucose response significantly lower in Quarter Horses than Thoroughbreds ($p<0.05$).

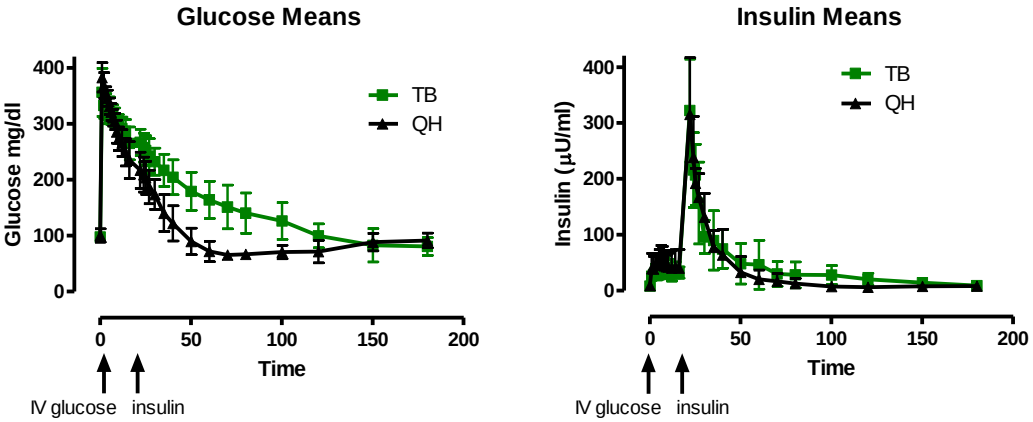


Figure 2. Regression of peak insulin and time to total consumption. Insulin units is in µU/ml, R-Squared=0.1359 and p<0.045

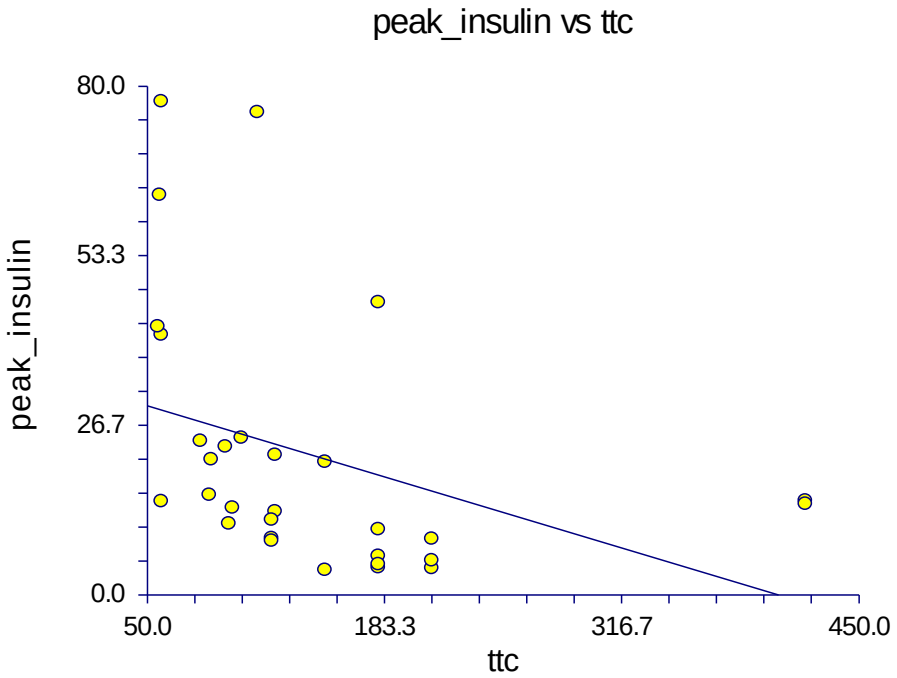
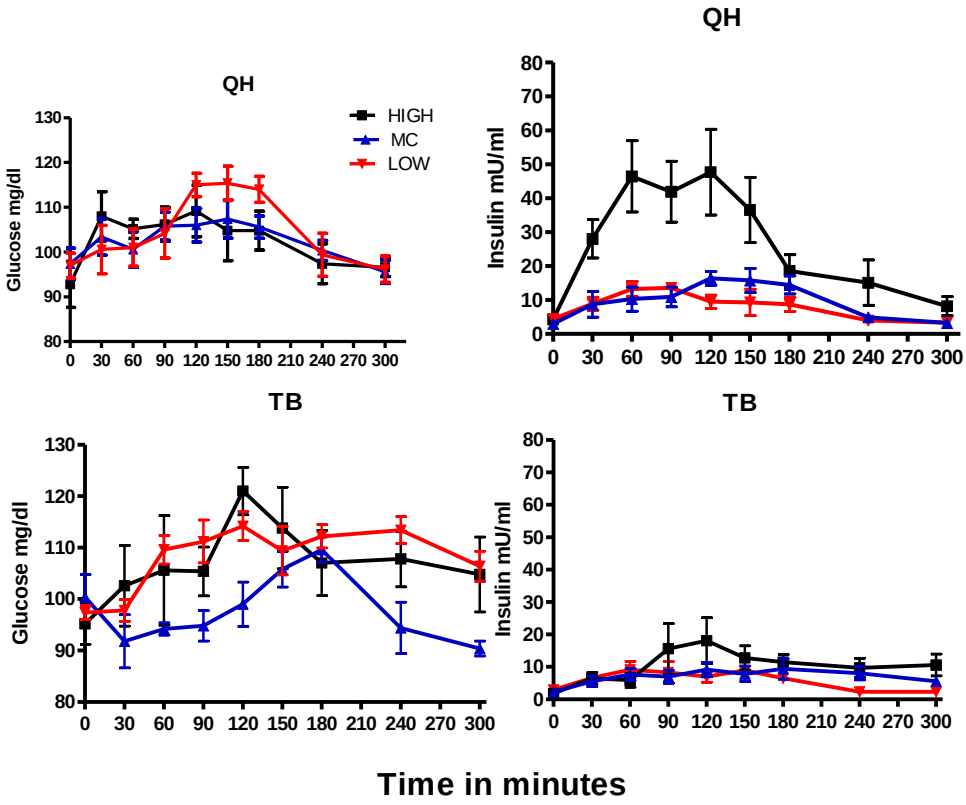


Figure 3. Glucose (mg/dl ± SEM) and Insulin (mμ/ml ± SEM) response curves for Quarter Horses and Thoroughbreds after feeding HC (17% NSC) solid black squares ■, MC (11% NSC) solid blue triangles▲ and LC (4% NSC) solid red triangles ▼ hay. Quarter Horses were significantly different for HC compared to LC and MC hay. Quarter Horse insulin response was significantly higher on HC than TB on HC.



CHAPTER 5

EFFECT OF DIETARY FATS WITH ODD OR EVEN NUMBERS OF CARBON ATOMS ON METABOLIC RESPONSE AMD MUSCLE DAMAGE WITH EXERCISE IN QUARTER HORSE-TYPE HORSES WITH TYPE 1 POLYSACCHARIDE STORAGE MYOPATHY

Effect of dietary fats with odd or even numbers of carbon atoms on metabolic response and muscle damage with exercise in Quarter Horse–type horses with type 1 polysaccharide storage myopathy

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Objective—Evaluate the effect of fats with odd and even numbers of carbon atoms on muscle metabolism in exercising horses with polysaccharide storage myopathy (PSSM).

Animals—8 horses with PSSM (6 females and 2 males; mean ± SD age, 6.3 ± 3.9 years).

Procedures—Isocaloric diets of grain, triheptanoin, corn oil, and high-fat, low-starch (HFLS) feed were fed for 3 weeks each; horses performed daily treadmill exercise. Grain was fed to establish an exercise target, and the HFLS fed as a negative control diet. Daily plasma samples were obtained. For each diet, a 15-minute exercise test was performed, and gluteus medius muscle specimens and blood samples were obtained before and after exercise.

Results—Feeding triheptanoin compared to corn oil diet resulted in exercise intolerance; higher plasma creatine kinase (CK) activity and higher plasma concentrations of C3:0- and C7:0-acylcarnitine and daily insulin; and lower concentrations of nonesterified fatty acids (NEFA) and C16:0-, C18:1-, and C18:2-acylcarnitine, without changes in concentrations of plasma glucose or resting muscle substrates and metabolites. Feeding grain induced higher CK activity and insulin concentrations and lower NEFA concentrations than corn oil or HFLS. Feeding grain induced higher glucose concentrations than triheptanoin and corn oil. In muscle, feeding grain resulted in lower glucose-6-phosphate, higher citrate, and higher postexercise lactate concentrations than the other 3 diets.

Conclusions and Clinical Relevance—Triheptanoin had a detrimental effect, reflecting decreased availability of NEFA, increased insulin stimulation of glycogen synthesis, and potential inhibition of lipid oxidation. Long-chain fats remain the best dietetic for PSSM.

ABBREVIATIONS

BW	Body weight
CAC	Citric acid cycle
CK	Creatine kinase
ER	Exertional rhabdomyolysis
G6P	Glucose-6-phosphate
GYS1	Glycogen synthase gene
HFLS	High fat, low starch
NEFA	Non esterified free fatty acids
PSSM	Polysaccharide storage myopathy
SET	Standardized exercise test

Polysaccharide storage myopathy is a form of ER that affects 12% of Quarter Horses.[1] It is characterized by high muscle glycogen concentrations (1.8 times as high as that for clinically normal horses) and accumulation of abnormal polysaccharide in muscle fibers.[2,3] Clinical signs develop after 10 to 30 minutes of submaximal exercise and include signs of muscle pain and stiffness, which is accompanied by high CK activity; these results are indicative of muscle damage.[4–6]

Most glycogen storage disorders are caused by deficiencies in enzymes involving glycogenolysis or glycolysis.[7] Excessive glycogen accumulation in the skeletal muscle of horses with PSSM has been investigated[8,9] and is not associated with a deficiency in glycolysis or glycogenolysis. Affected horses actually used more glycogen during near-maximal exercise than did clinically normal horses.[8] Results of

other studies[2,10,11] suggest that horses with PSSM may have heightened sensitivity to insulin and enhanced glucose uptake by skeletal muscle, which may further increase glycogen synthesis. Recently, PSSM in Quarter Horses was found to be attributable to a novel gain-of-function mutation in *GYS1*, which resulted in an increase in glycogen synthase activity.[12]

Despite the apparent availability of glycogen for energy metabolism, horses with PSSM have an energy deficit during submaximal exercise, as indicated by an abnormally high concentration of inosine monophosphate in muscle fibers after exercise.[4] The precise mechanism for the development of ER in horses with PSSM is not known; however, we postulate that disrupting the flux of glycogen to pyruvate and acetyl CoA may impair generation of CAC intermediates and energy, which results in muscle membrane damage and potential leakage of CAC intermediates from PSSM-affected muscle cells. This mechanism has been proposed in animals and in humans with chronic rhabdomyolysis and metabolic myopathies.[13–15]

Feeding a low-starch, fat -supplemented diet that includes corn oil or a HFLS commercially prepared feed can decrease the number of episodes of ER in horses with PSSM.[6,16] The recommended amount of dietary fat for horses with PSSM varies from 12% of digestible energy (determined on the basis of a dietary trial[16]) to 25% of digestible energy (determined on the basis of an anecdotal report[17]); however, there is little data that quantifies the amount and type of fat that is best utilized by horses with PSSM. Potential mechanisms for improvement when fed fat diets include decreased glucose uptake and decreased insulin stimulation of glycogen synthesis. Additionally, provision of dietary long-chain fatty acids with an even number of carbon atoms may

supply the CAC with an alternate energy source in the form of the 2-carbon acetyl-CoA moiety.

Triheptanoin, a 7-carbon fat, generates propionyl-CoA (in addition to acetyl CoA), and has been used in humans to replenish the CAC by providing succinyl-CoA.[14,18] Dietary supplementation with triheptanoin has been used to treat patients with long-chain fatty acid disorders, such as carnitine palmitoyltransferase II deficiency,[19] pyruvate decarboxlyase deficiency, and type II glycogen storage disease (ie, Pompe's disease), by supplying 5-carbon ketone bodies.[14,20]

Measuring plasma acylcarnitine concentrations is an accepted method of monitoring oxidation of fats of specific chain lengths. Excessive accumulation of acylcarnitine of a specific chain length indicates a metabolic disorder of the corresponding short-, medium-, or long-chain fatty acid.[14,20,21] Analysis of results of a recent study[22] indicated that triheptanoin was absorbed and metabolized by healthy Thoroughbreds on the basis of evaluation of corresponding plasma concentrations of acylcarnitines.

We hypothesized that CAC intermediates could become depleted in PSSM horses with chronic ER and that provision of a fat with an odd number of carbon atoms (such as triheptanoin) would increase these intermediates in skeletal muscle, thus potentially attenuating ER during submaximal exercise. The objectives of the study reported here were to determine whether dietary triheptanoin (compared with dietary corn oil) would reduce muscle damage in horses with PSSM as indicated by plasma CK activity after submaximal exercise and whether dietary triheptanoin or corn oil would affect substrate availability during submaximal exercise. Another objective was to

compare the metabolic responses for dietary triheptanoin and corn oil with those for grain (which is known to induce ER) and to a commercially available HFLS feed that has been reported[16] to decrease episodes of ER in horses with PSSM.

Materials and Methods

Horses—Eight Quarter Horse–type horses in which PSSM had been diagnosed were used in the study. Three of the horses were registered Quarter Horses, 1 was a registered Appaloosa, 2 were Quarter Horse–Arabian crossbred horses, and 2 were derived from mating a Quarter Horse mare with a Quarter Horse–Thoroughbred crossbred stallion. The diagnosis of PSSM was based on detection of amylase-resistant abnormal polysaccharide in gluteal muscle biopsy specimens.[3] Subsequent to the study, all 8 horses were found to be heterozygous for a dominant mutation in *GYS1*, which is known to cause PSSM.[12]

The horses (6 mares, 1 stallion, and 1 gelding) ranged from 2 to 14 years of age (mean ± SD, 6.3 ± 3.9 years). Body weight ranged from 277 to 567 kg (mean, 466 ± 104 kg). The horses were housed on a drylot and fed grass hay for at least 1 year prior to the study. During the study, all horses were housed in an accredited facility and were cared for in accordance with principles outlined by the Animal Use and Care Committee at the University of Minnesota.

Diets—Four isocaloric diets were formulated to provide a total digestible energy of 50 kcal/kg of BW/d and to meet minimum daily nutrient requirements **(Appendix A)**.

GRAIN

A high-starch grain diet that in other studies[10,16] induced subclinical increases in plasma CK activity in horses with PSSM was fed to determine an exercise target for each horse. The diet consisted of molasses-supplemented grain (3.4 g/kg of BW), calcium-balanced rice bran (1.8 g/kg of BW), and ration balancer (1g/kg of BW) and was divided into 2 feedings/d (7:30 AM and 4:30 PM). The grain consisted of 37% corn, 32% wheat middlings, 15% oats, 5.4% soy hulls, 4% soybean meal, 3.5% molasses, 1% limestone, and 0.5% vitamin-mineral premix (dry-weight basis). The rice bran was a commercially available rice bran pellet[a] and contained a minimum of 12.5% crude protein, a minimum of 20.0% crude fat, a maximum of 4.0% free fatty acids, a maximum of 13.0% crude fiber, 2.2% to 3.2% calcium, and a minimum of 1.8% phosphorus (dry-weight basis). The ration balancer was a soybean meal base formulated for grass hay and contained 30% protein, 1.0% fat, 5.0% crude fiber, 4.5% to 5.5% calcium, and 2.0% phosphorus (dry-weight basis) and additional vitamins and minerals to meet minimal daily requirements.

TRIHEPTANOIN AND CORN OIL

Both oils were in liquid form, and diets provided corn oil[b] or triheptanoin[c] (1.5 mL/kg of BW/d). Oil rations were allowed to soak into 1.5-kg cubes of timothy hay, which were split into 3 feedings/d (7:30 AM, 12:30 PM, and 4:30 PM). Horses were fed ration balancer (1g/kg of BW) and grass hay (15g/kg of BW/d) in addition to the oil-soaked hay cubes. The same individual prepared oil diets daily throughout the study, and all other personnel were not aware of the oil that the horses were consuming. All horses consumed the daily oil ration, although for 2 horses, the triheptanoin was palatable only when placed on dry (rather than moistened) hay cubes.

HFLS DIET

Horses were fed a commercially available HFLS diet[d] (5.4 g/kg of BW/d), which was split into 2 daily feedings (7:30 AM and 12:30 PM). Grass hay was fed at 17 g/kg of BW/d. The HFLS diet consisted of 12.5% protein, 12.5% fat, 22% fiber, and 10% starch (dry-weight basis). This diet was used for comparison with the other diets because it had been reported[16] to lower plasma CK activity in horses with PSSM, compared with results for the grain diet.

Exercise target—Horses were acclimated to a treadmill over a period of 6 to 11 days prior to the study, initially performing 4 minutes of walking (1.9 m/s) and then gradually adding alternating intervals of 2 minutes of walking and 2 minutes of trotting (3.0 to 3.8 m/s) on a day-by-day basis. Each horse had the same handler on the treadmill throughout the entire study, and horses were exercised in the same order at approximately the same time each day (between 7 AM and 3 PM). Because of the length of the study and variations in rate of consumption of feed among horses, it was not possible to exercise horses at a specific interval after feeding each day.

The handlers closely monitored each horse's adaptation to exercise to determine exercise tolerance on the treadmill. If horses moved with ease at an even gait, another exercise interval was added. If horses had an uneven gait, shortened hind limb stride, reluctance to continue, sweating, or a tucked up abdomen, the exercise session was terminated. All horses completed the acclimation period for treadmill exercise.

Grain diet—The grain diet was gradually introduced during the initial treadmill acclimation during a period of 5 days. When feeding of the complete grain diet was attained, the grain diet period began.

Exercise

An initial exercise period was performed by each horse, as established from the treadmill acclimation. This initial exercise period differed among horses and was 20 minutes (alternating intervals of 2 minutes of walking and 2 minutes of trotting) for horses that readily adapted to the treadmill; horses that experienced exercise intolerance with the treadmill exercise completed a shorter initial exercise period with fewer intervals of walking and trotting. The exercise target for each horse for the remainder of the study was established as the mean number of 2-minute walking-trotting intervals a horse was able to comfortably perform (up to a 20-minute maximum, including the warm-up interval [4 minutes of walking]), while being fed the grain diet. Horses were exercised daily Monday through Friday for 3 weeks and allowed to rest in box stalls on Saturday and Sunday.

Blood samples

Venous blood samples were collected into heparin-coated vacuum tubes Monday through Friday at 4 hours after exercise. Plasma for analysis of CK activity was separated immediately by use of centrifugation and analyzed within 24 hours. Aliquots of plasma samples were stored at –20°C for subsequent analysis of glucose, insulin, and NEFA concentrations.

Performance of an SET

At the end of each diet period (ie, on the 15th day of exercise), horses performed a 15-minute SET that consisted of 4 minutes of walking followed by 2-minute intervals of walking and trotting. Because of variations in the rate of consumption of feed, it was not possible to perform the SET for each horse at a specific interval after feeding. A catheter was placed in a jugular vein prior to the SET, and venous blood samples were obtained before and immediately after the SET. Blood samples were stored on ice and centrifuged within 30 minutes after collection; plasma was harvested, and aliquots were stored at –20°C for analysis of glucose and NEFA concentrations and at –80°C for analysis of insulin and acylcarnitine concentrations. Venous blood samples were collected 4 hours after the SET and analyzed to determine plasma CK activity.

Muscle biopsy specimens

Gluteal muscle biopsy specimens were obtained before and immediately after completion of the SET through the same incision by use of SC administration of lidocaine and a percutaneous needle biopsy technique.[23] Specimens were obtained within a 2-cm square on an 8 cm line from the highest point of the tuber coxae to the head of the tail at a depth of 6 cm. Biopsy specimens were immediately frozen in liquid nitrogen and stored at –80°C until biochemical analysis was performed.

Triheptanoin and corn oil diets—After the 3-week grain diet period, horses were allowed a 10-day washout period. Then, a randomized cross-over design was used, with half of the horses fed the triheptanoin diet and the other half fed the corn oil diet for 3 weeks. A 10-day washout period was then provided, and the horses were then fed the other oil-based diet. For the first 7 days of the washout period, the horses were

fed grass hay only, and then the alternate diet was gradually introduced over a period of 3 days.

The exercise target for each horse determined during feeding of the grain diet was used as the daily exercise format for each diet period. Each horse was closely monitored during exercise, and exercise was terminated if the horse had excessive sweating, stiffness, or an inability to maintain pace with the treadmill prior to reaching the exercise target. Daily blood samples were collected and processed as described previously. If overt signs of muscle stiffness were observed or plasma CK activity was > 50,000 U/L, the number of intervals a horse performed was decreased the following day to 4 minutes or less. Exercise was then gradually resumed over the subsequent days with the goal of returning to the exercise target within 4 days. The number of minutes exercised daily was recorded for each horse. The percentage of the exercise target achieved each day for each diet was calculated for each horse by use of the following equation: (minutes completed/target minutes) X 100. A mean percentage of the exercise target for each day was calculated for the triheptanoin, corn oil and HFLS diets.

For subsequent diets, collection of daily blood samples, performance of an SET and collection of blood samples before and after the SET, and collection of gluteal muscle biopsy specimens were performed as described for the grain diet. Gluteal muscle specimens were obtained from the opposite side (ie, left vs right) from the side used for the preceding SET.

HFLS diet—After both oil-based diets were fed, horses were allowed a 10-day washout period. The horses were fed grass hay only during the first 7 days of the washout period, and then the HFLS diet was gradually introduced over a period of 3 days. Horses were then fed the HFLS diet for 3 weeks. Horses were exercised daily and monitored (including daily blood samples) as described previously. Performance of an SET, collection of blood samples before and after the SET, and collection of gluteal muscle biopsy specimens was performed as described previously. The number of minutes for each exercise session and percentage of exercise target achieved was calculated as described for the triheptanoin and corn oil diets.

Analysis of plasma samples—Plasma CK activity was analyzed by use of an automated chemistry analyzer. Plasma glucose concentration was measured spectrophotometrically by use of the hexokinase method,[e] and NEFA concentration was assayed by use of the enzymatic colorimetric method.[f] Insulin concentration was measured by use of a radioimmunoassay validated for use in samples obtained from horses.[24] Concentrations of free, C2:0, C3:0, C5:0, C7:0, C16:0 (ie, hexadecanoic acid), C18:1 (ie, oleic acid), and C18:2 (ie, linoleic acid) acylcarnitines were assayed by use of mass spectrometry[24] on plasma samples obtained before and after exercise from horses only for the triheptanoin and corn oil diet periods.

Biochemical analysis of muscle biopsy specimens—Frozen gluteal muscle specimens were lyophilized; dissected free of blood, fat, and connective tissue; and then weighed. Glycogen concentration was determined fluorometrically in muscle biopsy specimens as glucose residues remaining after portions (1 to 2 mg) of muscle tissue were boiled for 2 hours in 1M HCl.[25] A comparison of glycogen concentrations

before and after exercise was not made because little difference was anticipated on the basis of analysis of concentrations determined after 20 minutes of submaximal exercise[26] and because variability of detected glycogen concentrations can be high when fibers with abnormal polysaccharide are included in the sample analyzed. A separate portion (4 to 6 mg) of muscle was homogenized by crushing with a glass rod in 1.5M perchloric acid and then cold centrifuged for 10 minutes at 9300 *g*. The supernatant was neutralized with 1M $KHCO_3$ and centrifuged again, and the remaining supernatant was used for analysis of lactate, G6P, citrate, pyruvate, and ATP concentrations via fluorometric techniques.[22,25]

Statistical analysis—Data were analyzed by use of commercially available software packages.[g,h] A repeated-measures ANOVA was used to analyze effects of diet on logarithmically transformed plasma CK activity, percentage of daily exercise target achieved among diets, and daily plasma glucose, insulin, and NEFA concentrations. A 2-way repeated-measures ANOVA was used to analyze effects of diet and exercise on CK activity; plasma concentrations of glucose, insulin, NEFA, and acylcarnitines; and muscle concentrations of ATP, lactate, G6P, pyruvate, and citrate in samples obtained before and after exercise. A 1-way ANOVA was used to determine the effect of diet on resting muscle glycogen concentrations. The post hoc Tukey-Kramer multiple comparison test was used. Significance was set at values of $P < 0.05$. Results were expressed as mean ± SD.

Results

Animals—All horses completed the daily exercise period during feeding of each of the diets; however, not all horses achieved the exercise target every day for all diets.

Daily blood samples could not be obtained by jugular venipuncture from 1 horse because of excitability, which necessitated placement of a long-term indwelling catheter during feeding of the grain, triheptanoin, and corn oil diets. Because of an inability to maintain a patent catheter in this horse during feeding of the HFLS diet, blood was only obtained before and after the SET. Values for daily blood samples for this horse were not included in the statistical analyses.

Weight gain— Mean ± SD weight gain during feeding of the grain diet was 8.0 ± 11.3 kg. Mean weight gain for horses during feeding of the triheptanoin and corn oil diets was 6.3 ± 9.4 kg and 5.9 ± 6.3 kg, respectively. Mean weight gain during feeding of the HFLS diet was 9.5 ± 3.2 kg. There was no significant difference in mean weight gain among the diets.

Daily exercise target and plasma CK activity—The exercise target established during the grain diet ranged from 12 to 18 minutes. There was a significant ($P < 0.001$) effect of diet on exercise intolerance. Horses completed less of their exercise target when fed the triheptanoin diet (achieved 83% of the exercise target) than when fed the corn oil diet (achieved 99% of the exercise target) (Figure 1). There was no difference in the exercise target achieved between horses when fed the corn oil diet and the HFLS diet (97%).

Mean daily plasma CK activities were higher than the reference range (96 to 620 U/L) during feeding of the triheptanoin and corn oil diets (Table 1). There was a significant ($P < 0.001$) effect of diet on log-transformed plasma CK activity. Log-transformed plasma CK activities were higher during feeding of the triheptanoin diet than during

feeding of the corn oil diet (Figure 1). Horses had higher log-transformed plasma CK activities when fed the grain diet than when they were fed the corn oil or HFLS diets; however, the highest CK activities were detected when horses were fed the triheptanoin diet. Horses had similar CK activities when fed the corn oil and HFLS diets. Day of exercise did not have a significant effect on log-transformed CK activity.

Daily plasma glucose, insulin, and NEFA concentrations—Diet had a significant ($P < 0.001$) effect on daily plasma glucose concentrations; however, concentrations were not significantly different between the triheptanoin and CO diets (Table 1). Feeding the grain diet results in significantly higher concentrations than when the HFLS diet was fed.

Diet had a significant ($P < 0.001$) effect on insulin concentrations. Daily plasma insulin concentrations were significantly higher for the triheptanoin diet than for the corn oil diet. Feeding the grain diet resulted in higher plasma insulin concentrations than for the corn oil and HFLS diets, but insulin concentrations did not differ between the corn oil and HLFS diets.

Diet had a significant ($P < 0.001$) effect on NEFA concentrations. Feeding corn oil resulted in higher NEFA concentrations than for all other diets (Table 1). Daily plasma NEFA concentrations were similar when grain and triheptanoin diets were fed.

Effect of SET on plasma CK activity—Mean ± SD log-transformed plasma CK activity in samples obtained 4 hours after SET was higher when horses were fed the

triheptanoin diet ($\log_{10}$ 3.3 ± 0.63) than when fed the corn oil ($\log_{10}$ 2.6 ± 0.18) or HFLS ($\log_{10}$ 2.7 ± 0.22) diets, and similar to the grain diet ($\log_{10}$ 3.1 ± 0.53) (Table 2).

Effect of SET on glucose, insulin, and NEFA concentrations—Diet had a significant ($P = 0.02$) effect on glucose concentrations. Plasma glucose and insulin concentrations obtained before and after the SET did not differ between the triheptanoin and corn oil diets. Feeding the triheptanoin diet resulted in lower blood glucose concentrations before the SET, compared with concentrations before the SET when the HFLS diet was fed. Glucose concentrations were significantly ($P < 0.001$) decreased after the SET; however, differences among horses between glucose concentrations obtained before and after the SET within the same diet were not identified in post hoc tests (Table 2). There was an overall significant ($P < 0.001$) decrease in insulin concentrations after the SET; however, only the HFLS diet had a significant change in concentrations determined in samples obtained before and after the SET. No differences were apparent among diets within insulin concentrations before and after the SET. Concentrations of NEFA were significantly ($P < 0.001$) affected by diet, and NEFA concentrations before the SET were lower for the triheptanoin diet than for the corn oil. No differences were found when comparing NEFA concentrations between the grain, triheptanoin, and HFLS diets for samples obtained before or after the SET. Concentrations of NEFA after the SET for the triheptanoin diet were lower than for the corn oil diet. Overall, the SET did not have a significant effect on NEFA concentrations.

Effect of SET on acylcarnitine concentrations—Concentrations of C2:0 acylcarnitines increased significantly ($P < 0.001$) after the SET, compared with

concentrations before the SET (Figure 2). Concentrations of C3:0 and C7:0 acylcarnitines were not significantly affected by the SET but were significantly ($P < 0.001$) higher for the triheptanoin diet, compared with concentrations for the corn oil diet. Mean ± SD concentrations of C16:0 acylcarnitine were not significantly affected by the SET but were significantly ($P = 0.03$) lower for the triheptanoin diet (0.0113 ± 0.004uM and 0.0138 ± 0.005uM before and after the SET, respectively) than for the corn oil diet (0.0188 ± 0.004uM and 0.0150 ± 0.008uM before and after the SET, respectively). Concentrations of C18:1 acylcarnitines were not significantly affected by the SET but were significantly ($P = 0.002$) lower after the SET for the triheptanoin diet than for the corn oil diet. Concentrations of C18:2 acylcarnitine concentrations were significantly ($P < 0.001$) lower for the triheptanoin diet than for the corn oil diet and increased significantly ($P = 0.006$) after the SET for the triheptanoin diet. Free and C5:0 acylcarnitine concentrations were not significantly affected by the SET and did not differ significantly between the triheptanoin and corn oil diets. Free acylcarnitine concentrations for the triheptanoin diet were 18.19 ± 6.95uM and 21.89 ± 8.42uM before and after the SET, respectively; free acylcarnitine concentrations for the corn oil diet were 21.04 ± 7.12uM and 23.22 ± 5.89uM before and after the SET, respectively.

Muscle biochemical analysis—Resting muscle glycogen concentrations were not significantly affected by diet **(Figure 3)**. Muscle ATP concentrations were not significantly affected by diet or by the SET. Concentrations of G6P were significantly lower for the grain diet than for any other diet, but G6P concentrations were not significantly different among the triheptanoin, corn oil, and HFLS diets. Muscle lactate concentrations were significantly ($P < 0.001$) affected by diet but not by the

SET. Lactate concentrations were not significantly different between the triheptanoin and corn oil diets. When horses were fed the grain diet, lactate concentrations after the SET were significantly higher than when horses were fed the other 3 diets. Muscle pyruvate concentrations were significantly ($P = 0.006$) affected by diet but not by the SET. Muscle pyruvate concentrations after the SET were significantly lower for the grain diet than for the HFLS diet. Muscle citrate concentrations were significantly ($P < 0.001$) affected by diet but not by the SET. Concentrations of citrate before the SET for the grain diet were higher than for the triheptanoin, corn oil and HFLS diets. Muscle citrate concentrations were not significantly different between the triheptanoin and corn oil diets. Muscle citrate concentrations before and after the SET were found to be significantly higher on the grain diet compared to the corn oil or HFLS diets.

Discussion

The study reported here clearly indicates that the type of fat ingested by horses with PSSM affects both exercise intolerance and muscle damage. Triheptanoin was selected for inclusion in the study because as a fat with an odd number of carbon atoms, it supplied acetyl-CoA (similar to the effect for long-chain fats) and also could potentially replenish the CAC through the provision of succinyl-CoA via methyl malonyl-CoA.[18] Triheptanoin appeared to be absorbed when fed, as indicated by higher concentrations of plasma C3:0 and C7:0 acylcarnitines, compared with results when horses were fed corn oil. Concentrations of C5:0 acylcarnitine, which is produced from C7:0 metabolism in the liver, were not different between corn oil and triheptanoin diets in the present study, but were significantly higher for triheptanoin versus corn oil in another study[22] in which oils were administered by nasogastric tube 120 minutes before exercise. It is possible that in the study reported here, the

variability in consumption of oils may have influenced the ability to detect differences in C5:0 acylcarnitines and that C5:0 arising from C7:0 consumption may be rapidly converted to acetyl CoA and propionyl CoA, which resulted in similar acylcarnitine concentrations between the oil diets. Rather than benefiting PSSM horses, provision of this alternate energy source exacerbated exercise intolerance and resulted in even greater muscle damage (as indicated by plasma CK activity) than for the grain diet. When fed to healthy Thoroughbreds in another study,[22] triheptanoin was tolerated well and only resulted in postexercise CK activity higher than the reference range in 1 of 8 horses.

Because triheptanoin appears to be of benefit for other metabolic myopathies, it is difficult to explain its unexpected detrimental effect in horses with PSSM. One potential explanation for its lack of a beneficial effect may be that the horses with PSSM in the present study did not have depletion of CAC intermediates. Muscle citrate concentrations in horses with PSSM in this study were higher than those measured in healthy Thoroughbreds in another study.[22] A further explanation for the potential detrimental effect of triheptanoin may relate to its ability to stimulate insulin secretion. Daily insulin concentrations were as high when horses with PSSM were fed the triheptanoin diet as when those same horses were fed the grain diet. In healthy horses, a dose of 217 mL of triheptanoin given via nasogastric tube resulted in a significant increase in the insulin concentration 2 hours later.[22] Thus, one of the detrimental properties of triheptanoin in horses with PSSM may be its ability to further increase glycogen synthase activity via insulin and glucose, which thereby disturbs energy flux in a manner similar to that for high-starch diets.[27] However, muscle metabolic responses when horses were fed triheptanoin differed from the

responses when horses were fed the grain diet in that lactate was not elevated after exercise and G6P concentrations were higher for the triheptanoin diet than for the grain diet.

Triheptanoin could have had a further negative impact on the supply of long-chain fats by inhibiting lipolysis through elevated insulin concentrations and by increasing malonyl-CoA concentrations (via postexercise citrate concentrations, similar to the effect for the grain diet), which further inhibits transport of long-chain fats.[28] Less utilization of long-chain fat in horses for the triheptanoin diet was suggested by lower plasma NEFA concentrations and lower C16:0 and C18:2 plasma acylcarnitine concentrations when horses were fed the triheptanoin diet, compared with the corresponding concentrations when horses were fed the corn oil diet. Triheptanoin, when fed to rats and human patients, is extremely gluconeogenic,[14,21] which provides additional glucose, an increased insulin response, and a decrease in lipolysis. Although these various mechanisms may all have contributed to impaired energy generation in horses with PSSM during submaximal exercise when fed the triheptanoin diet, we were unable to detect significant differences in muscle substrates or metabolites between horses when fed the triheptanoin or corn oil diet.

Feeding the grain diet in the study reported here resulted in exercise intolerance and rhabdomyolysis within 18 minutes after initiation of light exercise in horses with PSSM. There is evidence from other studies that horses with PSSM have impaired energy generation in muscle[4] and lower oxygen consumption during exercise,[29] compared with results for healthy horses. When horses with PSSM were fed the grain diet in the present study, they had a modest increase in lactate concentrations and did

not have a significant decrease in muscle pyruvate concentrations with exercise, which is in contrast to findings for humans with glycogen storage diseases (myophosphorylase or phosphofructokinases deficiencies).[15,30] Thus, if there were substrate limited–oxidative metabolism in horses with PSSM when fed the grain diet, it would appear that the limitation in flux lies downstream of the metabolism of glycogen to pyruvate. Detecting the precise site of substrate limitation may require study of individual muscle fibers. For example, the present study and another study[4,8] revealed that ATP concentrations in whole muscle homogenates did not decrease significantly after exercise. However, analysis of individual fibers dissected from the same whole muscle sample revealed accumulation of inosine monophosphate in some fibers of horses with PSSM horses.[4] It is likely that only a small number of muscle fibers are recruited after 15 to 20 minutes of exercise, and only those recruited fibers exhibited substrate limited–oxidative flux.

It is difficult to explain how a gain of function mutation in *GYS1*, which leads to enhanced glycogen synthase activity (even in the basal state),[12] would impair oxidative metabolism of substrates such as pyruvate or fatty acids. Based on results of the study reported here, we speculate that excessive stimulation of glycogen synthesis resulting from a gain of function mutation in glycogen synthase (stimulated further by insulin) might be interpreted by nutrient sensors in cells as an indication that glycogenolysis and lipolysis need not be activated. A potential scenario in horses with PSSM fed the grain diet could be that nutrient sensors, such as AMP kinase, did not fully activate enzymes, such as pyruvate dehydrogenase, during exercise to produce adequate amounts of acetyl CoA for oxidative metabolism. Although acetyl CoA could also be supplied by fatty acid oxidation, horses with PSSM, when fed the grain

diet, had low plasma NEFA concentrations, possibly as a result of suppression of lipolysis by high insulin concentrations.[31] An additional factor for reduction of fatty acid oxidation in horses with PSSM when fed the grain diet may have been the high muscle citrate concentrations identified. High muscle citrate concentrations have been reported[32] in muscle exposed to a high-glucose load. Citrate activates acetyl CoA carboxylase, which converts acetyl CoA to malonyl CoA, the committed step for fatty acid synthesis, thereby directing acetyl CoA away from the CAC cycle. Accumulation of malonyl CoA causes inhibition of carnitine palmytoyl transferase, which is the key enzyme necessary to transport long-chain fatty acids into the mitochondria for □-oxidation.[33,34] Thus, when horses with PSSM were fed the grain diet, they may have been unable to generate sufficient amounts of acetyl CoA from carbohydrate or fat metabolism to fuel muscle contraction during submaximal exercise.

The provision of long-chain fatty acids, such as those supplied by the corn oil diet, significantly decreased exercise intolerance and rhabdomyolysis. This is consistent with results of a randomized study[16] conducted to evaluate effects of the HFLS in exercising horses with PSSM. Feeding the corn oil diet increased plasma NEFA concentrations and C18:2 plasma acylcarnitine concentrations and thus the availability of fats for oxidation in skeletal muscle. Increased □-oxidation of long-chain fats with exercise when horses were fed the corn oil diet was suggested by increased C2:0 acyl carnitine concentrations after exercise. The long-chain fat diets also appeared to increase glycogenolytic-glycolytic and oxidative flux in muscle of horses with PSSM, as indicated by higher G6P concentrations, lower lactate concentrations, and higher pyruvate concentrations (HFLS diet only) in muscle,

compared with results for the grain diet. Thus, long-chain fat diets appear to provide ample energy for aerobic exercise in horses with PSSM.

The study reported here was designed as a randomized comparison to compare effects for the corn oil and triheptanoin diets. The HFLS diet was fed last as a negative control diet, which could bias the assessment of the HFLS diet because training is beneficial for horses with PSSM. Corn oil consists primarily of linoleic (54%), oleic (29%), and palmitic (13%) acids,[31] and the HFLS diet contained fats from rice bran (oleic [38%]), linoleic [34%], and palmitic [22%] acids)[35] and soy oil (linoleic [50%], oleic [20%], and palmitic [14%] acids).[36] Thus, the form of long-chain fats supplied by the corn oil and HFLS diets were relatively similar; however, the amount of digestible energy from fat supplied by the corn oil diet (30%) was twice that supplied by the HFLS diet (15%). Furthermore, plasma NEFA concentrations when horses were fed the corn oil diet were 50% higher than when they were fed the HFLS diet. The results of this study could indicate that in fit horses with PSSM, feeding half as much fat in the form of the HFLS diet has a similar beneficial effect as feeding 750 mL of corn oil. Although some veterinarians recommend 0.45 kg of fat/d for horses with PSSM,[17] results of the study reported here suggest that in fit horses, a reduced amount of dietary fat is similarly beneficial and may avoid weight gain, thereby averting further disruption of an already disturbed metabolism. Low amounts of dietary starch and sugar are also equally important for horses with PSSM.

Daily exercise is another important means by which to decrease exercise intolerance and rhabdomyolysis in horses with PSSM.[6,16] The significant effect of time on plasma CK activity among diets, with higher CK activities during the first week of exercise in

the present study, supports the benefit of daily exercise. Exercise may lower plasma insulin concentrations and increase plasma NEFA concentrations, and training may, over time, enhance uptake of fatty acids into skeletal muscle and improve muscle oxidative capacity.[16,37,38] It was notable that the plasma NEFA concentrations at 4 hours after exercise were at least double those in samples obtained before exercise. In addition, it is possible that daily exercise may improve nutrient sensing and thereby affect the balance of energy supplied as carbohydrate and fat for oxidative metabolism.[39,40] Regular exercise and diet do not appear to decrease muscle glycogen concentrations in horses with PSSM because these were unchanged in the present study as well as in horses in other studies.[8,11,29]

The study reported here revealed that a key factor in feeding horses with PSSM is the provision of long-chain fats, as compared to short-chain fats, as well as providing low-starch diets that maintain low daily plasma insulin concentrations. Although triheptanoin appears to be metabolized by horses with PSSM and is of benefit to humans with disorders of lipid metabolism and some glycogenoses, it did not have a beneficial effect for horses with the *GYS1* mutation.

a. EquiJewel, Kentucky Equine Research, Versailles, Ky.

b. ACH Food Co Inc, Memphis, Tenn.

c. Sasol, Witten, Germany.

d. Re-Leve, Kentucky Equine Research, Versailles, Ky.

e. Glucose/hexokinase, Marshfield Clinic Laboratories, Marshfield, Wis.

f. Waco NEFA C test kit, Waco Diagnostics, Richmond, Va.

g. NCSS, Kaysville, Utah.

h. Prism software, GraphPad Software Inc, San Diego, Calif.

Table 1—Mean (range) plasma CK activity and mean ± SD glucose, insulin, and NEFA concentrations in samples obtained after exercise daily for 3 weeks in 8 horses while they were fed a grain, triheptanoin, corn oil, or HFLS diet.

Diet	**CK* (U/L)**	**Glucose (mg/dl)**	**Insulin (µU/ml)**	**NEFA (mEq/L)**
Grain	2,758[a] (232–44,343)	103.8 ± 18.1[a]	17.0 ± 14.0[a]	0.084 ± 0.025[a,c]
Triheptanoin	5,623[b] (223–50,040)	89.1 ± 17.7[b]	15.0 ± 14.0[a,c]	0.077 ± 0.039[a,c]
Corn oil	856[c] (195–7,257)	88.9 ± 17.7[b]	8.4 ± 4.9[b]	0.139 ± 0.046[b]
HFLS†	1,491[c] (157–39,942)	96.6 ± 11.5[a,b]	11.0 ± 7.2[b,c]	0.095 ± 0.036[c]

*Statistical comparisons for CK activity were performed on log-transformed values to normalize the data. †Represents results for only 7 horses because the catheter for collection of blood samples did not remain patent in 1 horse.

[a-c]Within a column, values with different superscript letters differ significantly ($P < 0.05$).

Table 2—Mean ± SD values for plasma CK activity in samples obtained 4 hours after exercise and glucose, insulin, and NEFA concentrations in samples obtained before and after an SET for 8 horses while they were fed a grain, triheptanoin, corn oil, or HFLS diet.

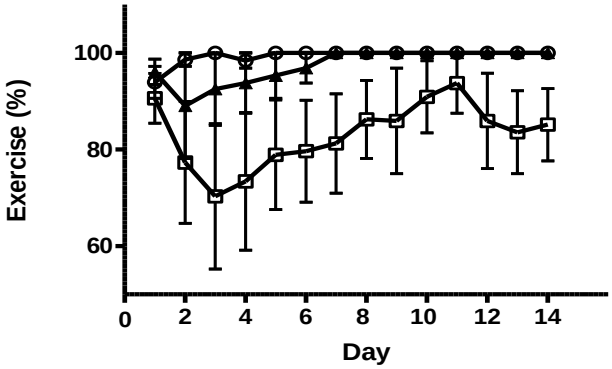
Diet	CK (U/L)	Glucose (mg/L)		Insulin (□U/mL)		NEFA (mEq/L)	
		Before	After	Before	After	Before	After
Grain	2,215 ± 2,153[a]	99.4 ± 4.1[a,b]	83.5 ± 13.3	21.7 ± 9.9	10.6 ± 4.2	0.030 ± 0.021[a,b]	0.046 ± 1.043[a,b]
Triheptanoin	4,114 ± 4,876[a,b]	82.4 ± 16.8[a]	82.1 ± 11.0	18.5 ± 8.4	9.2 ± 3.0	0.021 ± 0.006[a]	0.028 ± 0.010[a]
Corn oil	411 ± 151[a]	93.3 ± 8.6[a,b]	74.6 ± 13.9	12.3 ± 6.7	4.5 ± 1.3	0.080 ± 0.028[b]	0.091 ± 0.060[b]
HFLS	538 ± 308[a]	105.6 ± 18.3[b]	88.8 ± 21.1	23.0 ± 19.5	8.7 ± 4.9*	0.040 ± 0.019[a,b]	0.056 ± 0.035[a,b]

*Value differ significantly ($P < 0.05$) from value before exercise.

See Table 1 for remainder of key.

Figure 1. Mean ± SD percentage of individual exercise target completed by horses each day while fed the triheptanoin (white squares), corn oil (white circles), and HFLS (black triangles) diets (A) and mean plasma CK activity in samples obtained 4 hours after exercise in horses while they consumed the grain (black squares), triheptanoin, corn oil, and HFLS diets (B). In panel A, the individual exercise target for each of 8 horses was calculated as the mean amount of time each horse could exercise while being fed the grain diet. Mean percentage of exercise target completed was calculated as the mean of the amount of the daily exercise completed each day divided by the individual exercise target and multiplying the quotient by 100 for each of the other 3 diets. Horses completed significantly ($P < 0.05$) less of their exercise target while being fed the triheptanoin diet than while being fed the corn oil or HFLS diets; no other significant differences were detected among the other diets. In panel B, log-transformed plasma CK activity was significantly ($P < 0.05$) higher for the triheptanoin diet than for the corn oil or HFLS diets; no other significant differences were detected among the other diets. Plasma CK activity for the HFLS diet represents results for only 7 horses. Day 0 = First day on which the complete amount of the specified diet was fed; each diet was fed for 14 days with a 10 day washout period between subsequent diets.

A


Exercise (%)
100
80
60
0
2
4
6
8
10
12
14
Day

B

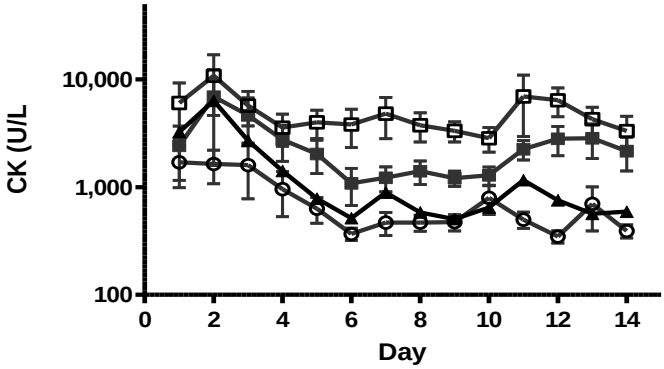

CK (U/L
10,000
1,000
100
0
2
4
6
8
10
12
14
Day

Figure 2—Mean ± SD concentrations of plasma acylcarnitines with various numbers of carbon atoms obtained before and after a 15-minute SET in horses when they were fed the triheptanoin (black bars) and corn oil (white bars) diets. Notice that scales on the y-axis differ among the graphs. a–cValues with different letters differ significantly ($P < 0.05$). When horses were fed the triheptanoin diet they had higher concentrations of propionyl and C7:0 acylcarnitines, whereas when horses were fed the corn oil diet, they had higher concentrations of long-chain fats (C18:2 acylcarnitine).

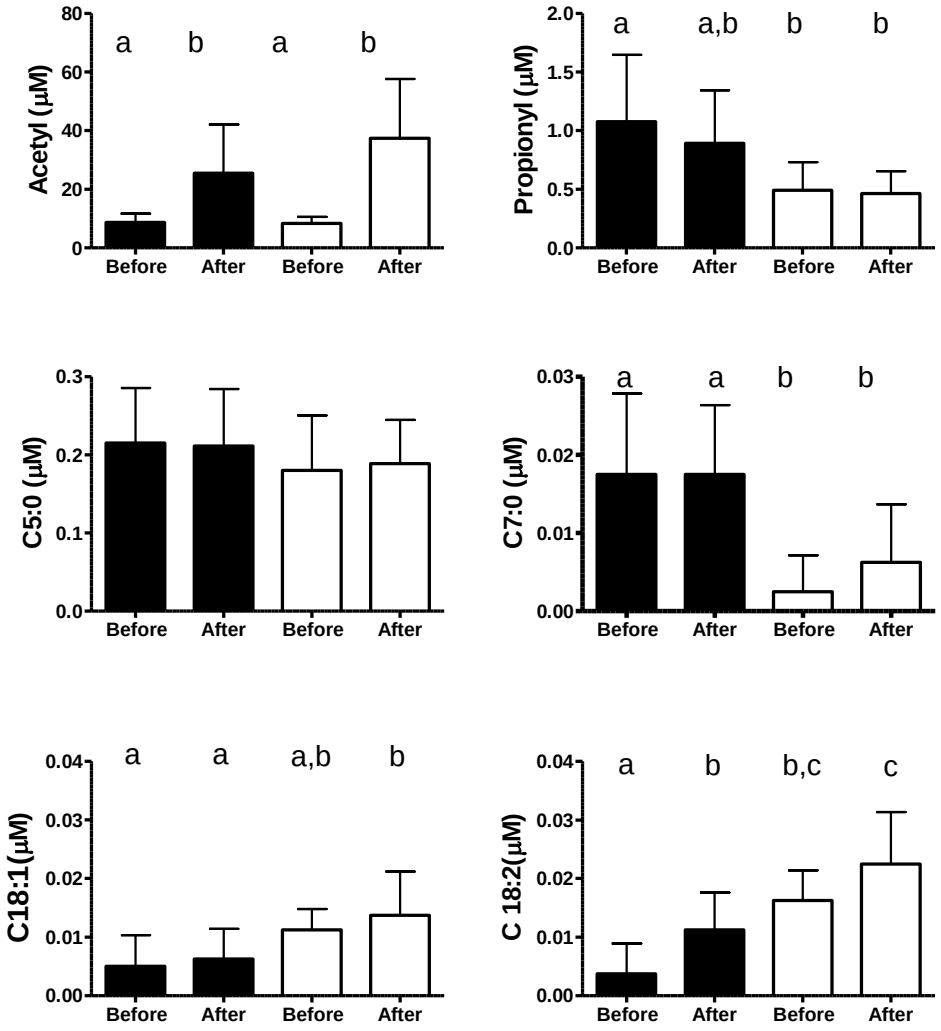
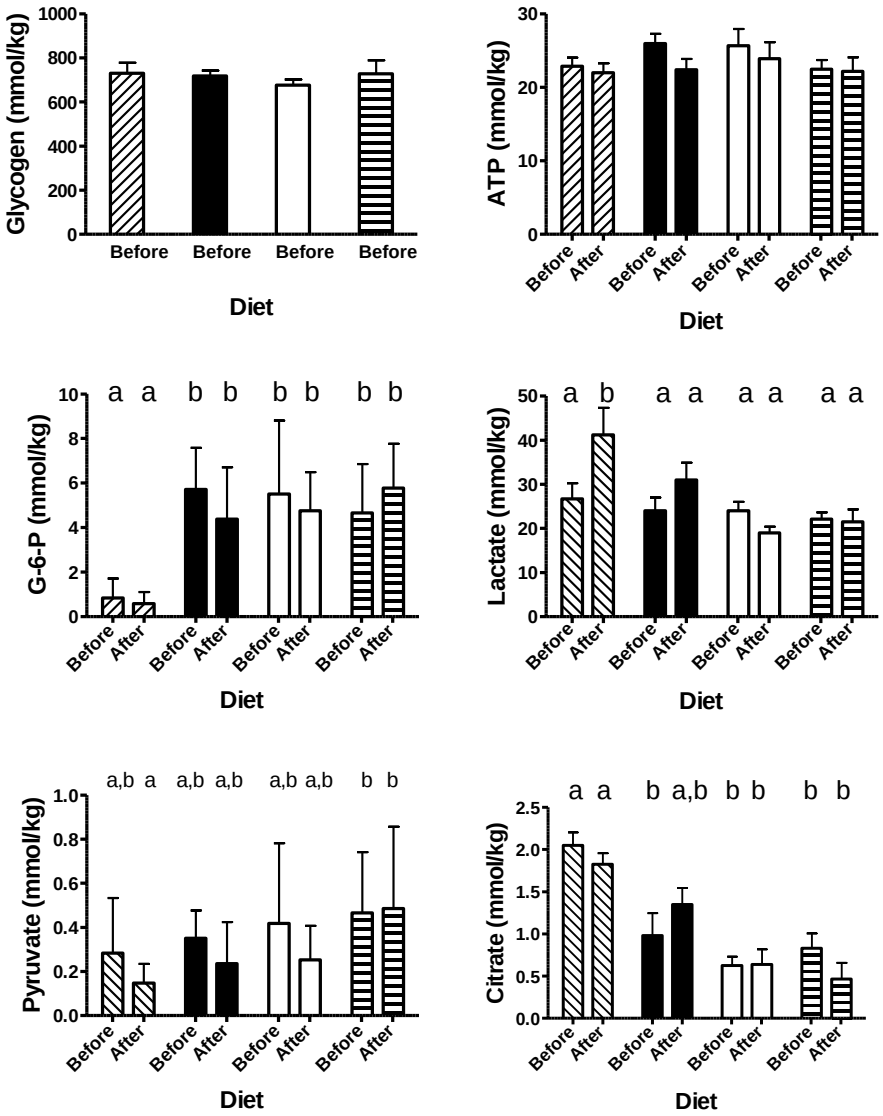


Figure 3—Mean ± SD concentrations of substrates and metabolites in gluteal muscle biopsy specimens obtained before and after a 15-minute SET in horses when fed the grain (diagonal-striped bars), triheptanoin (black bars), corn oil (white bars), and HFLS (horizontal-striped bars) diets. Values are reported on a dry-weight basis. *See* Figure 2 for remainder of key.



CHAPTER SIX

FUTURE DIRECTIONS

FUTURE DIRECTIONS :

The research contained within this thesis ranges from dietary management of PSSM to preliminary investigation into skeletal muscle response to resistance training; thus the results are broad and shallow, with much left to be understood. Three of the research endeavors were either pilot studies or first real attempts to identify factors involved, so many questions remain unanswered.

Water Treadmill: Very little research has been done to quantify the effectiveness of water treadmill therapy on conditioning and injury rehabilitation, although equine rehabilitation using this modality is frequently found in the industry. The water treadmill is used for rehabilitation and range of motion exercise, with the water acting to both support the horse and provide resistance. Horses suffering from injuries such as hyperextension of the superficial digital flexor tendon that would otherwise be unable to exercise may be able to use the water treadmill for rehabilitation purposes. The study done in this body of work did little to establish the efficacy of water treadmill as a rehabilitation tool for SDF tendon injury, and further investigation on the benefits of water treadmill use in the rehabilitation of injured horses is needed. A study on the effect of water level in the treadmill on equine joint kinematics (Trumble, personal communication) has been completed and is being readied for publication that will further the understanding of how water treadmill use can benefit equine rehabilitation.

The water treadmill protocol used in this study did not produce an increase in fitness as measured by V_{200} and by muscle oxidative enzyme activities. If increased fitness was the purpose for water treadmill use, then a more strenuous protocol would be needed to provide intended results. Furthermore, the finding of approximately 50% lower oxidative, glycolytic and substrate concentrations in the SDF than gluteal

muscle at rest suggest this muscle is either passive during locomotion or could be precariously predisposed to fatigue during maximal exercise.

A follow up study investigating the SDF muscle properties and to determine the cardiocirculatory response in healthy horses between water and land treadmill submaximal exercise is needed. The hypotheses to be investigated would include if training horses in a water treadmill would produce a greater increase in muscle oxidative capacity and improve markers of systemic fitness than training horses on a land treadmill. Also, an evaluation of the anaerobic metabolic response in the SDF muscle to see if it is more marked than previously recognized would be interesting. Further, it would be interesting to evaluate if the water treadmill produces a greater training effect in the SDF relative to the middle gluteal muscles than the land treadmill.

In order to test these hypotheses, the aims would be to determine if markers of fatigue are more pronounced in the SDF vs. the gluteus medius muscle after a SET. Heart rate and blood lactate responses as well as changes in substrate and metabolite concentrations in SDF and gluteal muscles may be different between water treadmill training and land treadmill training, as well as activity of markers of oxidative capacity in muscle after water treadmill training compared to training on a land treadmill. A longer training period would be used, as the present study did not produce as measurable cardiocirculatory effect.

Six Quarter Horse crosses would be used for this research after a minimum of 60 days detraining. After detraining, a SET would be performed, with gluteal and SDF muscle biopsies obtained at 5 min post-exercise. Horses would be randomly assigned to either land or water treadmill training for 8 weeks in a block design, with the same speed and duration of treadmill training for both the water and land treadmills. The

duration of training would increase by 1 min /day and then continue at 40 min per day until the end of 8 weeks, with a SET on the last day. A 60-day detraining period would occur between exercise blocks. The exercise intervals would be increased every 2 min until a heart rate of 200b/min is recorded for an entire exercise interval. Muscle biopsies would be repeated as before, 5 min after exercise on the opposite limb from the previous samples.

Horses would switch to train on the alternate treadmill and the same training protocol repeated, and at the conclusion of the second 8 week training period, each horse would repeat the SET.

The expectation is that after the near-maximal treadmill test the ATP concentrations in the SDF muscle would be more than 50% lower than the concentrations in the gluteal muscle. A training effect would be demonstrated by an increase in the oxidative capacity of skeletal muscle (higher CS activity) and with less depletion of glycogen and ATP, less lactate accumulation and lower heart rates and blood lactates with the SET. Further, a measurable difference in muscle oxidative capacity and metabolic response in horses with the 8-week training trial would be expected, and that this effect would be greater with water treadmill compared to the land treadmill training. ANOVA and post hoc tests would determine if there is a difference between training on the 2 different treadmills. Water treadmill training might be expected to increase oxidative enzyme activity more than equivalent training without any water resistance and the training effect might be greater in the muscle such as the SDF, which is below the water level as compared to the gluteal muscle, which is above water.

Glycemic and insulinemic differences between breeds: The effect of enhanced metabolic efficiency is considered the underlying cause of Equine Metabolic Syndrome (EMS). This syndrome represents a cluster of physiologic alterations that are important because of their association with laminitis (Geor and Frank 2009). Two important components of EMS are obesity and insulin resistance, and affected horses are often referred to as easy keepers, because they require fewer calories to maintain body condition. The influence of breed, sex, and age are compounding factors in determining the effect of diet on the health of these horses, but little is known about the exact effect of each variable (Frank et al. 2006; Hoffman et al. 2003a; Hoffman et al. 2003b; Williams et al. 2001).

A possible study that compares breeds using FSGIT results (Si, Sg, AIRg, Di) to quantify the difference between Thoroughbreds, Quarter Horses, and Standardbreds in response to a glucose challenge could be done via a collaborative effort with researchers. This investigation into the possible effect of breed on insulin sensitivity and glucose metabolism could be balanced for sex, which was not accounted for in the present study. Horses should be of approximately the same age if possible. The information yet to be realized on the factors of breed and sex on dietary management of horses facing metabolic imbalances will be invaluable.

The objectives of this investigation would be to estimate the glucose effectiveness and insulin sensitivity in horses to test the effect of breed and gender in normal horses of different breeds, specifically Quarter Horses, Thoroughbreds, and Standardbreds, since these breed already have FSGIT data available. Similar numbers of QH, SB and TB, evenly distributed between male and female and of similar physical attributes would be used.

Based on the present study, QH would be expected to be more insulin sensitive, and have a faster decline of blood glucose and higher concentration of insulin in response to the FSGIT compared to the other two breeds. The glucose nadir of the FSGIT would be lower for the QH compared to the other breeds and would not return to baseline values or the glucose AUC would be smaller for QH compared to the other breeds. It is unknown if TB and SB would differ. Differences between sexes are unknown at this time, so the expected results would be no difference between sexes for all measured variables.

The results seen in the present study comparing response to hay of different NSC could be related to the lack of fitness in the PSSM horses, differences in ages of horses, or a factor of individual variability in blood glucose and insulin responses. Another reason for the greater insulinemic response may be the higher rate of consumption of the higher NSC concentration forage compared to the lower NSC concentration forage. A more rapid rate of intake could increase the rate of passage into the small intestines and amount of glucose absorbed into the bloodstream. These possibilities should be subjected to further scrutiny in conjunction with the FSGIT study as proposed above.

Effect of feeding odd carbon fats to type 2 PSSM horses: Feeding odd-carbon, medium-chain length fats to horses with PSSM was conducted with the hope of discovering a treatment for acute episodes, in order to provide immediate relief and to reduce damage to muscle tissue. However, feeding triheptanion oil exacerbated muscle damage with exercise in type 1 PSSM horses, offering a new insight into how this disease produces rhabdomyolysis. The present study was conducted with Type 1 PSSM horses. However, the original impetus for investigation triheptanoin remains, specifically a clinical case showing improvement in an acute state. The disease status

of the foal from the case was a type 2 PSSM (unknown at the time of the present study). It is possible that there might still be a benefit of using odd-carbon medium-chain fat as a supplement in type 2 PSSM horses. A very small pilot study could be conducted using type 2 PSSM horses fed triheptanoin and exercised on a treadmill daily to investigate this possibility. The objective would be to determine if feeding odd-carbon, medium-chain fat to type 2 PSSM horses affects muscle metabolism with exercise and determine if feeding this fat reduces exercise intolerance as measured by CK activity.

This could be done with 8 horses with type 2 PSSM, 4 male and 4 female, with a mean age of 6.5 years and BCS of 4.5 – 6.5 to match the present study.

These horses would be fed isocaloric diets of triheptanoin, grain, and corn oil for 3 weeks each, with daily treadmill exercise (individual target exercise based on acclimation period). An incremental SET would be performed at the end of each three week period, with a wash out of one week between diets. The study would be cross over design with horses assigned diets randomly, and then switched.

Blood samples would be taken 4 hrs post exercise daily, and plasma CK activity determined. Aliquots of plasma would be stored for analysis of glucose, insulin, and NEFA concentrations. SET plasma samples would be taken pre and post exercise for glucose and NEFA concentration, and additional sample would be taken 4 hr post SET for CK activity. Gluteal muscle biopsy specimens would be taken pre- and post-SET for glycogen, lactate, G-6-P, citrate, pyruvate, and ATP concentrations.

A comparison could be made between the results of this proposed study and the present study to investigate differences between type 1 and type 2 PSSM. The expected results would be that no difference would be detected.

Conclusion: In conclusion, there are several interesting possibilities that could further out understanding of the differences between PSSM and healthy horses, and even within variations of PSSM in response to different carbohydrate loads and oil supplementation. There are still many factors such as breed, sex, and even age that could be assessed for influence on metabolic function in healthy horses. Finally, the amount of resistance training as provided by water treadmill use to induce cardiocirculatory conditioning is still unknown. The possible impact of differences between SDF and gluteal muscle properties as suggested by the present study is still unknown as well. These are all questions worthy of further examination.

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APPENDIX A

Percentage of digestible energy supplied by starch, fat, protein, and fiber in the grain, triheptanoin, corn oil, and HFLS concentrates.

Total digestible energy	***Grain***	***Triheptanoin***	***Corn oil***	***HFLS***
Starch (%)	33	19	19	24
Fat (%)	9	30	30	17
Protein (%)	22	18	18	22
Fiber (%)	36	33	33	37

All diets were isocaloric and provided 25 Mcal/500 kg of diet/d.