EFFECT OF EXERCISE AND OF MEALS OF DIFFERING STARCH CONTENT ON GLUCOSE KINETICS AND MUSCLE GLYCOGEN UTILIZATION AND REPLENISHMENT IN HORSES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree of Doctor of Philosophy in the

Graduate School of The Ohio State University

By

Eduard Jose-Cunilleras, DVM

* * * * *

The Ohio State University

2004

Dissertation Committee:

Kenneth W. Hinchcliff, Adviser

Steven T. Devor

Catherine W. Kohn

Richard A. Sams

Approved by

_____

Adviser
Veterinary Clinical Sciences
Graduate Program

ABSTRACT

A combination of plasma and muscle biochemical methods, indirect calorimetry, isotopic tracer studies ([6,6-^{2}H]glucose as constant rate infusion) and real-time reverse transcription polymerase chain reaction techniques were used to gain a better understanding of the effect of ingestion of meals of differing starch content prior to or after exercise by horses. In the first study (Chapter 3), horses were fed before exercise either (1) corn, (2) an isocaloric amount of alfalfa cubes (51.4 KJ/kg DE), or (3) not fed. The main finding was that meal type prior to exercise modestly altered substrate use during exercise such that corn feeding resulted in greater carbohydrate oxidation due to higher skeletal muscle utilization of blood-borne glucose, unchanged muscle glycogenolysis and lower whole body lipid oxidation. In the second study (Chapter 4), the glycemic response to ingestion of cereals (cracked corn, steamed oat groats or rolled barley) and intragastric administration of glucose was assessed by giving equal amounts of hydrolyzable carbohydrates. We determined that oat groats, corn and barley have similar areas under the plasma glucose concentration-time curve in horses, and compared with the glycemic index of 100, these cereals were approximately 60. In the third study (Chapter 5), horses with exercise-induced muscle glycogen depletion were either not fed

for 8 h, fed mixed alfalfa and grass hay (~15 Mcal, ~62 MJ DE), or fed an isocaloric amount of corn immediately and 4 h after exercise. The main findings were that corn feeding, when compared to feed withholding, resulted in mild to moderate hyperglycemia and hyperinsulinemia, and a 3-fold greater whole body availability and utilization of glucose. However, muscle glycogen replenishment was only minimally enhanced. In the last study (Chapter 6), we described the effect of glycogen-depleting exercise and of meal type after exercise (as in Chapter 5) on the insulin responsive glucose transporter (GLUT4) gene expression in skeletal muscle. We found that GLUT 4 gene expression in muscle increased by ~2-4 fold during 24 h after exercise, when compared to that prior to exercise but no differences were observed due to meal type fed after exercise.

To my wife, Sandra, for her love, patience and understanding throughout the years. To our families, specially my parents, for their unfailing support.

ACKNOWLEDGMENTS

I wish to acknowledge my advisor, Ken Hinchcliff, an outstanding mentor and scholar. He has always lead by example, always encouraging and honest, always supportive throughout my graduate and residency program. I feel indebted to him. It has been a privilege to have worked with him, and can only hope we continue to collaborate. Many thanks are also due to the rest of the members of my graduate committee, Drs. Steve Devor, Cathy Kohn, and Rick Sams. Steve was instrumental in providing a new perspective from within the Exercise Physiology field, Cathy was always supportive and gave her insight on some practical aspects of equine exercise physiology, and Rick's guidance during the assays of glucose isotopic tracers proved invaluable. I also want to acknowledge Dr. Lynn Taylor for her expertise and suggestions related to Equine nutrition, Dr. Ray Geor for his guidance in the technical aspects of glucose isotopic tracer analysis, and Dr. Kate Hayes, Ramiro Toribio and Larry Mathes for their expertise and support during the gene sequencing and expression experiments. I want to acknowledge Dr. Dave Horohov for providing the equine β-actin gene necessary in the gene expression experiments. I am also grateful to all graduate, technicians and undergraduate students that assisted me throughout these studies including Dr. Jeong-Su Kim, Leia Hill, Lori Avila, Josh Stern, Dr. Wayne Buck, Sue Ashcraft, Dr. Dave Hall, Aaron Ison and countless undergraduate students. This research studies were supported by grants from

the Equine Research Funds of The Ohio State University, the Grayson-Jockey Club Research Foundation, Inc., the USA Equestrian Inc., and from Purina Mills, St. Louis, Missouri. This financial support is gratefully acknowledged.

Finally, my deepest gratitude goes to my wife and my family. To my parents for motivating me to pursue my ambitions and being supportive all the way through. To my wife, Sandra, for her unfailing love and encouragement.

VITA

May 12, 1974……..Born - Barcelona, Spain

1992…… Baccalaureatte, Spain
Major: Biological Sciences

1997…… Doctor of Veterinary Medicine (equivalent)
Autonomous University of Barcelona, Spain

1997-1998……...Intenship, Equine Medicine
The Ohio State University

1998-2001……...Residency, Equine Internal Medicine
The Ohio State University

2001-2004……...Graduate Research Associate
The Ohio State University

PUBLICATIONS

Research Publications

1. Jose-Cunilleras E, Hinchcliff KW (2004) Carbohydrate metabolism in exercising horses. *Equine and Comparative Exercise Physiology* 1(1):23-32.

2. Nout Y, Hinchcliff KW, Jose-Cunilleras E, Dearth LR, Sivko GS, DeWille JW (2003) Moderate exercise and hyperglycemia after exercise do not alter muscle GLUT4 expression in horses. *Am J Vet Res*, 64(11): 1401-8.

3. Jose-Cunilleras E, Hinchcliff KW, Sams RA, Devor ST, Linderman J. (2002) Glycemic index of a meal fed before exercise alters substrate use and glucose flux in exercising horses. *J Appl Physiol*, 92(1):117-28.

4. Nout YS, Hinchcliff KW, Samii VF, Kohn CW, Jose-Cunilleras E, Reed SM. (2002) Chronic pulmonary disease with radiographic interstitial opacity (interstitial

pneumonia) in foals. *Equine Vet J*, 34(6):542-8.

5. Stewart AJ, Hinchcliff KW, Saville WJA, Jose-Cunilleras E, Hardy J, Kohn CW, Reed SM, Kowalski JJ (2001) *Actinobacillus* sp. bacteremia in foals: clinical signs and prognosis. *J Vet Intern Med*, 16(4):464-71.

6. Jose-Cunilleras E, Hinchcliff KW. (2001) *Listeria monocytogenes* septicaemia in foals. *Equine Vet J* 33(5):519-22.

7. Jose-Cunilleras E, Kohn CW, Hillier A, Saville WJA, Lorch G. (2001) Intradermal testing in healthy horses and horses with chronic obstructive pulmonary disease, recurrent urticaria, or allergic dermatitis. *J Am Vet Med Assoc* 219(8):1115-1121.

8. Lorch G, Hillier A, Kwochka K, Saville WJA, Kohn CW, Jose-Cunilleras E. (2001) Results of intradermal tests in horses without atopy and horses with chronic obstructive pulmonary disease *Am J Vet Res* 62(3):389-97.

Book chapters

1. Jose-Cunilleras E. (2004) Abnormalities of fluids and electrolytes in athletic horses. In: Hinchcliff KW, Geor RJ, Kaneps AJ (eds) Equine Sports Medicine and Surgery. Elsevier Science, Oxford, UK, pp:898-918.

2. Jose-Cunilleras E. (2004) Verminous encephalomyelitis. In: Reed SM, Bayly WM and Sellon DC (eds) Equine Internal Medicine, 2nd ed, WB Saunders, Philadelphia, PA, pp: 659-665.

3. Jose-Cunilleras E, Hinchcliff KW. (1999) Renal Pharmacology. *Vet Clin N Am (Equine Pract)* 15(3): 647-664.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

In this day and age, the horse's primary uses are various competitive or recreational athletic activities. For the same reasons that milk production and lean mass accretion are primary concerns in food animal sciences, athletic performance and welfare of working horses has been and continues to be of upmost importance in equine sports medicine. Therefore, studies of nutrition and management of horses resulting in enhanced athletic capacity and health are of great relevance to the equine industry.

Athletic events involving horses range from maximal intensity races of only a few seconds duration over a quarter mile distance, to endurance races completed over several hours or days. In all cases, physical activity requires mechanical work, which is fueled by chemical energy generated from endogenous sources. The chemical energy stored as high-energy phosphate bonds (in adenosine triphosphate and phosphocreatine) only supports the initial seconds of an exercise bout. Thereafter, carbohydrates and lipids are utilized to recycle the high-energy phosphate bonds and continue to transform chemical energy into chemical work and heat. Carbohydrates, in the form of muscle glycogen and blood-borne glucose (from hepatic glycogenolysis and gluconeogenesis, and from the gastrointestinal tract when carbohydrates are ingested), and lipids, in the form of fatty

acids released during lipolysis of triglycerides in adipocytes and within muscle fibers, are the substrates required during exercise. If caloric requirements are not met by utilization of a mixture of these substrates, muscular performance is impaired and fatigue may ensue.

The present series of investigations were undertaken to determine the effect of variation of glucose supply by dietary means on quantitative assessments of glucose and muscle glycogen homeostasis and carbohydrate utilization before, during and after exercise.

In the first study (Chapter 3), an isocaloric meal of grain or hay was given prior to exercise, or food was withheld, in order to determine the effects of dietary glucose supply on stable isotope tracer-determined whole body glucose appearance (glucose R_a) and disappearance (glucose R_d), and whole-body rates of carbohydrate and fat oxidation in horses during moderate-intensity exercise. Two hypothesis were tested in this study: (1) that feeding a starch-rich meal prior to exercise results in increased glucose R_a and glucose R_d during moderate-intensity exercise, and (2) that ingestion of a starch-rich meal prior to exercise would result in enhanced carbohydrate oxidation, attenuated lipid oxidation, greater relative contributions to energy expenditure from blood-borne glucose, and attenuated muscle glycogenolysis ("muscle glycogen sparing effect") during exercise. The second study (Chapter 4) aimed to better define the glycemic responses observed in resting horses given equal amounts of starch and sugar in the form of cracked corn, steamed oat groats and rolled barley, and compared these to intragastric administration of an equivalent amount of glucose. Given that previous studies suggested

that oat starch had higher small intestinal digestibility when compared to corn or barley, we hypothesized that oat groats would have higher glucose availability than corn and barley in horses, when assessed as the glycemic index compared with oral glucose. The third study (Chapter 5) examined the effect of post-exercise isocaloric meals of varying starch content (corn vs. hay), or feed withholding, on whole-body glucose turnover rates and muscle glycogen synthesis after exercise. We hypothesized that ingestion of starch-rich meals would result in increased glucose R_a and glucose R_d when compared to hay-fed or unfed horses. In addition, it was hypothesized that greater blood-borne glucose supply in grain-fed horses would result in enhanced muscle glycogen replenishment when compared to hay-fed or unfed horses. In the last study (Chapter 6), we aimed to better understand the cellular mechanisms involved in glucose homeostasis and muscle glycogen synthesis in horses by examining the effect of exercise-induced muscle glycogen depletion and post-exercise meal type on gene expression of the insulin-responsive glucose transporter in muscle after exercise. We hypothesized that exercise would increase GLUT-4 gene expression in equine skeletal muscle, and starch-rich meals would further enhance GLUT-4 gene expression when compared to isocaloric meals of roughage or feed withholding.

A detailed description of the aforementioned studies is presented in the following chapters.

CHAPTER 2

LITERATURE REVIEW

Carbohydrate and fat are the predominant sources of energy during exercise in mammals. Carbohydrates, such as muscle glycogen and plasma glucose, and fats from adipose tissue and intramuscular triglycerides are oxidized during exercise in amounts and proportions that vary depending on the exercise intensity, level of fitness and nutritional status. In horses, muscle glycogen, and to a lesser extent plasma glucose, are oxidized in substantial amounts during low, moderate and high intensity exercise. Carbohydrate availability to skeletal muscle affects exercise performance in humans, however this relationship is not well outlined in horses. Glucose supplementation by intravenous administration during exercise increases duration of moderate intensity exercise in horses. However, the effect of glucose supplementation by ingestion of a soluble carbohydrate-rich meal prior to exercise on athletic performance has not been established in horses. Low muscle glycogen concentrations prior to exercise in horses are associated with decreased time to exhaustion at moderate and high intensity exercise. Nutritional interventions intended to enhance muscle glycogen resynthesis have proven

less successful in horses than in other species. Replenishment of muscle glycogen after strenuous exercise in horses is not complete until 48-72 h after exercise, whereas in humans and laboratory animals it is complete by 24 h. The slower rate of muscle glycogen replenishment after exercise in horses may be related to an inherently lower ability to digest starch and other sources of glucose, a lower ability to synthesize muscle glycogen, or both. The aim of this review is to describe the current understanding of carbohydrate metabolism in the exercising horse, its implications for nutrition and athletic performance, and to contrast it to that in other species.

The athletic horse has a large capacity to perform muscular work compared to many other mammals, including humans. Energy to perform work is obtained from oxidation of carbohydrate and fat, and, to a minimal extent, protein. The energy requirements of athletic horses are met by ingestion of these nutrients. However, while metabolism and energy transduction in muscle fibers of mammalian species are similar, the energy requirements of different athletic species are met by ingestion of very different diets.

The horse is a herbivore that has adapted its gastrointestinal function for hindgut fermentation. Unlike ruminants, equids have a small simple stomach, followed by 60-70 feet of small intestine where digestion and absorption of soluble carbohydrates, fat and protein occurs. Microbial fermentation occurs in the cecum and large colon, which hold 80-100 liters of liquid and houses billions of bacteria and protozoa, that break down plant fiber releasing volatile fatty acids (VFAs), the most abundant being acetate, propionate and butyrate[1]. Energy intake of horses is primarily from carbohydrates in forages and grains. Fiber in

forages is fermented in the hindgut and acetate and butyrate are used for synthesis of fatty acids, and propionate used for synthesis of glucose. Starch and sugars in grains and molasses are digested primarily in the small intestine and absorbed as monosaccharides and eventually transformed to glucose or fat.

Glucose is stored as glycogen in liver and muscle. Glycogen is a branched polymer of glucose with a mixture of α-1,4 and α-1,6 linkages between glucose units. Muscle glycogen constitutes over 90% of the carbohydrate in the body (Table 2.1) and the amount of glycogen is muscle fiber type dependent. Fast-twitch muscle fibers (type II) have greater glycogen content than slow-twitch muscle fibers[2,3]. Fat is stored in both adipose tissue and in muscle, both at intracellular (myocyte) and extracellular sites. In contrast to carbohydrates, intramyocellular triglycerides make up only ≈5% of the fats in the body. Slow-twitch muscle fibers have greater lipid content than fast-twitch highly oxidative muscle fibers, and the amount of lipid in fast-twitch low oxidative muscle fibers is negligible[4,5]. Intracellular triglycerides are seen within muscle fibers as lipid droplets in close proximity to mitochondria.

Differential storage of glycogen and intramyocellular triglycerides among muscle fiber types is related to inherent metabolic differences of the muscle fibers. Muscle fiber types are classified based on: (1) differences in contractile properties and oxidative/glycolytic enzymatic profiles (slow twitch, fast twitch highly oxidative and fast twitch low oxidative), (2) based on differences in pH sensitivity of the myofibrillar ATPase (type I, IIA and IIB) and (3) based on myosin heavy-chain (MyHC) expression (type I, IIA and IIX). Type I fibers have a MyHC isoform that hydrolyzes ATP slowly, resulting in a

slow cross-bridge cycle, together with a small cross-sectional area, a high number of capillaries per fiber, greater storage of lipids and a high oxidative capacity. However, their glycolytic capacity and glycogen content is lower than that of other fiber types. In contrast, type II fibers have MyHC isoforms that create fast cross-bridge cycling and therefore develop force rapidly. Type IIX fibers are adapted for high power outputs for a limited time because they have a low oxidative capacity and limited oxygen availability (as reflected by their large cross sectional area and relatively low capillary supply). Type IIA fibers, however, have a considerable number of both capillaries and mitochondria, and rely on glycolytic and oxidative metabolism; they are therefore able to sustain high power outputs for longer than IIX fibers. Hybrid IIAX fibers are intermediate in their properties[6,7].

Glucose and fatty acids stored within or outside of the muscle are used as fuels during exercise in horses, as well as in other athletic species. However, quantitatively the contributions of different fuels vary among different athletic species due to inherent metabolic and nutritional differences. Our aim is to describe the present understanding of carbohydrate metabolism in the exercising horse, its implications on nutrition and athletic performance, and to contrast it to that in other species.

2.1 ENERGY METABOLISM AND FUEL SOURCES DURING EXERCISE

During exercise, skeletal muscles perform mechanical work using chemical energy obtained from fat and carbohydrate oxidation. The relative contributions of fat and carbohydrate used to fuel exercise depend on exercise intensity, level of fitness, sex, menstrual phase in the case of women, environmental conditions, type of meal ingested

and interval from ingestion to initiation of exercise. All of these factors influence muscle and liver glycogen concentrations and circulating hormone status (insulin, glucagon, catecholamines) and will dictate the mixture of substrates utilized to fuel exercise. Many, but not all, of these factors have been evaluated in horses.

Intensity of exercise is generally described relative to the percentage of maximal oxygen consumption (% $\dot{V}O_{2max}$). Increasing exercise intensities shift the predominant substrate contribution to energy expenditure from fat to carbohydrate. In addition, high intensity exercise increases reliance on intramyocellular stores of carbohydrate (glycogen granules) and fat (triglyceride droplets). Some of the mechanisms that limit fat oxidation with increasing exercise intensities include reduced non-esterified fatty acid mobilization from adipose tissues due to reduced blood flow to adipose tissue, despite maintained lypolysis[8], and decreased activity of carnitine palmitoyltransferase I, which is responsible for transport of long chain fatty acids from the sarcoplasm to the mitochondria, due to decreases in intracellular pH[9]. The increase in muscle glycogen degradation as exercise intensity increases is associated with activation of glycogen phosphorylase, which cleaves a single glucose molecule from glycogen. Increased activity of phosphorylase is in response to increased sarcoplasmic calcium concentration, associated with muscle contractions, and hormonal stimulation by epinephrine mediated via β-receptors and the intracellular second messenger 3',5'cyclic adenosine monophosphate[10].

Studies performed in trained male and female human athletes demonstrate that increasing exercise intensity from 25% $\dot{V}O_{2max}$ to 85% $\dot{V}O_{2max}$ alters the relative

contributions to energy expenditure of blood-borne glucose oxidation, muscle glycogenolysis, plasma non-esterified fatty acids (NEFA) and intramyocellular triglyceride (IMTG) oxidation[11,12]. At low exercise intensity (25% $\dot{V}O_{2max}$) oxidation of plasma NEFA account for most of the energy requirements. At moderate exercise intensity (65% $\dot{V}O_{2max}$) the contribution from oxidation of muscle glycogen, blood-borne glucose and IMTGs is greater than at lower exercise intensity. At moderate to high exercise intensity (85% $\dot{V}O_{2max}$) the contribution from muscle glycogenolysis increases exponentially and the relative contribution from the rest of the fuels decrease significantly[11,12]. In summary, greater energy fluxes required during high intensity exercise are met by increased reliance on intramuscular substrate stores, predominantly from muscle glycogen.

2.1.1 Horse studies:

Horses have a higher mass specific aerobic capacity than humans. Thoroughbred horses have a maximal oxygen consumption ($\dot{V}O_{2max}$) of approximately 160 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$ and Olympic-caliber human athletes have a $\dot{V}O_{2max}$ of approximately 70-80 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$[13-15]. As is the case in pronghorn antelopes, which have a $\dot{V}O_{2max}$ of ≈300 $O_2 \cdot kg^{-1} \cdot min^{-1}$[16], the high $\dot{V}O_{2max}$ of horses is likely associated with the pressure of natural selection on horses to become more athletic and aerobic animals, as well as selective breeding of those horses with greater athletic ability. Horses, when compared to humans, have a greater aerobic capacity because, relative to body mass, the former have a greater oxygen carrying capacity, greater cardiac output and greater oxygen conductance at a

capillary level[17]. Other reasons for the apparent greater mass specific aerobic capacity in horses compared to humans are differences in body composition, the proportion of muscles engaged during running in quadrupeds versus bipeds and differences in muscle mitochondrial density. Muscle mass as a percentage of body weight ranges from ≈52% in Thoroughbred racehorses to ≈42% in average horses of other breeds, and in humans from ≈47% in elite road-cyclists to ≈40% as the average for male young adults[18,19]. Not only is the muscle mass greater in horses than in humans but more importantly the proportion of muscles engaged during exercise in quadrupeds is much greater (≈70-80%) than in bipeds (≈30-40%). In addition, mitochondrial density, the volume of mitochondria per volume of muscle fiber, of equine muscle ranges from 6% to 8.5% depending on muscle group and fiber type, whereas that of humans ranges from 2% to 6%[20,21]. In summary, horses have a high aerobic capacity due to cardiovascular, muscular and metabolic adaptations and a greater mass of active skeletal muscle relative to body weight.

At the same exercise intensity relative to $\dot{V}O_{2max}$, the mass specific rates of oxygen consumption ($\dot{V}O_2$) and the energy expenditure are approximately 2-fold higher in horses than in humans. In addition higher energy fluxes in horses, when compared to humans, are associated with a greater contribution from carbohydrate (CHO) oxidation to energy expenditure. Other athletic (i.e. dogs) and non-athletic (i.e. goats) mammals have similar 2-fold difference in mass specific $\dot{V}O_2$ and energy expenditure. However, at approximately 60% $\dot{V}O_{2max}$ the contribution to energy expenditure from carbohydrate (57-60%) vs. fat (40-43%) oxidation is similar between dogs and goats, unlike the comparison between horses (75% from CHO, 25% from fat) and humans (40% from

CHO, 60% from fat)[22-24] (Table 2.2).

Horses and dogs, when compared to humans and goats exercising at similar % of $\dot{V}O_{2max}$, have a 2-fold higher energy flux. Horses, when compared to humans, dogs and goats exercising at the same intensity, have greater proportion of energy derived from carbohydrate. Therefore, observations made in humans or other animals about carbohydrate and fat metabolism and energy requirements during exercise at an apparent similar intensity (same % $\dot{V}O_{2max}$) may not apply to horses.

Estimation of substrate utilization during moderate to high intensity exercise in horses has limitations not encountered in humans exercising at the same % $\dot{V}O_{2max}$. Estimates of energy expenditure and contributions from fat and carbohydrate oxidation are obtained using indirect calorimetry and gaseous exchange measurement. Simultaneous infusion of stable isotopes of glucose or fatty acids allows a complete assessment of intramuscular versus extramuscular substrate oxidation. Estimates of whole body carbohydrate and fat oxidation during exercise by indirect calorimetry are based on some assumptions. One of the assumptions is that the rate of oxygen consumption ($\dot{V}O_2$) and the rate of carbon dioxide production ($\dot{V}CO_2$) as measured in exhaled gas reflect the consumption of O_2 and production of CO_2 at a cellular level. However, at high exercise intensities exhaled CO_2 may not accurately reflect gas exchange at a cellular level due to substantial loading of carbon dioxide into the arterial and venous blood and other body fluid compartments, as well as additional carbon dioxide produced from buffering of hydrogen ions by bicarbonate as concentrations of protons increase in muscle and plasma. Therefore, $\dot{V}CO_2$ as measured by indirect

calorimetry at high exercise intensity is an overestimation of the actual production of CO_2 at a mitochondrial level, and consequently the estimates of whole body carbohydrate and fat oxidation are erroneous. Horses running at exercise intensities at or below 60% $\dot{V}O_{2max}$ have a respiratory exchange ratio (RER= $\dot{V}CO_2/\dot{V}O_2$) of 0.9-0.96 and contributions from carbohydrate and fat oxidation can be estimated by indirect calorimetry[24,25]. However, in horses performing an incremental exercise test RER is above 1 at exercise intensities above 75% $\dot{V}O_{2max}$[26]. Therefore, indirect calorimetry estimation of contributions from carbohydrate and fat oxidation to energy expenditure cannot be reliably estimated at exercise intensities above 60% $\dot{V}O_{2max}$ in horses. Validation of estimates of carbohydrate and fat oxidation by indirect calorimetry during high-intensity exercise requires measurement of the absolute ratios of $^{13}C/^{12}C$ in expired air, in endogenous glucose, fat and protein in addition to $\dot{V}O_2$ to obtain carbohydrate and fat oxidation rates independently of CO_2 production[27]. In contrast, indirect calorimetry measurements of rates of substrate oxidation have been validated in humans exercising up to 85% $\dot{V}O_{2max}$, an exercise intensity that results in RER of ≈0.9[27]. The accuracy of estimates of fat and carbohydrate oxidation provided by indirect calorimetry likely declines as the RER approaches 1.0. Values are likely reliable at lower work intensities, but the intensity, or RER, at which the inaccuracy becomes important has not been determined in horses.

The regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity has not been formally investigated in horses. However, in comparing two studies a similar trend of increasing contribution from carbohydrate oxidation and

decreasing contribution from fat oxidation as a percentage of the energy used to fuel exercise, with increasing exercise intensity. Horses exercised at 35% $\dot{V}O_{2max}$ for 90 min have an energy expenditure of ≈210 cal·kg^{-1}·min^{-1} with relative contributions of energy of ≈42% from fat oxidation and ≈58% from carbohydrate[28]. Horses exercised at 50% $\dot{V}O_{2max}$ for 60 min have an energy expenditure of ≈325 cal·kg^{-1}·min^{-1} with relative contributions of energy of ≈30% from fat oxidation and ≈70% from carbohydrate[29]. In addition, at the same exercise intensity the pattern of substrate oxidation is dependent on duration of exercise. In horses exercised at 35% $\dot{V}O_{2max}$ for 90 minutes, during the 0-30 minute, 30-60 and 60-90 minute intervals, fat oxidation accounts for ≈43%, ≈55% and ≈68%, and carbohydrate oxidation accounts for ≈57%, ≈45% and ≈32%[28]. Therefore, there is a shift in the contribution to energy expenditure from carbohydrate to fat oxidation as duration of an exercise bout increases.

2.2 CARBOHYDRATE METABOLISM: MUSCLE GLYCOGEN, LIVER GLYCOGEN AND PLASMA GLUCOSE

The fuel reserves of a horse are in the form of fat, carbohydrates and protein. Protein is a quantitatively important source of energy production only in starvation. Since fats are more energy dense and fat reserves are large, horses have as much as 20-25 times more energy stored in the form of fat as compared to carbohydrate[30] (Table 2.1). Therefore, similar to other species, carbohydrate stores of horses are relatively limited when compared to fat stores. In addition, exercise performed by horses in athletic events requires concurrent oxidation of carbohydrate and fat. For these reasons carbohydrate

availability and oxidation by working skeletal muscle may become a limiting factor for exercise performance. The impact of carbohydrate availability and oxidation on exercise performance has been demonstrated in horses in two situations: 1) as an increase in the time to fatigue in horses administered supplemental glucose by intravenous infusion during moderate-intensity exercise[31], and 2) by depletion of muscle glycogen prior to exercise and demonstration of subsequent lower exercise performance[32,33]. These studies are discussed below in greater detail.

Carbohydrates are either absorbed from the gastrointestinal tract or synthesized *de novo* by liver gluconeogenesis. Carbohydrates are stored in the body in the form of glycogen. Other forms of carbohydrate that contribute to energy supply during exercise are plasma glucose and lactate. The rest of carbohydrates in the body are mostly in the form of glycosylated proteins or lipids and are not quantitatively relevant to exercise metabolism.

Liver glycogen is used to maintain normal glucose concentrations in periods when glucose availability from intestinal absorption is decreased. Those tissues dependent solely on glucose for their metabolism, such as neurons and red blood cells, require a constant supply of glucose from the liver via breakdown of glucose from glycogen or synthesis of glucose from gluconeogenic precursors, such as lactate, glycerol and most amino acids. Propionate, which is one of the volatile fatty acids absorbed in the hindgut from microbial fermentation of plant fiber, is considered a very important gluconeogenic precursor in resting horses, and it may account for as much as 50-60% of hepatic glucose production in non-exercising horses[34].

Unlike liver glycogen, muscle glycogen does not contribute to maintenance of normoglycemia. The majority of muscle glycogen is stored in fast-twitch high oxidative (Type IIA) and fast-twich low oxidative (Type IIX) muscle fibers. These muscle fiber types are those most dependent on glycogen for synthesis of ATP via oxidative phosphorylation and/or glycolysis resulting in lactic acid formation. Glycogen granules are particles of carbohydrate with complexed proteins (glycogenin, glycogen synthase, glycogen phosphorylase and phosphorylase kinase) found in subsarcolemmal and myofibrillar location. In the vastus lateralis of humans, most of the glycogen granules are found between myofibrils, but the subsarcolemmal space is also densely packed with glycogen granules[35].

2.3 GLUCOSE METABOLISM DURING EXERCISE IN HORSES

2.3.1 Plasma glucose concentrations and glucose kinetics during exercise

In horses, unlike humans and dogs, plasma glucose concentrations increase (2-4 mM) even during moderate intensity exercise (50% $\dot{V}O_{2max}$)[31]. This indicates a mismatch between the rate of appearance of glucose in blood (glucose R_a) and the rate of disappearance of glucose in blood (glucose R_d). Using stable isotope techniques it has been determined in horses that both glucose R_a (hepatic glucose production) and R_d (net glucose disposal by peripheral tissues) increase 4 fold during exercise at 35% $\dot{V}O_{2max}$ compared to resting values[28]. However at 50% $\dot{V}O_{2max}$ glucose R_a increases 7 fold but glucose R_d increases by only 4 fold compared to resting values[36]. This mismatch in the glucose turnover rates may be partly or completely due to sympathoadrenergic

mechanisms directly via hepatic sympathetic innervation or indirectly via circulating epinephrine, as discussed below.

2.3.2 Insulin, glucagon and insulin:glucagon ratio during exercise

Insulin and glucagon act to maintain glucose homeostasis and prevent hypoglycemia despite large increases in glucose uptake by working skeletal muscle. Similar to other species, during low and moderate intensity exercise, plasma insulin concentration of horses decreases, plasma glucagon concentration increases and the insulin/glucagon ratio decreases[24,28]. However, these responses will be altered if plasma glucose concentrations are elevated by oral or intravenous glucose administration. Plasma insulin concentration during exercise is higher and plasma glucagon is lower during exercise when plasma glucose concentration is high during exercise because of administration of glucose before or during exercise in horses[28,36].

2.3.3 Catecholamines and glucose turnover rates

Sympathoadrenergic mechanisms play an important role in the control of plasma glucose concentrations during exercise. The mismatch between glucose production and glucose oxidation during moderate intensity exercise leads to hyperglycemia in horses. An excessive rate of hepatic glucose production and lower rate of peripheral glucose disposal partly accounts for this phenomenon, as evidenced by β-adrenergic blockade by the non-selective β-blocker propranolol augmenting and epinephrine infusion inhibiting the rate of glucose disposal by skeletal muscle[24,36].

2.3.4 Effect of training status on glucose kinetics in horses:

In humans, training results in a lower rate of glucose oxidation during exercise at

the same absolute intensity when compared to pretraining values[37]. Similarly, training decreases reliance on glycogenolysis and blood-borne glucose oxidation during exercise in horses when compared to the response before training at the same absolute, but not relative exercise intensity[38].

2.3.5 Glucose availability and exercise performance in horses

Blood glucose is an important fuel for contracting muscle. Studies in human subjects have demonstrated that over 90% of the whole-body glucose uptake during moderate intensity exercise is oxidized by skeletal muscle[37]. Increased glucose availability during prolonged moderate intensity exercise by ingestion of glucose, glucose polymers, sugar rich snacks or carbohydrate meals prior and/or during exercise in humans enhances performance, measured as an increased time to fatigue or an improvement in time to complete a set distance[39]. Similarly, the time to fatigue is prolonged by 14-20% in horses exercised to exhaustion at 50-60% $\dot{V}O_{2max}$, when plasma glucose availability is enhanced by intravenous administration of glucose[31,40]. However, the relative contribution to energy expenditure from blood-borne glucose oxidation during exercise in horses is modest at best when compared to oxidation of muscle glycogen and intra/extramuscular sources of fat. At exercise intensities varying from 30-60% of $\dot{V}O_{2max}$ muscle glycogen oxidation and fat oxidation account for 32-68% and 25-56% respectively of the energy expenditure, whereas oxidation of blood-borne glucose only accounts for 6-12% of the energy expenditure during exercise[24,28,29].

Increasing glucose availability by intragastric administration of a glucose solution (2g/kg) prior to exercise increases the rate of blood-borne glucose oxidation and the rate

of whole body carbohydrate oxidation but muscle glycogenolysis is unchanged[36]. However, the effect of increased glucose availability by oral/intragastric administration on exercise performance in horses has not been determined.

Intravenous or oral glucose administration is not a practical intervention in athletic field events. A more practical strategy to increase glucose availability prior to exercise to provide a starch rich meal, such as corn or oats, prior to exercise in order to increase plasma glucose availability by intestinal digestion and absorption of glucose. Increasing glucose availability prior to exercise in horses by providing a grain meal does alter carbohydrate and fat metabolism but the effect of such a meal prior to exercise on athletic performance has not been determined in horses[29].

2.4 MUSCLE GLYCOGEN AND EXERCISE PERFORMANCE IN HORSES

2.4.1 Muscle glycogen depletion and rates of muscle glycogenolysis in exercising horses

Normal muscle glycogen concentration in horses is 130-140 mmol·kg^{-1}ww (560-600 mmol·kg^{-1}dw), which is greater than values in human athletes of 80-100 mmol·kg^{-1}ww (340-425 mmol·kg^{-1}dw)[41-44]. This greater concentration of muscle glycogen may contribute to the greater athletic capacity of horses. However, despite this greater muscle glycogen storage, concentrations of muscle glycogen are substantially depleted by exercise.

During a competitive 50 to 100 km endurance ride (≈4-9 h events) muscle glycogen depletion is 57-65% and the rate of muscle glycogenolysis during these events is 0.14-0.3 mmol·kg^{-1}ww·min^{-1} [4,5]. In a simulated 80 km endurance ride that lasted 4 h

muscle glycogen depletion was greater than 90% and the average rate of muscle glycogenolysis was 0.5 mmol·kg^{-1}ww·min^{-1} [44]. Therefore, substantial muscle glycogen depletion is observed in horses performing long distance low to moderate intensity exercise. During prolonged exercise activity glycogen depletion in different muscle fibre types occurs progressively, with initial depletion occurring in Type I fibers with depletion of glycogen in Type IIA fibres occurring later and depletion in Type IIX being the latest. This pattern of depletion is related to progressive recruitment of muscle fiber types[5,45].

High intensity exercise results in exponential increases in the rate of muscle glycogenolysis in humans. Similarly, in horses running an 800 m sprint (14.3 m/s, 32 mph for 50-60 sec) or a 2000 m sprint (13.4 m/s, 30 mph for 2.5 min) the rates of muscle glycogenolysis are 37 mmol·kg^{-1}ww·min^{-1} and 16 mmol·kg^{-1}ww·min^{-1}, respectively[43]. Muscle glycogen depletion at these exercise intensities varies from 20-40% of values before exercise[43,46-48]. The pattern of glycogen depletion during high intensity exercise in Thoroughbred and Standardbred races shows that significant glycogen depletion occurs in Type IIA and IIB muscle fibres[2,45]. These estimates of muscle glycogenolysis should approximate the rates of glycogen utilization in competitive Thoroughbred or Standardbred races.

The differences observed between humans and horses in resting muscle glycogen concentration, rate of muscle glycogenolysis during exercise and rate of muscle glycogen synthesis after exercise may be partly explained by differences in muscle fiber composition. The vastus lateralis muscle of human marathon runners has up to 80% of

Type I fibres, while that of elite sprinters contain up to 60% of the Type II fast-twitch fibres[49,50]. In contrast, muscle fiber type composition in the gluteus medius muscle of horses is not nearly as variable as in humans. Horses competing in endurance events have 18-32% type I, 36-46% type IIA, 20-38% type IIB muscle fibers in their gluteus medius[3-5], whereas thoroughbred racehorses have 7-11% type I, 57-61% Type IIA, and 32-28 Type IIB in their gluteus medius muscle[51]. Therefore, the high muscle glycogen concentration, fast rate of glycogenolysis during exercise and slow rates of muscle glycogen synthesis after exercise observed in horses when compared to humans may be partly related to a lower percentage of slow twitch type I muscle fibers in major locomotory muscles.

2.4.2 Exercise performance and muscle glycogen

In human athletes low muscle glycogen concentrations before exercise are associated with decreased exercise performance and conversely, high muscle glycogen concentrations enhance performance[41]. Increased muscle glycogen availability prior to exercise has been shown to enhance endurance exercise performance in humans[39,52], and it is common practice in cyclists and marathon runners to reduce training and increase carbohydrate intake the days prior to a competitive event. However, each gram of glycogen is stored with ≈2.7 g of water, and glycogen loading may result in a weight gain, which may be detrimental for high intensity exercise.

The relationship between muscle glycogen and exercise performance has not been as extensively investigated in horses. However, low muscle glycogen concentrations prior to exercise in horses appear to decrease exercise performance at moderate and high-

intensity exercise. Time to exhaustion in horses trotting at 6.5 mph decreases by 35% when muscle glycogen prior to exercise is 70% lower than normal[53]. Anaerobic work performance, as measured by pulling increasing weight loads on a sled, decreases by 31% in horses when muscle glycogen is 42% lower than normal[54]. Anaerobic work performance tests are designed to estimate the capacity to perform brief maximal intensity exercise that relies mostly on utilization of the intramuscular ATP-Phosphocreatine pool and anaerobic glycolysis to fuel the work of high intensity exercise. In a different study under controlled laboratory conditions, horses undergoing 3 consecutive days of aerobic intense exercise followed by 1 min sprints had depletion of muscle glycogen by 55-75%[32,33]. When muscle glycogen remained decreased by 60%, maximum accumulated oxygen deficit, which is another estimate of anaerobic capacity, and run time to fatigue during an "all out" sprint of 2 minutes at 25mph decreased by 26% and 28%, respectively[33].

In summary, exercise and dietary induced muscle glycogen depletion is associated with decreased exercise performance both at low and high intensity exercise in horses. However, attempts to increase muscle glycogen concentration above that usually found have proved unfruitful and the effect of high muscle glycogen concentrations on exercise performance in horses is unknown.

2.4.3 Muscle glycogen replenishment

Muscle glycogen replenishment after exercise will depend on substrate availability and the interval from completion of the exercise bout to delivery of substrate. Muscle glycogen replenishment is enhanced in horses when supplemental glucose is

administered as an IV infusion (6 g/kg)[55]. However, intragastric administration of an oral glucose polymer at 3g/kg does not enhance muscle glycogen resynthesis in horses[56], unlike ingestion of the same dosage of glucose in humans. In these studies, muscle glycogen did not return by 24 h after exercise to concentrations found prior to exercise, even for those horses administered supplemental oral or intravenous glucose[55,56]. In contrast, humans with similar degrees of glycogen depletion have replenished or even supercompensated muscle glycogen stores by 24 h when glucose is ingested as a solution or as meals with a high glycemic index.

In Thoroughbreds and trotting Standardbreds, the rate of muscle glycogen synthesis after high intensity sprinting exercise, which lowers muscle glycogen concentration by 30-40% prior to exercise, vary between 0.6-1.5 mmols·kg^{-1}ww·h^{-1} [47,48,57]. Unlike other animals, horses fed increasing amounts of digestible carbohydrate have only a minimal increase in the rate of muscle glycogen resynthesis[58], and the rate of glycogen resynthesis after high intensity sprint exercise is ≈1.5 mmols·kg^{-1}ww·h^{-1}[47] which is 4 times lower than values observed in human athletes[59,60]. In a controlled laboratory study, horses that undertook 3 consecutive days of aerobic intense exercise followed by 1 min sprints, had depletion of muscle glycogen by 55-75%[32] and were subsequently fed one of three isocaloric diets containing varying amounts of soluble carbohydrates for 3 days[58]. For those horses fed a diet high in grain and low in roughage, rate of muscle glycogen resynthesis was higher and replenishment was complete by 3 days, whereas those horses fed a mixed hay and grain diet or mostly hay did not attain complete replenishment of muscle glycogen by 3 days after exercise. The rate of muscle

glycogen resynthesis was ≈1.5 mmols·kg^{-1}ww·h^{-1}[58]. Therefore, muscle glycogen resynthesis in horses is complete in 3 days when the diet fed after exercise contains sufficient starch. However, chronic feeding of such a high proportion of starch may not be well tolerated by horses.

The reasons for the relatively slower rate of muscle glycogen replenishment after exercise in horses, when compared to other species, have not been elucidated. One possibility, as discussed below, is that the gastrointestinal function of the horse is not well suited to digest starch and other soluble carbohydrates that will be a source of glucose for glycogen replenishment. If this is the case, the limiting factor is glucose availability from the gastrointestinal tract. This possibility is supported by the fact that intravenous glucose supplementation enhances muscle glycogen resynthesis in horses after exercise, whereas oral glucose polymer administration does not[55,56]. A second possibility is that those molecular mechanisms involved in insulin-stimulated glycogen synthesis are not as functional as in other species. Some of the mechanisms involved in glycogen synthesis are the insulin-stimulated translocation of glucose transporters (glucose transporter type 4) from intracellular vesicle pools to the sarcolemma, as well as the activity of glycogen synthase. The molecular mechanisms underlying glycogen synthesis in horses is an active area of research but there are no published studies at this time.

2.5 NUTRITIONAL INTERVENTIONS TO ALTER/OPTIMIZE CARBOHYDRATE METABOLISM DURING EXERCISE IN HORSES

2.5.1 Effect of meal type prior to exercise on carbohydrate metabolism during

exercise

Studies performed in horses have described the effects of varying meal types prior to exercise on the concentrations of a number of plasma substrates and hormones during and after moderate intensity exercise[61-67]. In summary, ingestion of a high-glycemic meal, such as corn, 2-4 hours prior to a moderate-intensity exercise bout results in a transient decrease in plasma glucose concentration during exercise, attenuation of exercise-induced increase of non-esterified fatty acid concentration, and increased serum insulin concentration during exercise when compared to horses not fed or fed a hay meal. These alterations in plasma and serum concentrations of substrates and hormones have been hypothesized to be deleterious for performance in horses, presumably because they lead to impaired substrate availability during exercise. However, a complete quantitative analysis by indirect calorimetry and stable isotopic tracer methods of the effects of meal type prior to exercise on substrate metabolism has not been conducted until recently[29]. Horses fed a corn meal one hour prior to exercise at 50% $\dot{V}O_{2max}$ for 60 minutes, have greater rates of blood-borne glucose oxidation and whole-body carbohydrate oxidation when compared to rates of horses eating an isocaloric meal of hay or not eating prior to exercise. However, ingestion of a meal of corn did not have a sparing effect on muscle glycogen utilization[29]. In addition, neither the latter[29] or previous studies[61-67] have determined the effect of meal type prior or withholding feed prior to exercise on athletic performance.

2.5.2 Dietary manipulations intended to minimize muscle glycogenolysis during exercise and/or optimize muscle glycogen resynthesis after exercise

Horses fed a fat supplemented diet (10% of fat by weight) for 3 weeks have greater

muscle glycogen concentration prior to exercise and greater muscle glycogenolysis during sprinting exercise when compared to a control diet of hay and grain[68]. However, the diets fed in this study appear not to be isocaloric. In a study in which horses were fed a diet of 15% added-fat, when compared to no fat feeding (diets were not isocaloric), muscle glycogen concentration was lower before and after exercise and net muscle glycogenolysis was not different[69]. In another study in which horses were fed isocaloric diets after exercise, one of the two diets containing 5% of fat, muscle glycogen resynthesis was not different[70]. In summary, some authors claim that feeding horses a high-fat diet has a muscle glycogen sparing effect or increases muscle glycogen concentration, however the evidence is conflicting partly due to differences in study design and further study is required.

One of the causes of the horse's limited ability to digest starch in grains may be its low rate of amylase secretion. Amylase is the pancreatic enzyme responsible for degradation of starch. The output of amylase per unit weight of pancreatic tissue in horses is only 5-6% as much as it is in pigs, therefore the amount of starch that horses can tolerate is relatively small when compared to other monogastric animals[71,72]. Similarly, horses, when compared to cattle, have a pancreatic flow that is 3 times higher but amylase concentration in pancreatic secretion is 1/10 of that in cattle[73,74]. Therefore, the horse's ability to secrete amylase into the small intestine is lower than that of other herbivores or other monogastric animals. The maximal starch ingestion tolerated by horses is 0.3-0.4% of body weight, in other words a 450-kg horse (≈1000 lb) has a maximal starch digestibility of 1.4-1.8 kg (3-4 lb) of starch, which is equal to 2.3-3 kg (5-6.7 lb) of corn[75].

As described previously under “Muscle glycogen resynthesis”, horses that undergo 3 consecutive days of muscle glycogen depleting exercise will have an enhanced rate of muscle glycogen resynthesis when fed a diet high in starch. In order to avoid the gastrointestinal complications of feeding excessive amounts of grain to horses, one possibility is to increase the number of feedings per day and decrease the amount of each meal. In doing so, one would minimize the risks of diets high in grain intake without compromising even further muscle glycogen resynthesis.

In summary: 1) energy metabolism of horses during exercise is different to that of humans and dogs, therefore data obtained in other athletic species should not be directly extrapolated; 2) carbohydrate availability may limit performance in horses as demonstrated by decreased exercise performance when muscle glycogen concentrations are low, as well as by increased exercise performance when glucose is supplemented intravenously; and 3) horses have a limited capacity for rapid muscle glycogen resynthesis when compared to other species, which may be related to differences in diets and in adaptations of the gastrointestinal tract.

2.6. REFERENCES

1. Lewis LD. Water, energy, protein, carbohydrates, and fats for horses. *Equine Clinical Nutrition: feeding and care*. Baltimore, USA: Williams and Wilkins, 1995;3-24.

2. Hodgson DR, Rose RJ, Allen JR, et al. Glycogen depletion patterns in horses performing maximal exercise. *Res Vet Sci* 1984;36:169-173.

3. Hodgson DR, Rose RJ, Allen JR. Muscle glycogen depletion and repletion patterns in horses performing various distances of endurance exercise. Equine Exercise Physiology 1983;229-236.

4. Essen-Gustavsson B, Karlstrom K, Lindholm A. Fibre types, enzyme activities and substrate utilisation in skeletal muscles of horses competing in endurance rides. *Equine Vet J* 1984;16:197-202.

5. Snow DH, Baxter P, Rose RJ. Muscle fibre composition and glycogen depletion in horses competing in an endurance ride. *Vet Rec* 1981;108:374-378.

6. Rivero JL, Talmadge RJ, Edgerton VR. Correlation between myofibrillar ATPase activity and myosin heavy chain composition in equine skeletal muscle and the influence of training. *Anat Rec* 1996;246:195-207.

7. Quiroz-Rothe E, Rivero JL. Co-ordinated expression of contractile and non-contractile features of control equine muscle fibre types characterised by immunostaining of myosin heavy chains. *Histochem Cell Biol* 2001;116:299-312.

8. Romijn JA, Coyle EF, Sidossis LS, et al. Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. *J Appl Physiol* 1995;79:1939-1945.

9. Starritt EC, Howlett RA, Heigenhauser GJ, et al. Sensitivity of CPT I to malonyl-CoA in trained and untrained human skeletal muscle. *Am J Physiol Endocrinol Metab* 2000;278:E462-468.

10. Richter EA, Ruderman NB, Gavras H, et al. Muscle glycogenolysis during exercise: dual control by epinephrine and contractions. *Am J Physiol* 1982;242:E25-32.

11. Romijn JA, Coyle EF, Sidossis LS, et al. Substrate metabolism during different exercise intensities in endurance-trained women. *J Appl Physiol* 2000;88:1707-1714.

12. Romijn JA, Klein S, Coyle EF, et al. Strenuous endurance training increases lipolysis and triglyceride-fatty acid cycling at rest. *J Appl Physiol* 1993;75:108-113.

13. Rose RJ, Hodgson DR, Kelso TB, et al. Maximum O2 uptake, O2 debt and deficit, and muscle metabolites in Thoroughbred horses. *J Appl Physiol* 1988;64:781-788.

14. Saltin B, Astrand PO. Maximal oxygen uptake in athletes. *J Appl Physiol* 1967;23:353-358.

15. Seeherman HJ, Morris EA. Methodology and repeatability of a standardised treadmill exercise test for clinical evaluation of fitness in horses. *Equine Vet J Suppl* 1990:20-25.

16. Lindstedt SL, Hokanson JF, Wells DJ, et al. Running energetics in the pronghorn antelope. *Nature* 1991;353:748-750.

17. Wagner PD. Determinants of VO_{2max}: man vs. horse. *Journal of Equine Veterinary Science* 1995;15:398-404.

18. Boileau RA, Horswill CA. Body composition in Sports: Measurement and Applications for Weight Loss and Gain In: Garrett WE,Kirkendall DT, eds. *Exercise and sport science*. Philadelphia: Lippincott Williams & Wilkins, 2000;319-338.

19. Gunn HM. Muscle, bone and fat proportions and muscle distribution of thoroughbreds and other horses. Equine Exercise Physiology 2 1987;253-264.

20. Hoppeler H, Jones JH, Lindstedt SL, et al. Relating maximal oxygen consumption to skeletal muscle mitochondria in horses. Equine Exercise Physiology 2 1987;278-289.

21. Hoppeler H. Muscle aerobic potential in the animal kingdom. In: Saltin B, ed. *Biochemistry of Exercise VI, Intenational Series on Sport Sciences*. Champaign, IL: Human Kinetics Publishers, 1985;417-434.

22. Romijn JA, Coyle EF, Sidossis LS, et al. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol* 1993;265:E380-391.

23. Roberts TJ, Weber JM, Hoppeler H, et al. Design of the oxygen and substrate pathways. II. Defining the upper limits of carbohydrate and fat oxidation. *J Exp Biol* 1996;199 (Pt 8):1651-1658.

24. Geor RJ, Hinchcliff KW, Sams RA. β-adrenergic blockade augments glucose utilization in horses during graded exercise. *J Appl Physiol* 2000;89:1086-1098.

25. Davie AJ, Evans DL, Hodgson DR, et al. Effects of muscle glycogen depletion on some metabolic and physiological responses to submaximal treadmill exercise. *Can J Vet Res* 1999;63:241-247.

26. Eaton MD, Evans DL, Hodgson DR, et al. Effect of treadmill incline and speed on metabolic rate during exercise in thoroughbred horses. *J Appl Physiol* 1995;79:951-957.

27. Romijn JA, Coyle EF, Hibbert J, et al. Comparison of indirect calorimetry and a new breath 13C/12C ratio method during strenuous exercise. *Am J Physiol* 1992;263:E64-71.

28. Geor RJ, Hinchcliff KW, Sams RA. Glucose infusion attenuates endogenous glucose production and enhances glucose use of horses during exercise. *J Appl Physiol* 2000;88:1765-1776.

29. Jose-Cunilleras E, Hinchcliff KW, Sams RA, et al. Glycemic index of a meal fed before exercise alters substrate use and glucose flux in exercising horses. *J Appl Physiol* 2002;92:117-128.

30. Harris P. Energy sources and requirements of the exercising horse. *Annu Rev Nutr* 1997;17:185-210.

31. Farris JW, Hinchcliff KW, McKeever KH, et al. Glucose infusion increases maximal duration of prolonged treadmill exercise in Standardbred horses. *Equine Vet J Suppl* 1995:357-361.

32. Lacombe V, Hinchcliff KW, Geor RJ, et al. Exercise that induces substantial muscle glycogen depletion impairs subsequent anaerobic capacity. *Equine Vet J Suppl* 1999;30:293-297.

33. Lacombe VA, Hinchcliff KW, Geor RJ, et al. Muscle glycogen depletion and subsequent replenishment affect anaerobic capacity of horses. *J Appl Physiol* 2001;91:1782-1790.

34. Simmons HA, Ford EJH. Gluconeogenesis from Propionate Produced in the Colon of the Horse. *British Veterinary Journal* 1991;147:340-345.

35. Marchand I, Chorneyko K, Tarnopolsky M, et al. Quantification of subcellular glycogen in resting human muscle: granule size, number, and location. *J Appl Physiol* 2002;93:1598-1607.

36. Geor RJ, Hinchcliff KW, McCutcheon LJ, et al. Epinephrine inhibits exogenous glucose utilization in exercising horses. *J Appl Physiol* 2000;88:1777-1790.

37. Coggan AR, Kohrt WM, Spina RJ, et al. Endurance training decreases plasma glucose turnover and oxidation during moderate-intensity exercise in men. *J Appl Physiol* 1990;68:990-996.

38. Geor RJ, McCutcheon LJ, Hinchcliff KW, et al. Training-induced alterations in glucose metabolism during moderate-intensity exercise. *Equine Vet J Suppl* 2002:22-28.

39. Jacobs KA, Sherman WM. The efficacy of carbohydrate supplementation and chronic high- carbohydrate diets for improving endurance performance. *International Journal of Sport Nutrition* 1999;9:92-115.

40. Farris JW, Hinchcliff KW, McKeever KH, et al. Effect of tryptophan and of glucose on exercise capacity of horses. *J Appl Physiol* 1998;85:807-816.

41. Bergström J, Hermansen L, Hultman E, et al. Diet, muscle glycogen and physical performance. *Acta Physiol Scand* 1967;71:140-150.

42. Essen-Gustavsson B, McMiken D, Karlstrom K, et al. Muscular adaptation of horses during intensive training and detraining. *Equine Vet J* 1989;21:27-33.

43. Harris RC, Marlin DJ, Snow DH. Metabolic response to maximal exercise of 800 and 2,000 m in the thoroughbred horse. *J Appl Physiol* 1987;63:12-19.

44. Snow DH, Kerr MG, Nimmo MA, et al. Alterations in blood, sweat, urine and muscle composition during prolonged exercise in the horse. *Vet Rec* 1982;110:377-384.

45. Valberg S. Glycogen depletion patterns in the muscle of standardbred trotters after exercise of varying intensities and durations. *Equine Vet J* 1986;18:479-484.

46. Nimmo MA, Snow SH. Changes in muscle glycogen, lactate and pyruvate concentrations in the thoroughbred horse following maximal exercise. Equine Exercise Physiology 1983;237-244.

47. Snow DH, Harris RC, Harman JC, et al. Glycogen repletion following different diets. Equine Exercise Physiology 2 1987;701-706.

48. Snow DH, Harris RC. Effects of daily exercise on muscle glycogen in the Thoroughbred horse. Equine Exercise Physiology 3 1991;299-304.

49. Komi PV, Karlsson J. Skeletal muscle fibre types, enzyme activities and physical performance in young males and females. *Acta Physiol Scand* 1978;103:210-218.

50. Bergh U, Thorstensson A, Sjodin B, et al. Maximal oxygen uptake and muscle fiber types in trained and untrained humans. *Med Sci Sports* 1978;10:151-154.

51. Snow DH, Guy PS. Fibre type and enzyme activities of the gluteus medius in different breeds of horse. In: Poortmans J,Niset G, eds. *Biochemistry of Exercise IV-B*. Baltimore,: University Park Press, 1981;275-282.

52. Hawley JA, Schabort EJ, Noakes TD, et al. Carbohydrate-loading and exercise performance. An update. *Sports Med* 1997;24:73-81.

53. Topliff DR, Potter GD, Dutson TR, et al. Diet manipulation and muscle glycogen in the equine. 8th Equine Nutrition and Physiology Symposium 1983;119-124.

54. Topliff DR, Potter GD, Kreider JL, et al. Diet manipulation, muscle glycogen metabolism and anaerobic work performance in the equine. 9th Equine Nutrition and Physiology Symposium 1985;224-229.

55. Davie AJ, Evans DL, Hodgson DR, et al. Effects of intravenous dextrose infusion on muscle glycogen resynthesis after intense exercise. *Equine Veterinary Journal* 1995;18:195-198.

56. Davie AJ, Evans DL, Hodgson DR, et al. The effects of an oral glucose polymer on muscle glycogen resynthesis in standardbred horses. *J Nutr* 1994;124:2740S-2741S.

57. Hyyppä S, Rasanen LA, Pösö AR. Resynthesis of glycogen in skeletal muscle from standardbred trotters after repeated bouts of exercise. *Am J Vet Res* 1997;58:162-166.

58. Lacombe VA, Hinchcliff KW, Kohn CW, et al. Post-exercise feeding of meals of varying glycemic index affects muscle glycogen resynthesis in horses. *J Vet Intern Med* 2002;16:336.

59. Costill DL, Sherman WM, Fink WJ, et al. The Role of Dietary Carbohydrates in Muscle Glycogen Resynthesis after Strenuous Running. *American Journal of Clinical Nutrition* 1981;34:1831-1836.

60. Ivy JL. Glycogen resynthesis after exercise: effect of carbohydrate intake. *Int J Sports Med* 1998;19 Suppl 2:S142-145.

61. Essén-Gustavsson B, Blomstrand E, Karlström K, et al. Influence of diet on substrate metabolism during exercise. Equine Exercise Physiology 3 1991;288-298.

62. Lawrence L, Soderholm LV, Roberts A, et al. Feeding Status Affects Glucose-Metabolism in Exercising Horses. *Journal of Nutrition* 1993;123:2152-2157.

63. Lawrence LM, Williams J, Soderholm LV, et al. Effect of feeding state on the response of horses to repeated bouts of intense exercise. *Equine Vet J* 1995;27:27-30.

64. Pagan JD, Burger I, Jackson SG. The influence of time of feeding on exercise response in Thoroughbreds fed a fat supplemented or high carbohydrate diet. 14th Equine Nutrition and Physiology Symposium 1995;92-93.

65. Pagan JD, Harris PA. The effects of timing and amount of forage and grain on exercise response in thoroughbred horses. *Equine Vet J Suppl* 1999;30:451-457.

66. Rodiek A, Bonvicin S, Stull C, et al. Glycemic and endocrine responses to corn or alfalfa fed prior to exercise. Equine Exercise Physiology 3 1991;323-330.

67. Stull C, Rodiek A. Effects of postprandial interval and feed type on substrate availability during exercise. *Equine Vet J Suppl* 1995;18:362-366.

68. Oldham Sl, Potter GD, Evans JW, et al. Storage and mobilization of muscle glycogen in exercising horses fed a fat-supplemented diet. 11th Equine Nutrition and Physiology Symposium 1989;57-62.

69. Pagan JD, Essén-Gustavsson B, Lindholm A, et al. The effect of dietary energy source on exercise performance in Standardbred horses. Equine Exercise Physiology 2 1987;686-700.

70. Hyyppä S, Saastamoinen M, Pösö AR. Effect of a post exercise fat-supplemented diet on muscle glycogen repletion. *Equine Veterinary Journal Suppl* 1999;30:493-498.

71. Alexander F, Chowdhury AK. Enzymes in the ileal juice of the horse. *Nature* 1958;181:190.

72. Radicke S, Landes E, Kienzle E, et al. Aktivitat der amylase im Dunndarmchymus in Abhangigkeit von der Futterart [in German, English summary]. *Pferdeheilkunde, Proceedings of the 1st European Conference of Horse Nutrition* 1992:99.

73. Comline RS, Hall LW, Hickson JCD, et al. Pancreatic secretion in the horse. *Journal of Physiology-London* 1969:10P-11P.

74. Walker JA, Krehbiel CR, Harmon DL, et al. Effects of slaframine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4DAMP) on pancreatic exocrine secretion in the bovine. *Can J Physiol Pharmacol* 1994;72:39-44.

75. Potter GD, Arnold FF, Householder DD, et al. Digestion of starch in the small or large intestine of the equine. *Pferdeheilkunde, Proceedings of the 1st European Conference of Horse Nutrition* 1992:107-111.

Fuel	Tissue	Grams	Energy (kcal)
Triglycerides	Adipose tissue	40,000	360,000
Triglycerides	Muscle	1,400-2,800	12,600-25,200
Glycogen	Muscle	3,150-4,095	13,230-17,200
Glycogen	Liver	90-300	380-1260
Glucose	Plasma	27	110

Table 2.1 Fuel stores, distribution within the body and energy storage in a 450 kg horse[30].

	Horses[23] 60%$\dot{V}O_{2max}$	Humans[11] 65%$\dot{V}O_{2max}$	Dogs[22] 60%$\dot{V}O_{2max}$	Goats[22] 60%$\dot{V}O_{2max}$
$\dot{V}O_{2max}$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	137	67	146	68
$\dot{V}O_2$ ($ml \cdot kg^{-1} \cdot min^{-1}$)	77	44	84	39
TEE ($cal \cdot kg^{-1} \cdot min^{-1}$)	400	200	410	190
Contribution from CHO ox	75%	40%	60%	57%
Contribution from Fat ox	25%	60%	40%	43%

Table 2.2 Mass specific maximal rates of oxygen consumption ($\dot{V}O_{2max}$), as well as rates of oxygen consumption ($\dot{V}O_2$), total energy expenditure (TEE) and contributions from carbohydrate (CHO) and fat oxidation during moderate intensity exercise in athletic (horses and dogs) and non-athletic mammals (humans and goats)[22-24].

CHAPTER 3

GLYCEMIC INDEX OF A MEAL FED BEFORE EXERCISE ALTERS SUBSTRATE USE AND GLUCOSE FLUX IN EXERCISING HORSES.

3.1 SUMMARY

In a randomized, balanced, crossover study, each of 6 fit, adult horses ran on a treadmill at 50% $\dot{V}O_{2max}$ for 60 min after being denied access to food for 18 hours. Horses were the: (1) fed corn (51.4 KJ/kg digestible energy), or (2) fed an isocaloric amount of alfalfa 2-3 hours before exercise, or (3) not fed before exercise. Feeding corn, when compared with fasting, resulted in higher plasma glucose and serum insulin and lower serum non-esterified fatty acid concentrations prior to exercise ($P<0.05$), and lower plasma glucose, serum glycerol and serum non-esterified fatty acid concentrations, and higher skeletal muscle utilization of blood-borne glucose during exercise ($P<0.05$). Feeding corn, compared to feeding alfalfa, resulted in higher carbohydrate oxidation and lower lipid oxidation during exercise ($P<0.05$). Feeding a soluble carbohydrate-rich meal (corn) to horses prior to exercise results in increased muscle utilization of blood-borne

glucose and carbohydrate oxidation, and decreased lipid oxidation compared to a meal of insoluble carbohydrate (alfalfa) or not feeding. Carbohydrate feedings were not associated with a sparing of muscle glycogen compared to fasting.

3.2. INTRODUCTION

Muscle glycogen and blood-borne glucose are important substrates for contracting skeletal muscle during moderate-intensity exercise bouts. Carbohydrate (CHO) metabolism in exercising muscle and the factors that influence CHO metabolism during exercise in horses are assumed to be similar to humans. However, this may not necessarily be the case. Horses have a greater aerobic capacity than humans and therefore at the same exercise intensity, relative to the maximal rate of oxygen consumption ($\dot{V}O_{2max}$), the rate of oxygen consumption ($\dot{V}O_2$) and the energy expenditure are much higher in horses than in humans[1]. In addition to the greater aerobic capacity of horses, their gastrointestinal tract anatomy and physiology is adapted to a herbivorous diet based on ingestion of structural carbohydrates of plants (mainly cellulose and hemicellulose) and bacterial fermentation of structural carbohydrates in the hindgut with subsequent absorption of volatile fatty acids [VFAs] (acetate, propionate and butyrate). Horses obtain a substantial proportion of their energy requirements by absorption of VFAs during resting as well as during low intensity exercise; however, the contribution of VFAs to energy expenditure during exercise is unknown. Thus, the observations made in human subjects regarding CHO metabolism in exercising skeletal muscle and the influence of

nutritional status on CHO metabolism may not apply to horses because of the aforementioned physiological differences between horses and humans.

Among the numerous factors that influence carbohydrate metabolism in exercising muscle, there is evidence, in studies in human subjects, that diet composition and interval prior to exercise can affect both uptake of glucose by muscle and the rate of muscle glycogen utilization. In studies performed in human subjects, ingestion of glucose or a high-glycemic meal (HGM) prior to exercise results in enhanced carbohydrate oxidation (CHO_{ox}) and utilization of blood-borne glucose, and the rate of glycogenolysis is enhanced[2] or unchanged[3-6]. In humans, ingestion of a HGM or CHO supplementation prior and/or during endurance and moderate-intensity exercise has resulted in enhanced[3-16], decreased[17] or unchanged submaximal exercise performance[3,4,18-20]. Current recommendations of feeding prior to and during endurance exercise in human athletes include ingestion of carbohydrate solutions in order to maintain the rate of CHO_{ox}[21].

Studies performed in horses have determined the effect of meal composition and interval before exercise on the blood concentrations of substrates and hormones and muscle glycogen utilization during exercise[22-28]; however the relative contributions of carbohydrate oxidation, in the form of blood-borne glucose and muscle glycogen utilization, and lipid oxidation to energy production during exercise have not been determined. In brief, ingestion of a high-glycemic meal (HGM) 2-4 hours prior to moderate-intensity exercise results in a transient decrease in plasma glucose concentration during exercise, attenuation of exercise-induced increase of nonesterified fatty acid concentration, and increased serum insulin concentration during exercise when compared to horses withheld from food or fed a hay meal. These alterations in plasma

and serum concentrations of substrates and hormones have been hypothesized to be deleterious for performance in horses, presumably because these alterations are associated with impaired substrate use during exercise. However, none of these studies have determined the effect of meal composition prior to exercise on substrate use during exercise, or on measurements of exercise performance in horses.

If similar mechanisms regulate CHO metabolism in working skeletal muscle in horses and in humans, it is plausible that preexercise ingestion of a HGM in horses may enhance glucose availability to skeletal muscle and alter the hormonal milieu so that CHO_{ox} is maintained and lipid oxidation is suppressed, as compared to ingestion of a low-glycemic meal or not feeding prior to exercise. To date, however, this hypothesis has not been tested. The present study was, therefore, undertaken to determine the effects of glucose supply (pre-exercise ingestion of a high-glycemic meal [corn], vs. pre-exercise ingestion of a low-glycemic meal [hay], vs. withholding feed) on stable isotope tracer-determined whole body glucose appearance and uptake in horses during moderate-intensity exercise. It was hypothesized that ingestion of a high-glycemic meal prior to exercise would result in enhanced whole body glucose appearance and uptake. A further objective of this study was to determine the effects of glucose supply on whole-body rates of carbohydrate and lipid oxidation and the relative contribution of these fuels to energy expenditure, as well as, the rate of muscle glycogenolysis. It was hypothesized that ingestion of a high-glycemic meal prior to exercise would result in enhanced carbohydrate oxidation, attenuated lipid oxidation, greater relative contributions to energy expenditure from blood-borne glucose, and attenuated muscle glycogenolysis ("muscle glycogen sparing effect").

3.3 MATERIALS AND METHODS

All animal experiments were conducted after approval by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University and were performed in compliance with their guidelines and recommendations.

Experimental design. The effects of feeding a meal of corn or alfalfa before exercise on glucose kinetics, whole body substrate utilization, and muscle glycogen utilization during moderate intensity exercise were examined in a balanced, randomized, three-way cross-over study. Six horses undertook 60 min of treadmill exercise at a workload requiring 50% $\dot{V}O_{2max}$ in each of three experimental conditions: *1)* 90 min after consuming a meal of cracked corn (1.7 kg in a 450 kg horse; 12.4 kcal/kg [51.8 kJ/kg] digestible energy)(Grain); *2)* 90 min after consuming a meal of an isocaloric amount of alfalfa cubes (3.0 kg in a 450 kg horse)(Hay); *3)* after having feed withheld for 18 hours (Feed withholding). In preliminary studies it was determined that horses offered hay were not able to consume the entire meal in 60-min. For this reason horses were allowed to eat the grain meal from 0800 to 0900 (60-min), and the hay meal from 0700 to 0900 (120-min). Trials were separated by 7 days, and the order of the trials was randomized for individual horses but balanced among treatments.

Horses. Six Standardbred horses (5 mares and 1 gelding), 4-11 years (7.8 ± 2.5 [SD]) of age and 404-485 kg (425 ± 30 [SD]) body mass were studied. All horses were housed indoors during the experimental period, fed a diet of timothy grass-alfalfa hay, alfalfa cubes, mixed pelleted grain and cracked corn, and had unlimited access to a salt-mineral block and water. All horses were conditioned and undertaking regular treadmill exercise

for 6 weeks before the study. Horses were not exercised the day before an experimental trial, and after an experimental trial received 4 days of light treadmill exercise (30 min of trotting at 4-4.5 m/s with the treadmill set at 4º incline).

Preliminary testing. For each horse, $\dot{V}O_{2max}$ and the relationship between rate of O_2 consumption ($\dot{V}O_2$) and speed were determined during an incremental exercise test 1 week before the first experiment. The incremental exercise test consisted of the horse running on a high-speed treadmill (Sato, Uppsala, Sweden) inclined at 4º for 90 s at 4 m/s; the treadmill speed was then increased by 1 m/s every 90 s until the horse was no longer able to maintain its position on the treadmill. $\dot{V}O_2$ was measured every 10 s during the exercise test. $\dot{V}O_{2max}$ was defined as the value at which $\dot{V}O_2$ reached a plateau, despite further increases in speed. A plateau was defined as a change in $\dot{V}O_2$ of <4 $ml \cdot kg^{-1} \cdot min^{-1}$ with an increase in speed. The running speed that elicited 50% $\dot{V}O_{2max}$ was calculated for each horse using linear regression analysis of speeds below $\dot{V}O_{2max}$.

Diet composition. Horses were fed a basal diet of mixed alfalfa and grass hay and pelleted concentrate to maintain ideal body weight (condition score 5-6 out of 9). Corn and alfalfa cubes were fed beginning 4 weeks before the start of the experimental trials so that horses were accustomed to the diet. The diet consisted of 4.8 kg of alfalfa hay cubes, 2.9 kg of mixed hay, 1.4 kg of pelleted concentrate and 1 kg of cracked corn per day for a 450-kg horse. The amounts of hay and grain were given following the guidelines published by the National Research Council for horses in moderate work[29]. The equation used for estimation of daily requirements of digestible energy (Mcal of DE/day) was:

$DE = 1.5 \cdot (1.4 + 0.03 \cdot BW)$

where DE is the daily requirement of digestible energy (Mcal/day) and BW is the body weight (kg). The roughage and concentrates in the diet were analyzed by a commercial laboratory (Holmes laboratory Inc., Millersburg, OH) (Table 3.1).

Experimental protocol. Before each experimental trial, food was withheld for 18 h, and the horses were confined to their stalls for 24 h. All experiments began at 0700 when horses were offered alfalfa cubes, or at 0800 when horses were offered cracked corn. After aseptic preparation and injection of local anesthesia of the overlying skin, catheters (14 gauge, 5.25 in.; Angiocath, Becton Dickinson) were inserted into the right and left jugular veins for isotope infusion and blood sample collection, respectively. Thereafter, a blood sample was obtained for subsequent determination of background isotopic enrichment. In the Grain trial, horses were fed, at 0800 in their stall, a meal of cracked corn corresponding to ¼ of the daily energy requirements as previously established for horses on moderate work[29]. In the Hay trial, horses were fed, at 0700 in their stall, an isocaloric meal of alfalfa cubes. In the Feed withholding trial, horses were not fed before exercise. In all trials, horses were kept undisturbed (with the exception of blood sampling from the jugular catheter) in the stall until 0900. Horses were then moved to stockades in a temperature controlled building. Glucose kinetics was determined by a primed (17.5 $\mu mol \cdot kg^{-1}$), continuous (0.22 ± 0.01 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) infusion of [6,6-^{2}H]glucose (99% enriched; Cambridge Isotopes, Cambridge, MA) in 0.9% saline. The [6,6-^{2}H]glucose was infused for 90 min. with a calibrated infusion pump while the horses stood quietly in the stocks. Fifteen minutes before initiation of exercise a sample of the middle gluteal muscle was obtained by percutaneous biopsy. Thereafter, the horses were positioned on an inclined treadmill (4º incline), and a loose-fitting face mask for measurement of

respiratory gas exchange was applied. The horses completed a 5-min warm-up (3 m/s treadmill belt speed) followed by 60 min of running at a speed calculated to elicit 50% $\dot{V}O_{2max}$. The rate of [6,6-^{2}H]glucose infusion was tripled (0.66 ± 0.02 μmol·kg^{-1}·min^{-1}) during the warm-up. During the exercise test, fans mounted 0.5 m in front and to the sides of the treadmill were used to maintain an air velocity of approximately 4 m/s over the horse. Ambient conditions were similar for all trials (room temperature and relative humidity were 15-20 °C and 50-60%, respectively).

Respiratory exchange measurements. Rates of oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), and respiratory exchange ratio (RER) were measured with an open-circuit calorimeter (Oxymax-XL, Columbus Instruments, Columbus, OH) as previously described[30]. Flow through the system was ~1,500 l/min STP when the horse was stationary and 9,000 l/min during running. Data for expired O_2 (electrochemical cell, Columbus Instruments, Columbus, OH) and CO_2 (single-beam nondispersive infrared sensor, Columbus Instruments, Columbus, OH) concentrations were measured continuously and were reported at 10-s intervals. The gas-analysis system was calibrated before the start of each exercise test by using gas mixtures with O_2 and CO_2 concentrations that spanned the measurement range. The overall accuracy of the system for the measurement of oxygen consumption was verified for each run by the nitrogen dilution method and measured values corrected for any discrepancy[31]. The accuracy of the system for the measurement of respiratory exchange ratio was verified for each run by burning of propane, which has a respiratory quotient of 0.6, and measured values

corrected for any discrepancy. Standard equations were used to calculate $\dot{V}O_2$ and $\dot{V}CO_2$, and RER was obtained by dividing $\dot{V}CO_2$ by $\dot{V}O_2$.

Sampling procedures. Blood samples were obtained at –60, and –30 min for Hay trials and at 0, 30, 60, 90, 120, 135, 150, 155, 165, 180, 195 and 210 min for all trials (where the "0" min sample was collected at 0800 when the first sample was obtained in Grain and Feed withholding trials). Blood samples were divided (6-ml aliquots) into four different tubes for subsequent analysis. Two aliquots of each sample were placed in evacuated tubes containing EDTA. These samples were later analyzed for plasma isotopic enrichment, hematocrit, plasma total protein, nonesterified fatty acid (NEFA) and glycerol concentrations. A 6-ml aliquot was placed in a tube containing sodium fluoride-potassium oxalate for subsequent determination of plasma glucose and lactate concentration. The final aliquot was placed in a tube containing no additive for measurement of serum insulin concentration. Plasma or serum was obtained by centrifugation (1,500xg for 20 min at 4ºC) within 30 min of collection and frozen at –80ºC until analysis.

Muscle biopsy samples were collected percutaneously from the middle gluteal muscle via the needle biopsy technique[32]. Muscle biopsies were obtained 15 min before commencement of exercise and within 3 min of cessation of exercise. Muscle samples were immediately placed in liquid nitrogen and stored at –80ºC until analysis.

Plasma isotopic enrichment. Plasma [6,6-^{2}H]glucose enrichment was determined as previously described by our laboratory [33]. The intra- and interassay coefficients of variation were 1.6 ± 0.1 and 2.7 ± 0.4 %, respectively. To control for between-day

variability in plasma [6,6-^{2}H]glucose enrichment determination, all samples for a given horse (Feed withholding, Hay and Grain trials) were analyzed during the same analytic session. All samples were analyzed in duplicate.

Plasma biochemical analyses. Plasma glucose concentration was measured spectrophotometrically using a commercial kit that employs the hexokinase reaction (Glucose Hexokinase kit, Sigma Diagnostics, St. Louis, MO), and plasma lactate concentration was measured using an automated lactate oxidase method (Sport 1500 lactate analyzer, Yellow Springs Instruments, Yellow Springs, OH). Plasma NEFA concentration was determined using a commercial kit that employs an enzymatic colorimetric method (NEFA test kit, Wako Chemicals, Dallas, TX). Plasma glycerol concentration was measured by using an enzymatic spectrophotometric method (triglycerides kit 337A [without triglyceride hydrolysis step], Sigma Diagnostics, St. Louis, MO). Plasma glucose, NEFA and glycerol concentrations were measured using a microplate reader spectrophotometer (Versamax, Molecular Devices Corporation, Sunnyvale, CA). Intra- and interassay coefficients of variation for measurement of plasma glucose, lactate, NEFA and glycerol were, ~1.0 and ~2.5%, respectively. Hematocrit was measured by the microhematocrit technique. Plasma total protein was measured by refractometry (Cambridge Instruments, Buffalo, NY). To control for between-day variability, all samples for a given horse (Feed withholding, Hay and Grain trials) were analyzed during the same analytic session. All samples were analyzed in duplicate.

Plasma hormone analyses. Serum immunoreactive insulin was determined in duplicate by use of a commercially available RIA (insulin kit, Coat-a-Count Diagnostics.

Los Angeles, CA) that has been validated for horse blood[34]. Intra- and interassay coefficients of variation were ~6 and ~8%.

Muscle glycogen. Muscle samples were dissected free of any blood and connective tissue and duplicate samples, of 20 mg each, were analyzed. The samples were extracted, and analyzed for muscle glycogen concentration (as glucosyl units per kg of wet weight) according to the procedure of Passoneau and Lauderdale[35]. Bovine liver glycogen (G0885, Sigma Diagnostics, St. Louis, MO) was used to obtain known standard concentrations of 200 mM and 50 mM, as a quality control for the acid hydrolysis procedure. Intra- and interassay coefficients of variation were 9 and 12%, respectively.

Calculations of glucose kinetics. Glucose rate of appearance (R_a) and rate of disappearance (R_d) at rest were calculated by using the steady-state tracer dilution equation [36]:

$$R_a = R_d = F \cdot \left[\left(IE_i / IE_p \right) - 1 \right]$$

where F is the infusion rate of the isotope ($\mu mol \cdot kg^{-1} \cdot min^{-1}$), IE_i and IE_p are the stable isotopic enrichment of the infusate and plasma, respectively, and – 1 accounts for the tracer's contribution to the turnover rate of the substrate[36]. The rate of infusion was calculated by multiplying the infusion pump rate by the concentration of glucose in the infusate. During exercise, glucose R_a and R_d were calculated using the non-steady-state equation developed by Steele and modified for use with stable isotopes[37]. This equation is modified for use with stable isotopes as the amount of tracer infused is no longer negligible

$$R_a = \frac{F - V_d \frac{C_m}{1+E} \frac{dE}{dt}}{E}$$

and

$$R_d = R_a - V_d \frac{\frac{dC_m}{dt}(1+E) - C_m \frac{dE}{dt}}{(1+E)^2}$$

where V_d is the effective volume of distribution, E is the plasma isotopic enrichment, C_m is the measured plasma concentration of the tracee, and dE/dt and dC_m/dt are maximum rates of change in enrichment and glucose concentration, respectively, as a function of time. With use of this fixed, one-compartment model of Steele, it is assumed that 1) the apparent glucose space is 25% of body weight and 2) 65% of this space represents the rapidly mixing portion of the glucose pool. Therefore, the effective V_d for glucose was assumed to be 162 ml/kg. Glucose metabolic clearance rate (MCR) was calculated by dividing glucose R_d by the plasma glucose concentration. Glucose R_a was assumed to represent glucose absorption from the gastrointestinal tract plus hepatic glucose production, although a small contribution from renal glycogenolysis and gluconeogenesis is possible.

Rates of energy expenditure and whole body substrate oxidation. Total energy expenditure (TEE) and absolute rates of carbohydrate (CHO) and lipid oxidation were calculated as follows[38,39]:

$$TEE\ (kcal/min) = 3.9\ \dot{V}O_2 + 1.1\ \dot{V}CO_2$$

$$CHO_{ox}\ (g/min) = 4.585\ \dot{V}CO_2 - 3.2255\ \dot{V}O_2$$

$$Lipid\ oxidation\ (g/min) = 1.7012\ \dot{V}O_2 - 1.694\ \dot{V}CO_2$$

where $\dot{V}O_2$ is in liters per minute and it was assumed that protein oxidation made a

negligible contribution to $\dot{V}O_2$ and $\dot{V}CO_2$ (i.e., nonprotein RER). The calculated values were based on respiratory gas exchange values averaged over 5-min intervals. Carbohydrate oxidation (CHO_{ox}) in grams per minute was converted to micromoles per kilogram per minute by dividing by the molecular weight of glucose (mol wt 180.16) and the horse's body weight. Similarly, rates of fat oxidation were converted to micromoles per kilogram per minute by dividing by the molecular weight of palmitate (mol wt 256.43) and the horse's body weight. Muscle glycogen (plus lactate) oxidation was calculated as the difference between total CHO_{ox} and glucose R_d. Coggan et al.[40] reported that, in human subjects, >95% of glucose R_d is oxidized during submaximal exercise. Therefore, glucose R_d provides a reasonable estimate of plasma glucose oxidation during exercise. Finally, the absolute and relative contributions by plasma glucose, other CHO sources (muscle glycogen and lactate), and lipid to the total energy expenditure during the 0- to 30- and 35- to 60-min periods of exercise were estimated using standard caloric equivalents (4.2 kcal/g CHO, 9.0 kcal/g lipid).

Statistical analyses. Values are expressed as means ± SE. The data for all dependent measures were analyzed using a two-way ANOVA for repeated measures, with treatment (Feed withholding, Hay and Grain) and time as independent factors. Percent data were subject to arcsine transformation before ANOVA. The null hypothesis was rejected at $\alpha \leq 0.05$ for the main effects (treatment and time) and $\alpha \leq 0.10$ for the interaction. Significant differences identified by ANOVA were isolated using the Tukey post hoc test. The only dependent variable analyzed with a paired Student t-test was the difference

in the relative feed consumptions between hay and grain meals. The Sigmastat 2.0 software package (Jandel Scientific, San Rafael, CA) was used for statistical computations.

3.4 RESULTS

Individual values for $\dot{V}O_{2max}$ ranged from 109 to 125 ml·kg^{-1}·min^{-1} (mean 117 ± 2.7 ml·kg^{-1}·min^{-1}). Mean running speed on a 4° inclined treadmill during the exercise protocol was 4.9 ± 0.2 m/s (range, 4.3-5.5 m/s) which corresponded to a relative workload of 50 ± 0.7% of $\dot{V}O_{2max}$ (range, 46.9-52.4%).

Preexercise feed consumption. Before exercise in hay meal trials, horses were offered 2.9 ± 0.1 kg [6.3 ± 0.2 lb] (range, 2.7-3.3 kg) and consumed 82 ± 8% (range, 64-100%) of the meal. In grain meal trials, horses were offered 1.6 ± 0.04 kg [3.5 ± 0.1 lb] (range, 1.5-1.8 kg) and all horses consumed all of the meal. The difference in caloric intake between the two meals was approximately 10 ± 3.9 kJ DE/kg of body weight [2.4 ± 0.9 kcal/kg] (range, 0-19 kJ/kg). The meal consumption in Hay trials had a tendency to be lower than in Grain trials ($P = 0.06$).

Plasma glucose concentration. Feeding type prior to exercise significantly affected plasma glucose concentrations before and during exercise. Before ingestion of a meal, plasma glucose was similar among the three trials (Fig. 3.1). During the 150-min period after allowing horses to eat a grain meal equivalent to one quarter of the daily energy requirements, glucose concentration steadily increased from 4.4 ± 0.1 mM before the meal to 6.6 ± 0.7 mM at 150-min (Fig. 3.1). Plasma glucose concentration remained

unchanged in the Hay meal and in horses in which the feed was withheld. During exercise in Feed withholding trials, plasma glucose concentration increased steadily to reach a peak of 9.0 ± 0.1 mM, whereas in Grain trials plasma glucose concentration decreased from preexercise values during the first 15 min of exercise and were subsequently similar to values in Hay trials. Plasma glucose concentrations were higher ($P < 0.05$) in Feed withholding trials than in Grain or Hay meal trials during the second half of the exercise trial.

Serum insulin. Feeding type prior to exercise significantly altered serum immunoreactive insulin (IRI) concentrations before and during exercise. Allowing horses to eat a Hay meal induced an insulinemic response in which insulin increased over 60% (127.1 ± 17.5 pM at 30-min) from insulin concentrations before feeding (72.7 ± 6.1 pM at –60-min; $P < 0.001$, Fig. 3.1). Serum immunoreactive insulin concentrations steadily increased after a Grain meal from 67.2 ± 4.9 pM at 0-min to reach a peak of 175.6 ± 17.4 pM 150-min after offering the meal ($P < 0.001$). During exercise, serum IRI decreased in all trials but in Grain meal trials remained elevated above values in Feed withholding trials for the first 15 min of exercise ($P < 0.05$). No differences in serum IRI concentration were found among trials between 30 and 60 min of exercise.

NEFA and glycerol. Plasma glycerol concentrations were affected by feeding type during exercise but not before exercise. Plasma glycerol concentrations during the second half of exercise were significantly lower in Grain trials than in Hay or Feed withholding trials. Plasma glycerol concentration increased steadily during exercise in all trials from ~0.1 ± 0.0 mM to reach a peak of 1.22 ± 0.1, 1.13 ± 0.1 and 0.96 ± 0.1 mM at 60 min of

exercise for Feed withholding, Hay and Grain, respectively ($P < 0.001$, 150 vs. 210 min; Fig. 3.2). Feeding type prior to exercise altered plasma NEFA concentrations before and during exercise. During the 120-min period in which horses were allowed to eat hay, plasma NEFA steadily decreased from 0.90 ± 0.2 mM before the hay meal to 0.27 ± 0.1 mM at 60 min ($P < 0.001$). Non-esterified fatty acids gradually decreased after both Hay and Grain meal whereas in Fasting trials it initially remained unchanged and gradually increased (Fig. 3.2). Plasma NEFA concentration decreased sharply at commencement of exercise in Feed withholding trials (0.95 ± 0.2 mM immediately before exercise and 0.40 ± 0.1 mM at 5-min of exercise, $P = 0.003$, Fig. 3.2). Plasma NEFA concentrations during exercise were significantly lower in the Grain trial than in the Feed withholding and Hay trials ($P < 0.05$).

Respiratory gas exchange and whole body substrate oxidation. Feeding type prior to exercise altered RER, and the whole body rates of CHO_{ox} and Lipid oxidation during exercise. There was a small but significant ($P < 0.001$) increase in $\dot{V}O_2$ during the 60-min of treadmill exercise, and $\dot{V}O_2$ was similar among the three trials (Table 3.3). The respiratory exchange ratio decreased significantly in all three trials over the 60-min of exercise ($P < 0.001$), and at 60-min of exercise Grain trials had a significantly higher RER compared to Hay trials (0.89 ± 0.01 and 0.84 ±0.01, Grain vs. Hay, $P = 0.021$). Total energy expenditure steadily increased over time from 126.1 ± 3.3, 127.8 ± 5.6, 129.1 ± 4.1 kcal/min at 5 min to 143.1 ± 2.9, 138.3 ± 2.6, 140.2 ± 2.6 kcal/min at 60 min for Feed withholding, Hay and Grain trials, respectively ($P < 0.001$). Total CHO_{ox} decreased during exercise in Feed withholding and Hay trials, but remained stable in Grain trials (Table 3.4). Total CHO_{ox} was higher in Grain than in Hay trials at 60 min of

exercise (327 ± 26 μmol·kg^{-1}·min^{-1} vs. 239 ± 26 μmol·kg^{-1}·min^{-1}, Grain vs. Hay, P = 0.023). Conversely, fat oxidation was suppressed in Grain trials compared to Feed withholding and Hay trials and at 60 min the rate of fat oxidation was significantly lower in Grain trials (49 ± 4 μmol·kg^{-1}·min^{-1}) compared to Hay trials (72 ± 8 μmol·kg^{-1}·min^{-1}, P = 0.016).

Glucose kinetics. Feeding type prior to exercise altered plasma glucose isotopic enrichment and glucose R_d before and during exercise, and altered glucose R_a before exercise as well as glucose MCR during exercise. Before exercise, plasma isotopic enrichment (Fig. 3.3) was lower in Grain trials than in Feed withholding and Hay trials (P < 0.001). During the first 5 min of exercise, plasma isotopic enrichment increased in all trials, however in Grain trials plasma isotopic enrichment values remained stable whereas in Feed withholding and Hay trials plasma isotopic enrichment steadily decreased despite the threefold increase in tracer infusion rate.

At rest, the mean glucose R_a (Fig. 3.4A) and R_d (Fig. 3.4B) during the Grain trials were both approximately 70% greater than during Feed withholding and Hay trials (both R_a and R_d, P < 0.005). Glucose R_a increased steadily in all trials from 7.9 ± 0.7, 8.1 ± 0.6 and 13.6 ± 1.6 μmol·kg^{-1}·min^{-1}to reach a peak of 60 ± 6, 52 ± 4 and 53 ± 7 μmol·kg^{-1}·min^{-1} for Feed withholding, Hay and Grain trials, respectively, at 30-min of exercise (P < 0.001). During the second half of the exercise trial R_a remained stable or decreased slightly in all trials.

During exercise, there was a significant trial effect (P = 0.007) for glucose R_d. Whereas glucose R_d increased with the onset of exercise in all trials, the increase was much larger in Grain trials (Fig. 3.4B). In Feed withholding and Hay trials glucose R_d

increased from 7.9 ± 0.7 and 8.1 ± 0.6 μmol·kg^{-1}·min^{-1} before exercise to reach a peak of 37 ± 5 and 40 ± 3 μmol·kg^{-1}·min^{-1}, respectively, at 15-min of exercise ($P < 0.001$), and remained stable or decreased slightly during the rest of the exercise period. In Grain trials glucose R_d, increased sharply from 13.6 ± 1.6 μmol·kg^{-1}·min^{-1} before exercise to reach a peak of 56 ± 4 μmol·kg^{-1}·min^{-1} at 15-min of exercise ($P < 0.001$), and it steadily decreased during the rest of the exercise. Glucose MCR demonstrated a similar pattern (Fig. 3.5). During exercise MCR was ~50-100% higher in Grain trials than in Feed withholding trials ($P < 0.05$ at 5, 15 and 30-min), whereas only at 5-min was MCR higher in Hay trials compared to Feed withholding trials ($P < 0.05$).

Muscle glycogen. Preexercise muscle glycogen concentration was similar among the three trials (Fig. 3.6). Net muscle glycogen utilization during exercise was similar ($P = 0.6$) among the three trials (47 ± 9, 44 ± 11, 35 ± 8 mmol·kg^{-1} wet muscle in Feed withholding, Hay and Grain trials, respectively). Postexercise muscle glycogen concentration was lower when compared to preexercise samples in all trials ($P < 0.001$). The calculated rates of muscle glycogen oxidation (total CHO_{ox} – glucose R_d) during exercise were not affected by meal type (Table 3.4).

Hematocrit, plasma total protein, and lactate. Feeding type prior to exercise altered hematocrit and plasma total protein concentration before exercise but did not affect plasma lactate concentration. Hematocrit was significantly increased by 5 min of exercise and remained elevated throughout exercise in all trials (35 to 37% immediately before exercise and 49 to 50% at 5-min of exercise, $P < 0.001$, Table 3.2). During the resting period in Hay meal trials, hematocrit was higher than in Grain meal and Feed withholding trials (Feeding x Time, $P < 0.059$). Plasma total protein concentration

followed a pattern similar to that for hematocrit. Plasma total protein concentration increased significantly by 5 min of exercise and remained elevated throughout exercise in all trials (6.8 to 7.0 g/dL immediately before exercise and 7.3 to 7.6 g/dL at 5-min of exercise, $P < 0.001$, Table 3.2). During the period immediately following Hay meal feeding, plasma total protein concentration increased from 7.1 ± 0.1 g/dL to 7.5 ± 0.1, whereas in Grain meal and Feed withholding trials plasma total protein concentration remained essentially unchanged (6.9 ± 0.1 and 7.1 ± 0.1, respectively for Grain and Feed withholding; Feeding x Time $P < 0.001$). Plasma lactate concentration increased steadily in all trials to reach a peak of 3.9 ± 0.4, 4.3 ± 0.6 and 4.2 ± 0.5 mM at 60 min of exercise for Feed withholding, Hay and Grain, respectively ($P < 0.001$, 150 vs. 210 min; Table 3.2).

Overall pattern of substrate utilization. Feeding type prior to exercise altered the overall pattern of substrate utilization during exercise. Figure 3.7 depicts the estimated relative caloric contribution from oxidation of muscle glycogen and lactate (Other CHO_{ox}), lipid, and blood glucose during the first and second half of exercise. The total rate of energy expenditure was similar among the three trials (325 ± 7, 322 ± 5, 324 ± 5 $cal \cdot kg^{-1} \cdot min^{-1}$ for Feed withholding, Hay, and Grain trials, respectively). During the first half of exercise, no differences were detected among the three trials in the relative contributions to energy expenditure from lipid and muscle glycogen oxidation. The contribution of blood glucose during the first half of exercise was ~85% and ~35% higher in Grain when compared to Feed withholding and Hay trials, respectively ($P < 0.05$), and ~40% higher in Hay when compared to Feed withholding trials ($P < 0.05$). During the second half of exercise, contribution of blood glucose was similar among all trials. The

contribution of muscle glycogen decreased significantly during the second half of exercise; however the decrease in the contribution of muscle glycogen was attenuated in Grain trials, and a tendency was observed in Grain trials to have a higher muscle glycogen contribution when compared to Hay trials ($P = 0.11$). In Hay trials, during the second half of exercise, the relative caloric contribution of lipid oxidation was higher when compared to Grain trials ($48 \pm 4\%$ and $34 \pm 4\%$, $P < 0.05$, Hay vs. Grain).

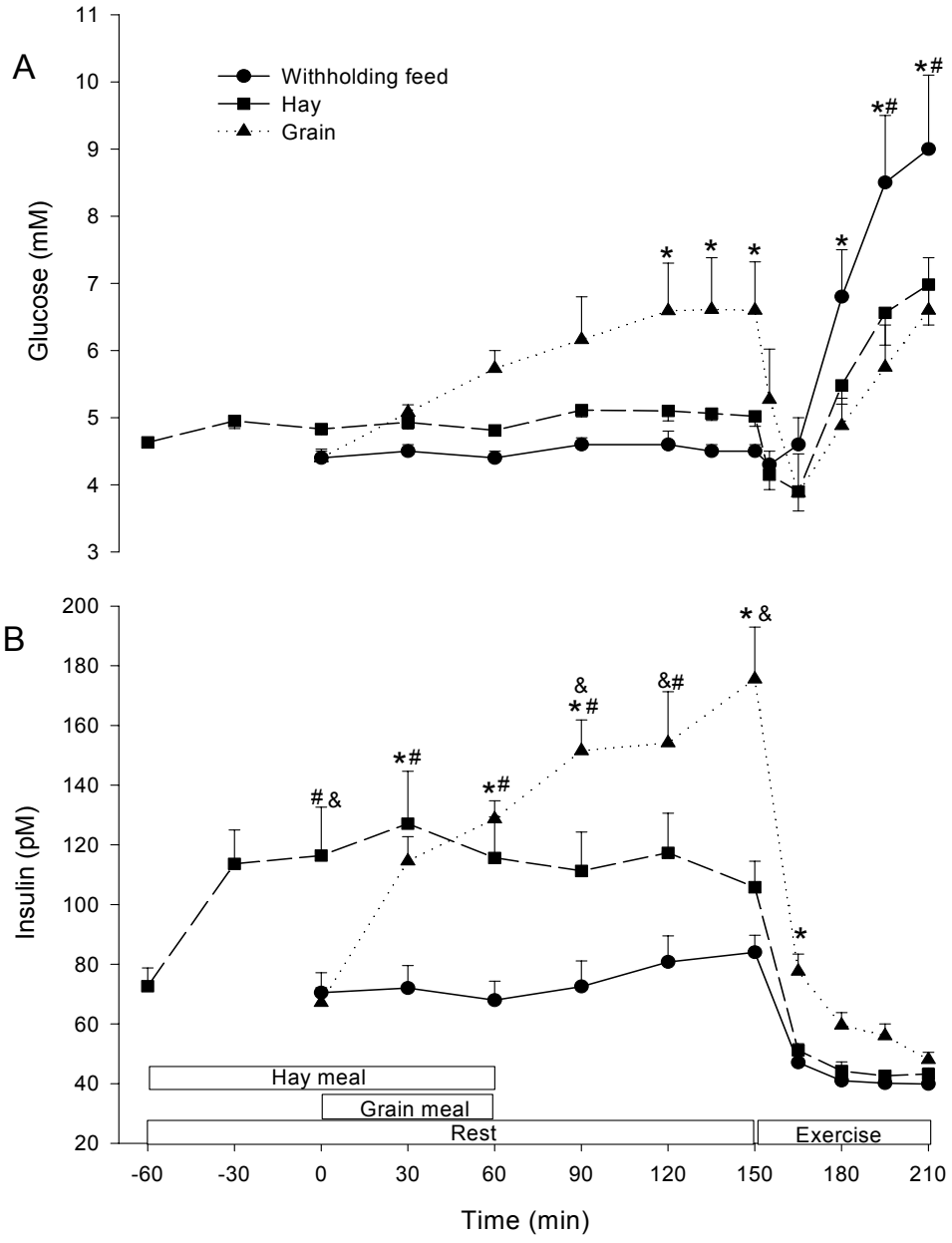

Figure 3.1. Plasma glucose (A) and serum immunoreactive insulin (B) at rest and during 60 min of exercise at 50 ± 0.7% of $\dot{V}O_{2max}$ after withholding feed, feeding a hay meal (Hay) or feeding an approximately isocaloric grain meal (Grain) 90-min before exercise. Values are means ± SE for 6 horses. * Grain meal significantly different from withholding feed, $P<0.05$; # Hay meal significantly different from withholding feed, $P<0.05$; & Grain meal significantly different from hay meal, $P<0.05$.

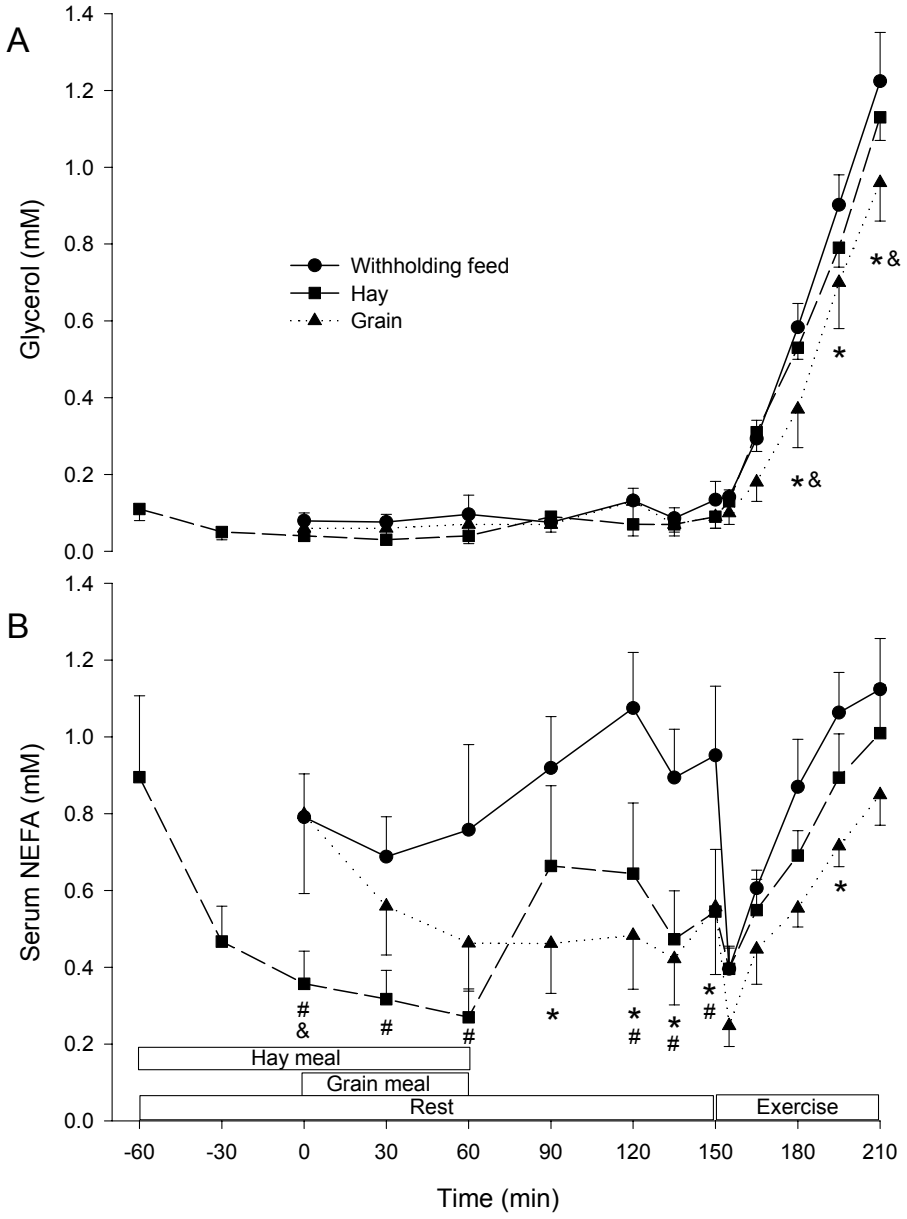

Figure 3.2. Plasma glycerol (A) and nonesterified fatty acids (NEFA; B) at rest and during 60 min of exercise at 50 ± 0.7% of $\dot{V}O_{2max}$ after withholding feed, feeding a hay meal (Hay) or feeding an approximately isocaloric grain meal (Grain) 90-min before exercise. Values are means ± SE for 6 horses. * Grain meal significantly different from withholding feed, $P<0.05$; # Hay meal significantly different from withholding feed, $P<0.05$; & Grain meal significantly different from hay meal, $P<0.05$.

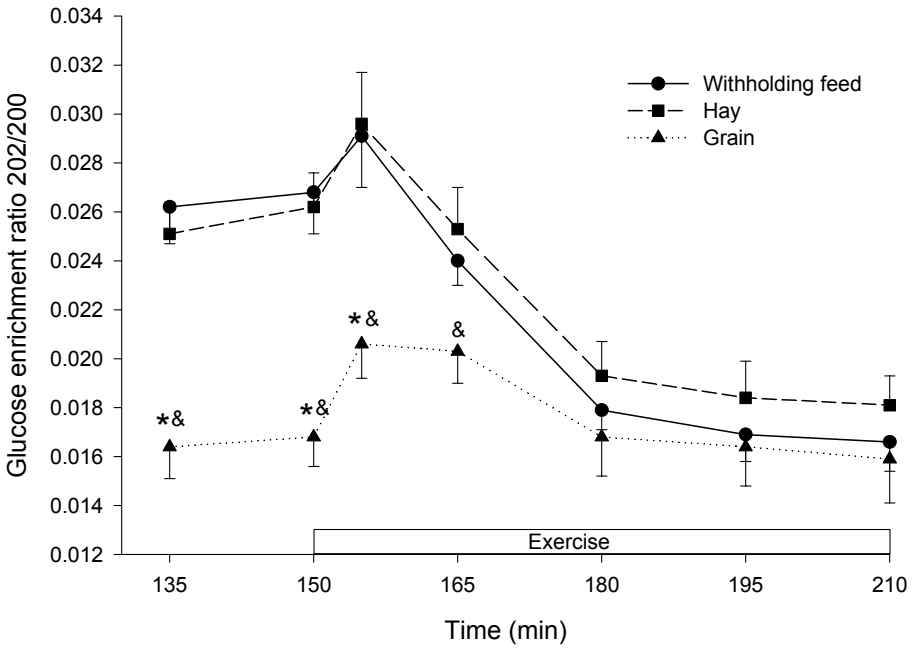

Figure 3.3. Plasma isotopic enrichment at rest and during 60 min of exercise at 50 ± 0.7% of $\dot{V}O_{2max}$ after withholding feed, feeding a hay meal (Hay) or feeding an approximately isocaloric grain meal (Grain) 90-min before exercise. Values are means ± SE for 6 horses. * Grain meal significantly different from withholding feed, $P<0.05$; & Grain meal significantly different from hay meal, $P<0.05$.

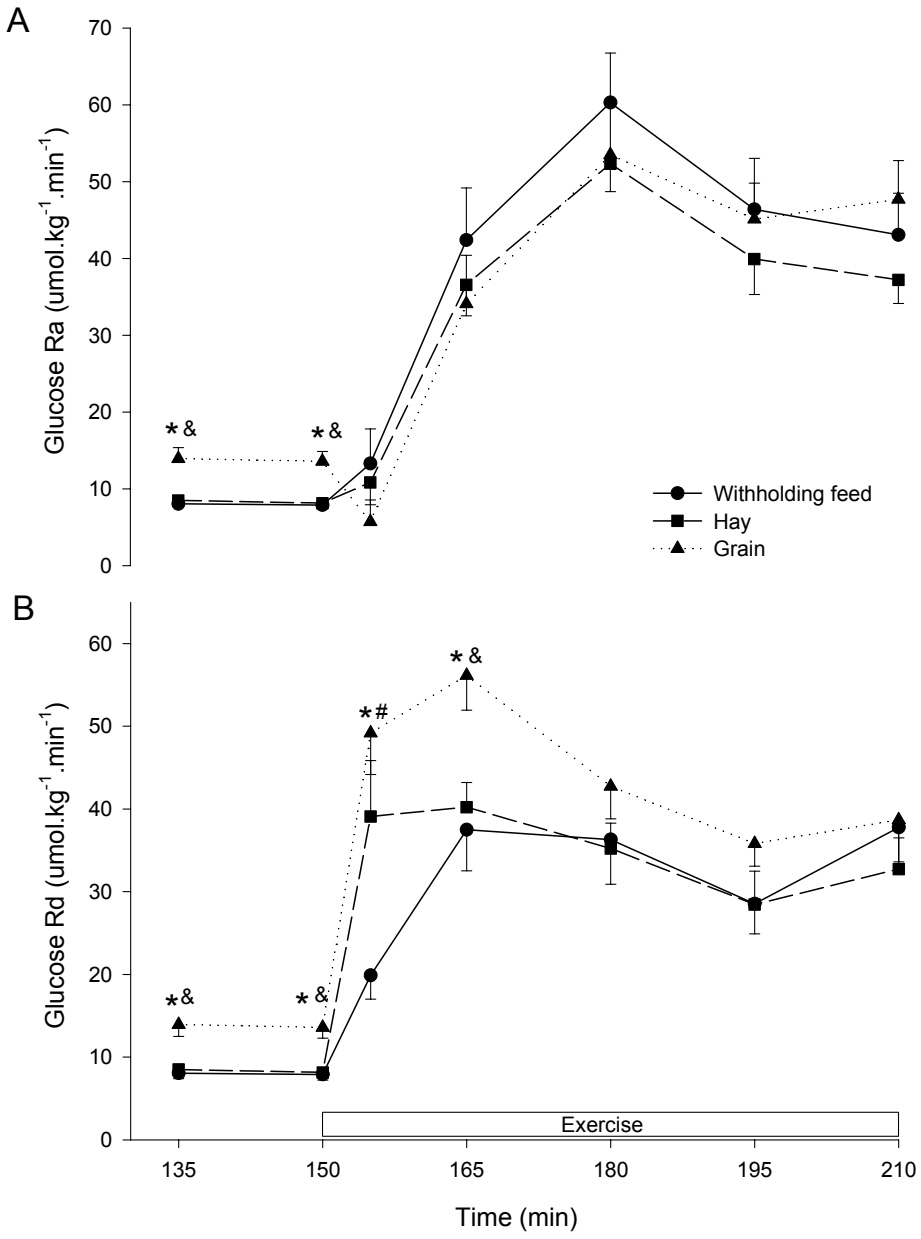

Figure 3.4. Glucose rate of appearance (R_a; A) and disappearance (R_d; B) at rest and during 60 min of exercise at 50 ± 0.7% of $\dot{V}O_{2max}$ after withholding feed, feeding a hay meal (Hay) or feeding an approximately isocaloric grain meal (Grain) 90-min before exercise. Values are means ± SE for 6 horses. * Grain meal significantly different from withholding feed, $P<0.05$; # Hay meal significantly different from withholding feed, $P<0.05$; & Grain meal significantly different from hay meal, $P<0.05$.

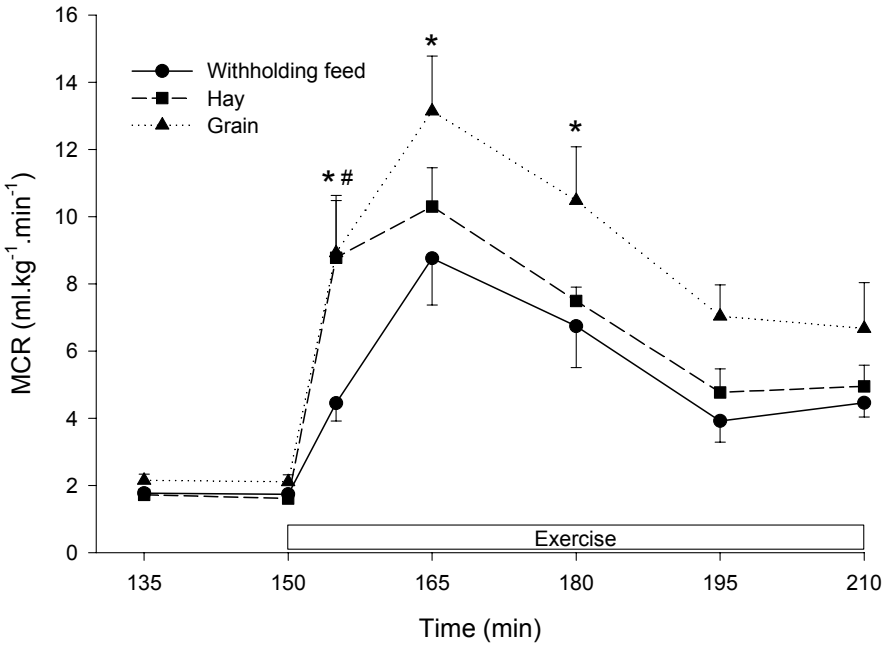

Figure 3.5. Glucose metabolic clearance rate (MCR) at rest and during 60 min of exercise at 50 ± 0.7% of $\dot{V}O_{2max}$ after withholding feed, feeding a hay meal (Hay) or feeding an approximately isocaloric grain meal (Grain) 90-min before exercise. Values are means ± SE for 6 horses. * Grain meal significantly different from withholding feed, $P<0.05$; # Hay meal significantly different from withholding feed, $P<0.05$.

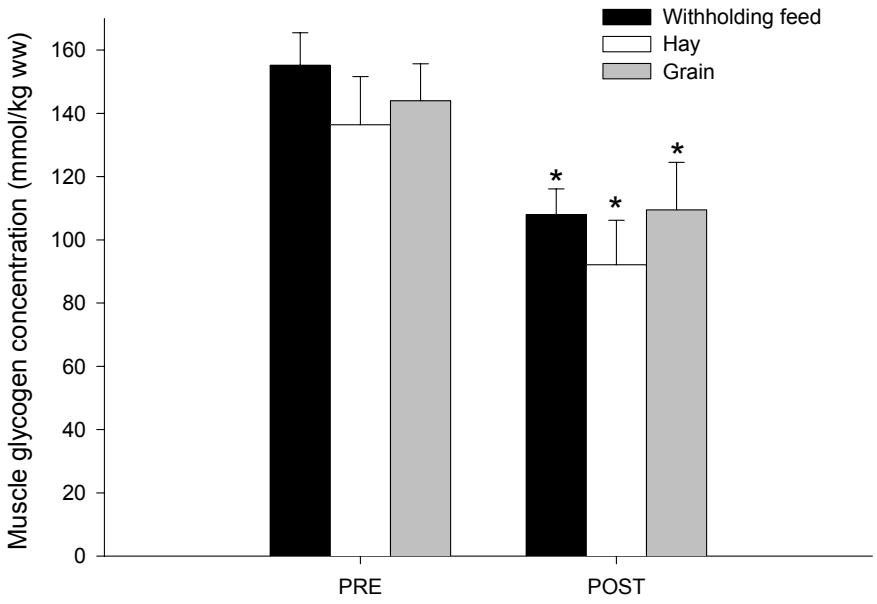

Figure 3.6. Muscle glycogen concentrations before (PRE) and after (POST) 60 min of exercise at 50 ± 0.7% of $\dot{V}O_{2max}$ after withholding feed, feeding a hay meal (Hay) or feeding an approximately isocaloric grain meal (Grain) 90-min before exercise. Values are means ± SE for 6 horses; ww, wet weight. * Significant difference compared to pre-exercise values, $P<0.05$.

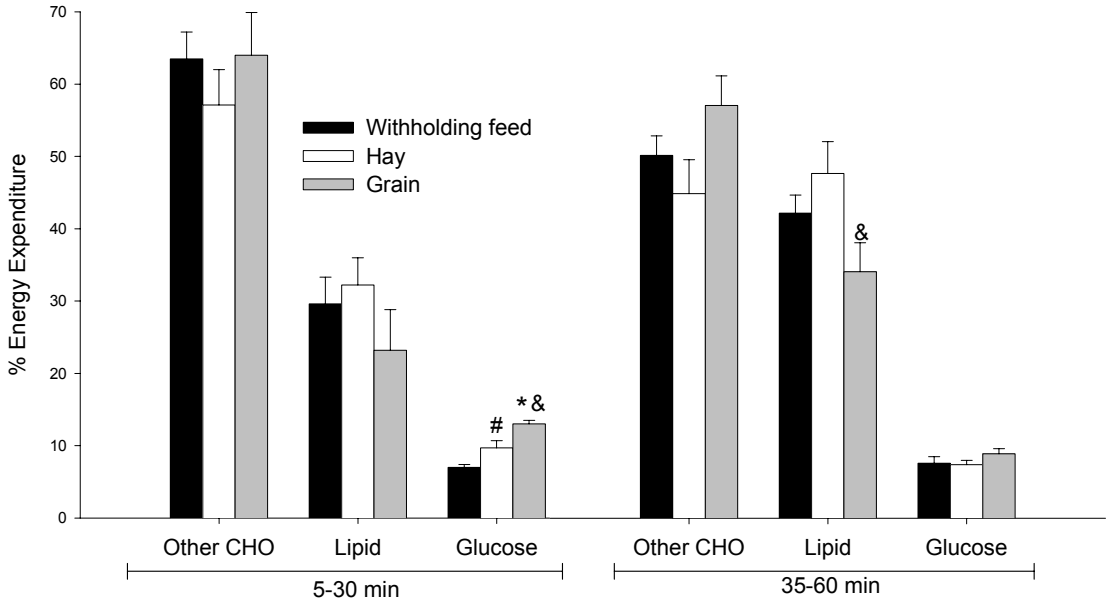

Figure 3.7. Relative caloric contributions from oxidation of Other CHO (muscle glycogen and lactate), lipid, and blood glucose in 6 horses during the 5- to 30-min and 35- to 60-min periods of exercise. * Grain meal significantly different from withholding feed, $P<0.05$; # Hay meal significantly different from withholding feed, $P<0.05$; & Grain meal significantly different from hay meal, $P<0.05$. For all trials the % energy expenditure of each substrate oxidation is significantly different at 5-30 min vs. 35-60 min ($P<0.05$), except glucose utilization in trials after withholding feed.

	Alfalfa cubes	Cracked Corn	Mixed hay	Pelleted concentrate
Dry matter, %	86.1	85.9	87.3	86.4
Crude Protein, %	13.4 (15.6)	8.04 (9.36)	16.2 (18.5)	12.7 (14.7)
ADF, %	34.0 (39.8)	2.76 (3.21)	34.4 (39.4)	11.5 (13.3)
NDF, %	43.3 (50.2)	7.48 (8.7)	40.3 (46.2)	20.6 (23.8)
Lignin, %	6.11 (7.09)	0.15 (0.17)	5.27 (6.04)	2.02 (2.34)
Starch (est.), %	3.26 (3.79)	64.1 (74.6)	3.69 (4.23)	61.8 (71.5)
Crude Fat, %	2.33 (2.7)	3.44 (4.0)	2.36 (2.7)	3.02 (3.5)
Ash, %	8.08 (9.38)	0.86 (1.0)	5.74 (6.58)	5.22 (6.04)
Calcium, %	1.01 (1.17)	0.02 (0.02)	1.12 (1.28)	0.53 (0.61)
Phosphorus, %	0.28 (0.32)	0.21 (0.25)	0.23 (0.26)	0.54 (0.62)
DE, Mcal/kg	1.85 (2.16)	3.32 (3.87)	2.09 (2.40)	2.73 (3.15)

Table 3.1. Result of analysis of the dietary components.
Values of crude protein, Acid detergent fiber (ADF), Neutral detergent fiber (NDF), Lignin, Starch (estimated), Crude fat, Ash, Calcium and Phosphorus are expressed as %; Digestible energy (DE) is expressed as Mcal/kg of feed. Values are as sampled and, in parenthesis, as a dry matter basis.

Time, min	0	90	150	155	165	180	195	210
	Rest			Exercise				
Hematocrit %								
Fasted	38 ± 2	35 ± 1	35 ± 2	49 ± 1	50 ± 1	49 ± 1	48 ± 1	49 ±1
Hay	39 ± 1	39 ± 1‡	37 ± 1	49 ± 1	50 ± 1	49 ± 1	49 ± 1	49 ± 1
Grain	36 ± 1	34 ± 2*	35 ± 1	50 ±1	49 ±1	48 ±1	49 ± 1	50 ± 1
Plasma total protein, g/dL								
Fasted	7.2 ± 0.1	7.1 ± 0.1	7.0 ± 0.1	7.6 ± 0.1	7.5 ± 0.1	7.4 ± 0.2	7.4 ± 0.1	7.7 ± 0.2
Hay	7.5 ± 0.2‡	7.3 ± 0.2	6.9 ± 0.1	7.3 ±0.1	7.4 ± 0.1	7.3 ± 0.2	7.5 ± 0.1	7.6 ± 0.2
Grain	6.9 ± 0.1*	6.9 ± 0.1*	6.8 ± 0.1	7.4 ± 0.2	7.2 ± 0.2	7.1 ± 0.2	7.3 ± 0.1	7.4 ± 0.2
Lactate, mM								
Fasted	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	1.6 ± 0.1†	2.9 ± 0.3†	3.9 ± 0.4†	3.9 ± 0.4†
Hay	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	1.0 ± 0.1	2.0 ± 0.2†	3.6 ± 0.6†	4.2 ± 0.7†	4.3 ± 0.6†
Grain	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.9 ± 0.1	1.5 ± 0.2†	3.1 ± 0.4†	4.0 ± 0.5†	4.2 ± 0.5†

Table 3.2. Hematocrit and plasma total protein and lactate concentrations before and during 60 min of exercise at 50 ± 0.7% of $\dot{V}O_{2max}$. Values during exercise are from 155- to 210-min. Expressed as means ± SE, n=6. $\dot{V}O_{2max}$, maximal rate of oxygen uptake; * $P<0.05$, Grain vs. Hay; ‡$P<0.05$, Hay vs. Feed withholding; †$P<0.05$, 165- to 210-min vs. 150 min. Hematocrit and Total solids from 155- to 210-min are higher than 150-min ($P<0.001$) in all trials.

Time, min	5	15	30	45	60
$\dot{V}O_2$, ml·kg^{-1}·min^{-1}					
Fasted	60±1	64±1	67±2	67±2	69±2
Hay	62±3	63±2	66±1	68±1	68±1
Grain	61±2	63±2	66±2	67±2	67±2
RER					
Fasted	0.92±0.02	0.90±0.01	0.88±0.01	0.87±0.01	0.87±0.01
Hay	0.90±0.01	0.89±0.01	0.87±0.01	0.85±0.01	0.84±0.01
Grain	0.93±0.02	0.92±0.02	0.90±0.02	0.89±0.01	0.89±0.01*

Table 3.3. Rate of oxygen consumption ($\dot{V}O_2$), and respiratory exchange ratio (RER) during 60 min of exercise at 50 ± 0.7% of $\dot{V}O_{2max}$. Values are means ± SE, n=6. Rate of oxygen consumption and respiratory exchange ratio were calculated from 5-min average values. * P<0.05, Grain vs. Hay.

Time, min	5	15	30	45	60
CHO Oxidation, μmol·kg^{-1}·min^{-1}, (cal·kg^{-1}·min^{-1})					
Fasted	339±28 (226±18)	318±24 (212±16)	297±27 (198±18)	289±18 (193±12)	287±18 (191±12)
Hay	313±19 (209±12)	309±28 (206±18)	282±24 (188±16)	258±26 (172±17)	239±26 (159±17)
Grain	352±30 (234±20)	357±32 (238±21)	337±33 (225±22)	325±26 (217±17)	327±26 (218±18)*
Lipid Oxidation, μmol·kg^{-1}·min^{-1}, (cal·kg^{-1}·min^{-1})					
Fasted	32±6 (76±14)	44±4 (106±9)	56±5 (134±11)	59±3 (140±8)	63±1 (150±3)
Hay	41±4 (97±9)	46±6 (109±13)	58±5 (139±13)	67±6 (160±14)	72±8 (172±19)
Grain	30±8 (72±19)	33±7 (79±17)	43±6 (103±15)	48±4 (115±11)	49±4 (117±10)*
Glycogen (and lactate) Oxidation, μmol·kg^{-1}·min^{-1}, (cal·kg^{-1}·min^{-1})					
Fasted	319±26 (211±18)	281±25 (184±17)	261±27 (171±18)	260±18 (171±12)	246±17 (161±11)
Hay	284±15 (185±8)	269±29 (175±22)	247±25 (161±16)	230±28 (151±19)	207±26 (135±19)
Grain	320±31 (209±17)	301±33 (195±22)	294±32 (192±21)	290±26 (190±17)	288±24 (189±17)
Glucose Rate of Disappearance, μmol·kg^{-1}·min^{-1}, (cal·kg^{-1}·min^{-1})					
Fasted	20±3 (15±2)	37±5 (28±4)	36±5 (27±4)	29±4 (22±3)	38±4 (29±3)
Hay	39±7 (30±5)‡	40±3 (30±2)	35±3 (27±2)	28±4 (22±3)	33±4 (25±3)
Grain	49±5 (37±3)†	56±4 (42±3)*†	43±4 (32±3)	36±3 (27±2)	39±6 (39±4)

Table 3.4 Rate of oxygen consumption ($\dot{V}O_2$), respiratory exchange ratio (RER), rate of carbohydrate (CHO) and lipid oxidation, calculated muscle glycogen (and lactate) oxidation and rate of glucose disappearance (R_d) during 60 min of exercise at 50 ± 0.7% of $\dot{V}O2max$. Values are means ± SE, n=6. Rates of carbohydrate and fat oxidation were calculated from respiratory gas exchange data, averaged over 5-min. Muscle glycogen (plus lactate) oxidation was estimated from the difference between total carbohydrate oxidation and rate of glucose disappearance. Values in parenthesis are the relative caloric contributions to energy expenditure from oxidation of carbohydrate, lipid, muscle glycogen (and lactate) and blood-borne glucose. * P<0.05, Grain vs. Hay; †P<0.05, Grain vs. Feed withholding; ‡P<0.05, Hay vs. Feed withholding.

3.5 DISCUSSION

The present study examined the effects of a high-glycemic meal (Grain), and an approximately isocaloric low-glycemic meal (Hay) or feed withholding prior to exercise on carbohydrate and lipid metabolism in horses during moderate-intensity exercise. The main findings were 1) a ~50% increase in whole body glucose uptake during the first 30-min of moderate intensity exercise after feeding a corn meal prior to exercise, compared with exercise after withholding feed for 18-h; 2) feeding a hay or grain meal, but not the glycemic response elicited by the meal fed prior to exercise, decreases plasma glucose concentration during exercise, compared with exercise after withholding feed; 3) augmentation of CHO_{ox} and attenuation of lipid oxidation during exercise in trials preceded by corn feeding; and *4*) feeding status prior to exercise does not affect net muscle glycogen utilization.

As expected, feeding a high-glycemic meal (HGM) prior to exercise resulted in marked hyperglycemia (Fig. 3.1A) and hyperinsulinemia (Fig. 3.1B) before the exercise bout. These results are consistent with findings of previous studies of the effect of feeding of different grains and/or roughages on plasma metabolite and hormone concentrations in horses[25-28]. Stull and Rodiek showed that a corn meal that provides 25% of the energy requirements induces a ~50% increase in plasma glucose concentration that peaks between 2 and 3 hours after eating, and a 6-fold increase in serum insulin concentration that peaks 2 hours after eating[41]. However, in the same study, an isocaloric amount of alfalfa hay did not induce an increase in plasma glucose or serum insulin concentrations. This is in contrast to our findings, in which hay feeding did not result in an increase in plasma glucose but did result in a 60% increase in serum insulin

concentration after hay consumption. The meals fed in this study achieved our aim of offering meals of different glycemic indices to horses prior to exercise.

In this study, the increase in plasma glucose concentration induced by corn feeding was accompanied by increments in glucose R_a and R_d. Glucose R_a was similar in horses held off feed and fed hay, and was similar to previous estimations by intravenous [2-^{3}H]glucose infusion of the total glucose production in two ponies fed hay (9.8 $\mu mol \cdot kg^{-1} \cdot min^{-1}$)[42]. During exercise, glucose R_d was significantly higher in trials preceded by ingestion of a grain meal compared with trials preceded by a hay meal or withholding feed. Similarly, in humans, ingestion of a high glycemic meal (HGM)(mashed potatoes) or glucose solution prior to moderate intensity exercise results in higher glucose R_a and R_d before and during exercise, compared to low glycemic meal (LGM) (muesli) or placebo or fasting[5,18,43]. However, preexercise glucose ingestion markedly decreases hepatic glucose production during exercise, and the increase in glucose R_a reflects ongoing intestinal uptake of glucose[43]. In the present study, we did not measure the contribution of gut-derived R_a (intestinal absorption of glucose) to the total R_a. Nonetheless, it is likely that continued absorption of glucose from the gastrointestinal tract contributed to the higher glucose R_a prior to exercise in horses fed a grain meal. During the first half of exercise in horses fed grain, as the increase in glucose R_d was not accompanied by a similar increase in glucose R_a, plasma glucose concentration decreased sharply. However, plasma glucose concentrations during exercise in horses fed grain were not different than in horses fed hay prior to exercise. This is in contrast to previous

studies performed in horses[27,28] in which horses fed corn prior to exercise had lower plasma glucose concentrations during exercise compared to those of horses fed alfalfa hay prior to exercise.

In horses that had feed withheld, blood glucose concentrations steadily increased during the exercise bout and were higher than in horses fed prior to exercise. These findings are consistent with previous studies in horses[23-26,28] but not in humans [6,13,14,17-19]. Endurance trained human athletes cycling at a higher relative exercise intensity (85% $V_{O_{2max}}$) but a similar absolute exercise intensity (58 ml $O_2 \cdot kg^{-1} \cdot min^{-1}$) had similar responses to exercise than the horses in this study[44]. Human athletes exercising at an intensity that elicits a similar V_{O_2} as for the horses in this study, also had: (1) a steady increase in blood glucose concentrations during the exercise bout (~3.9 mM prior and ~8.2 mM during exercise), (2) had similar glucose R_d before and during exercise (~10 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ prior and ~50 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ during exercise) and (3) similar relative and absolute contributions from different substrates to energy expenditure during exercise (~180 $cal \cdot kg^{-1} \cdot min^{-1}$ from muscle glycogen oxidation, ~80 $cal \cdot kg^{-1} \cdot min^{-1}$ from muscle triglyceride and plasma NEFA oxidation, and ~40 $cal \cdot kg^{-1} \cdot min^{-1}$ from plasma glucose oxidation)[44]. These differences between human subjects and horses when comparing them at the same exercise intensity relative to % $V_{O_{2max}}$ (e.g. 50% $V_{O_{2max}}$) may be associated with the greater aerobic capacity of horses. In other words, when horses exercise at the same work intensity relative to $V_{O_{2max}}$ as human athletes, the V_{O_2} and the workload is much greater in horses than in human athletes[1]. Therefore when comparing some metabolic responses to exercise it may be more meaningful to compare human athletes and horses when performing exercise tasks that elicit similar V_{O_2}.

Previous studies in horses have given recommendations of feeding prior to exercise based on changes in substrate and hormone concentrations in plasma or serum[25,26]. Because of the exercise-induced decrease in plasma glucose concentration and reduced NEFA availability in horses fed grain prior to exercise, it has been recommended not to feed a grain meal prior to exercise[25,26]. In humans, ingestion of a HGM prior and/or during exercise has resulted in enhanced[6,11,13,14], decreased[17] or unchanged submaximal exercise performance[3,4,18-20]. In horses, the effect of CHO ingestion on performance during moderate-intensity exercise has not been determined. Therefore, recommendations on interval and type of feeding prior to exercise can only be offered based upon the metabolic responses induced by the interval of feeding and the composition of the meal. Determination of plasma or serum concentration of metabolites and substrates has been the sole means for providing recommendations of dietary manipulations in horses prior to exercise. These studies have provided little insight into the influence of feeding status prior to exercise on substrate supply and utilization during exercise. The combination of estimation of whole body carbohydrate and fat oxidation rates (by indirect calorimetry measurements) and estimation of rates of appearance and disappearance of metabolites in plasma (by measurement of infused metabolites labeled with stable isotopes) is the only means to determine the contribution of extramuscular and intramuscular substrates to energy production. To the best of our knowledge, the present study is the first one that estimates the contribution of extramuscular and intramuscular substrates to energy production in horses fed different meals or denied access to food prior to exercise.

Ingestion of corn before exercise resulted in a significant decrease in plasma glycerol concentration during exercise (Fig. 3.2A) and plasma NEFA concentration before and

during exercise (Fig. 3.2B). Lower plasma NEFA concentrations in grain trials also may have contributed to the higher rate of glucose utilization in this trial compared to hay or feed withholding trials. In humans, preexercise carbohydrate ingestion inhibits lipolysis during moderate-intensity exercise and suppresses the increase in plasma NEFA concentration[45,46]. Similarly, in horses, preexercise ingestion of corn attenuates the increase in fatty acid concentration during exercise[24,28].

Feeding corn prior to exercise resulted in a greater relative contribution to energy expenditure from blood-borne glucose during the initial half of a 60-min moderate intensity exercise event, as compared to withholding feed; and resulted in greater carbohydrate oxidation and lower lipid oxidation during the second half of the exercise bout, as compared to hay feeding prior to exercise (Fig. 3.7). Similarly to the results in our present study, increased glucose availability during 60-min of running at 55% O_{2max} by pre-exercise intragastric administration of glucose (2 g/kg, 10 kcal/kg DE [41.6 KJ/kg DE]) to horses resulted in enhanced CHO_{ox} and utilization of blood-borne glucose during moderate-intensity exercise, but did not alter muscle glycogen utilization[47]. In studies performed in human subjects, ingestion of glucose or high-glycemic meals prior to exercise results in enhanced CHO_{ox} and utilization of blood-borne glucose, and the rate of glycogenolysis may be enhanced[2] or unchanged[3-6]. The same controversy is apparent in regards to the effect of increased glucose availability on muscle glycogenolysis in studies performed in horses. Lawrence reported that increased glucose availability by ingestion of a HGM prior to exercise enhances muscle glycogenolysis in exercising horses, as compared to feed withholding (~46 mmol/kg ww vs. 18 mmol/kg ww in ~15 min exercise test of 1.6 km at 6 m/s, 0.8 km at 1.9 m/s (treadmill grade changed to 2%), 0.4

km speed-up period and a final 1.6 km at 11 m/s)[23]. This is in contrast to the findings of this study (Fig. 3.6), previous studies performed in our laboratory[33,47] and by others[22], in which increased glucose availability by pre-exercise ingestion of grain or intragastric glucose administration or CHO-rich diet, respectively, did not alter muscle glycogenolysis during exercise. However, in our study a nonsignificant reduction of muscle glycogenolysis of ~25% was noted when horses were fed grain before exercise compared to feed withholding.

The total energy expenditure during 60 min of exercise was ~19.3 Kcal/kg (~80.4 KJ/kg), which is ~33.8 Kcal/kg DE (~141 KJ/kg DE) assuming an efficiency of utilization of DE of 57%[48]. When this is translated into National Research Council (NRC) nutritional requirements[29] and the maintenance energy requirements are added (~33.1 Kcal/kg DE [~138 KJ/kg DE]), there is an average total daily energy requirement of ~66.9 Kcal/kg DE (~278.8 KJ/kg DE), an increase over maintenance of ~102%. Rose et al. reported similar increases in daily energy requirement (~112%) for horses exercised at 50% O_{2max} for 75 min[49], and Pagan et al. reported lower increases in daily energy requirement over maintenance (~67%) for 450-kg horses exercised for 2 h on a flat surface at 4.2 m/sec by a 75-kg rider[48]. These estimates of the increase in energy requirements for exercising horses may be useful in the future to develop more accurate guidelines of the nutritional requirements of these animals.

In summary, this study has demonstrated that feeding of a high glycemic meal before exercise augments CHO_{ox} and utilization of blood-borne glucose in horses during moderate-intensity exercise but does not alter muscle glycogen usage. Conversely, feeding of an isocaloric low glycemic roughage meal before exercise, when compared to

a high glycemic meal, augments lipid oxidation in horses during moderate-intensity exercise but does not alter muscle glycogen usage. These findings suggests that feeding a high glycemic meal two hours prior to exercise might be indicated in exercise bouts of moderate intensity in which carbohydrate oxidation is responsible for supplying more than half of the energy requirement for energy transduction during exercise.

3.6 REFERENCES

1. Wagner PD. Determinants of VO_{2max}: man vs. horse. *Journal of Equine Veterinary Science* 1995;15:398-404.

2. Hargreaves M, Costill DL, Fink WJ, et al. Effect of Pre-Exercise Carbohydrate Feedings on Endurance Cycling Performance. *Medicine and Science in Sports and Exercise* 1987;19:33-36.

3. Febbraio MA, Keenan J, Angus DJ, et al. Preexercise carbohydrate ingestion, glucose kinetics, and muscle glycogen use: effect of the glycemic index. *Journal of Applied Physiology* 2000;89:1845-1851.

4. Hargreaves M, Costill DL, Katz A, et al. Effect of Fructose Ingestion on Muscle Glycogen Usage During Exercise. *Medicine and Science in Sports and Exercise* 1985;17:360-363.

5. Jeukendrup AE, Raben A, Gijsen A, et al. Glucose kinetics during prolonged exercise in highly trained human subjects: effect of glucose ingestion. *Journal of Physiology-London* 1999;515:579-589.

6. Kirwan JP, O'Gorman D, Evans WJ. A moderate glycemic meal before endurance exercise can enhance performance. *Journal of Applied Physiology* 1998;84:53-59.

7. Coggan AR, Coyle EF. Effect of Carbohydrate Feedings During High-Intensity Exercise. *Journal of Applied Physiology* 1988;65:1703-1709.

8. Coggan AR, Coyle EF. Reversal of Fatigue During Prolonged Exercise by Carbohydrate Infusion or Ingestion. *Journal of Applied Physiology* 1987;63:2388-2395.

9. Coyle EF, Coggan AR, Hemmert MK, et al. Muscle Glycogen Utilization During Prolonged Strenuous Exercise When Fed Carbohydrate. *Journal of Applied Physiology* 1986;61:165-172.

10. Coyle EF, Hagberg JM, Hurley BF, et al. Carbohydrate Feeding During Prolonged Strenuous Exercise Can Delay Fatigue. *Journal of Applied Physiology* 1983;55:230-235.

11. Gleeson M, Maughan RJ, Greenhaff PL. Comparison of the Effects of Pre-Exercise Feeding of Glucose, Glycerol and Placebo on Endurance and Fuel Homeostasis in Man. *European Journal of Applied Physiology and Occupational Physiology* 1986;55:645-653.

12. Goodpaster BH, Costill DL, Fink WJ, et al. The effects of pre-exercise starch ingestion on endurance performance. *International Journal of Sports Medicine* 1996;17:366-372.

13. Sherman WM, Brodowicz G, Wright DA, et al. Effects of 4 h Preexercise

Carbohydrate Feedings on Cycling Performance. *Medicine and Science in Sports and Exercise* 1989;21:598-604.

14. Sherman WM, Peden MC, Wright DA. Carbohydrate Feedings 1-H before Exercise Improves Cycling Performance. *American Journal of Clinical Nutrition* 1991;54:866-870.

15. Tsintzas OK, Williams C, Wilson W, et al. Influence of carbohydrate supplementation early in exercise on endurance running capacity. *Medicine and Science in Sports and Exercise* 1996;28:1373-1379.

16. Wright DA, Sherman WM, Dernbach AR. Carbohydrate Feedings before, During, or in Combination Improve Cycling Endurance Performance. *Journal of Applied Physiology* 1991;71:1082-1088.

17. Thomas DE, Brotherhood JR, Brand JC. Carbohydrate Feeding before Exercise - Effect of Glycemic Index. *International Journal of Sports Medicine* 1991;12:180-186.

18. Febbraio MA, Stewart KL. CHO feeding before prolonged exercise: Effect of glycemic index on muscle glycogenolysis and exercise performance. *Journal of Applied Physiology* 1996;81:1115-1120.

19. Sparks MJ, Selig SS, Febbraio MA. Pre-exercise carbohydrate ingestion: effect of the glycemic index on endurance exercise performance. *Medicine and Science in Sports and Exercise* 1998;30:844-849.

20. Wee SL, Williams C, Gray S, et al. Influence of high and low glycemic index meals on endurance running capacity. *Medicine and Science in Sports and Exercise* 1999;31:393-399.

21. Jacobs KA, Sherman WM. The efficacy of carbohydrate supplementation and chronic high- carbohydrate diets for improving endurance performance. *International Journal of Sport Nutrition* 1999;9:92-115.

22. Essén-Gustavsson B, Blomstrand E, Karlström K, et al. Influence of diet on substrate metabolism during exercise. Equine Exercise Physiology 3 1991;288-298.

23. Lawrence L, Soderholm LV, Roberts A, et al. Feeding Status Affects Glucose-Metabolism in Exercising Horses. *Journal of Nutrition* 1993;123:2152-2157.

24. Lawrence LM, Williams J, Soderholm LV, et al. Effect of feeding state on the response of horses to repeated bouts of intense exercise. *Equine Vet J* 1995;27:27-30.

25. Pagan JD, Harris PA. The effects of timing and amount of forage and grain on exercise response in thoroughbred horses. *Equine Vet J Suppl* 1999;30:451-457.

26. Pagan JD, Burger I, Jackson SG. The influence of time of feeding on exercise response in Thoroughbreds fed a fat supplemented or high carbohydrate diet. 14th Equine Nutrition and Physiology Symposium 1995;92-93.

27. Rodiek A, Bonvicin S, Stull C, et al. Glycemic and endocrine responses to corn or alfalfa fed prior to exercise. Equine Exercise Physiology 3 1991;323-330.

28. Stull CL, Rodiek AV. Responses of blood glucose, insulin and cortisol concentrations to common equine diets. *J Nutr* 1988;118:206-213.

29. National Research Council (U.S.). Subcommittee on Horse Nutrition. *Nutrient requirements of horses*. 5th rev. ed. Washington, D.C.: National Academy Press, 1989.

30. Hinchcliff KW, McKeever KH, Muir WW, 3rd, et al. Effect of furosemide and weight carriage on energetic responses of horses to incremental exertion. *Am J Vet Res* 1993;54:1500-1504.

31. Fedak MA, Rome L, Seeherman HJ. One-Step N2-Dilution Technique for Calibrating Open-Circuit Vo2 Measuring Systems. *Journal of Applied Physiology* 1981;51:772-776.

32. Lindholm A, Piehl K. Fibre composition, enzyme activity and concentrations of metabolites and electrolytes in muscles of standardbred horses. *Acta Vet Scand* 1974;15:287-309.

33. Geor RJ, Hinchcliff KW, McCutcheon LJ, et al. Epinephrine inhibits exogenous glucose utilization in exercising horses. *J Appl Physiol* 2000;88:1777-1790.

34. Freestone JF, Shoemaker K, Bessin R, et al. Insulin and glucose response following oral glucose administration in well-conditioned ponies. *Equine Vet J Suppl* 1992:13-17.

35. Passonneau JV, Lauderdale VR. A comparison of three methods of glycogen measurement in tissues. *Anal Biochem* 1974;60:405-412.

36. Wolfe RR. *Radioactive and stable isotope tracers in biomedicine : principles and practice of kinetic analysis*. New York: Wiley-Liss, 1992.

37. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci* 1959;82:420-430.

38. Frayn KN. Calculation of Substrate Oxidation Rates In vivo from Gaseous Exchange. *Journal of Applied Physiology* 1983;55:628-634.

39. Ferrannini E. The Theoretical Bases of Indirect Calorimetry - a Review. *Metabolism-Clinical and Experimental* 1988;37:287-301.

40. Coggan AR, Kohrt WM, Spina RJ, et al. Endurance training decreases plasma

glucose turnover and oxidation during moderate-intensity exercise in men. *J Appl Physiol* 1990;68:990-996.

41. Stull C, Rodiek A. Effects of postprandial interval and feed type on substrate availability during exercise. *Equine Vet J Suppl* 1995;18:362-366.

42. Simmons HA, Ford EJH. Gluconeogenesis from Propionate Produced in the Colon of the Horse. *British Veterinary Journal* 1991;147:340-345.

43. Marmy-Conus N, Fabris S, Proietto J, et al. Preexercise glucose ingestion and glucose kinetics during exercise. *Journal of Applied Physiology* 1996;81:853-857.

44. Romijn JA, Coyle EF, Sidossis LS, et al. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol* 1993;265:E380-391.

45. Coyle EF, Jeukendrup AE, Wagenmakers AJM, et al. Fatty acid oxidation is directly regulated by carbohydrate metabolism during exercise. *American Journal of Physiology-Endocrinology and Metabolism* 1997;36:E268-E275.

46. Horowitz JF, MoraRodriguez R, Byerley LO, et al. Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. *American Journal of Physiology-Endocrinology and Metabolism* 1997;36:E768-E775.

47. Geor RJ, Hinchcliff KW, Sams RA. Glucose infusion attenuates endogenous glucose production and enhances glucose use of horses during exercise. *J Appl Physiol* 2000;88:1765-1776.

48. Pagan JD, Hintz HF. Equine Energetics. II. Energy-Expenditure in Horses During Submaximal Exercise. *Journal of Animal Science* 1986;63:822-830.

49. Rose RJ, Knight PK, Bryden WL. Energy use and cardiorespiratory responses to prolonged submaximal exercise. Equine Exercise Physiology 3 1991;281-287.

CHAPTER 4

GLYCEMIC INDEX OF CRACKED CORN, OAT GROATS AND ROLLED BARLEY IN HORSES.

4.1 SUMMARY

Muscle glycogen synthesis depends on glucose availability. This study was undertaken to determine the glycemic and insulinemic response of horses to equal amounts of hydrolyzable carbohydrates (starch and sugar) in the form of one of three grain meals or intragastric administration of a glucose solution. In a randomized cross-over design, seven horses were fed each of three grain meals (cracked corn, steamed oat groats or rolled barley) or were infused intragastrically with glucose solution, at 2 g of hydrolyzable carbohydrate (starch plus sugar) per kg BW. The amount of hydrolyzable carbohydrate ingested was not different among all treatments ($P = 0.7$). Plasma glucose concentration peaked in all four treatments by 1.5 to 2 h after feeding. Plasma glucose concentration remained higher than baseline in oat groats or barley-fed horses throughout 8 h, whereas plasma glucose returned to baseline by 5 to 6 h in corn-fed horses or after glucose administration. Meal consumption was slower in oat-groats fed horses than in

corn-fed ones, which may confound the glycemic and insulinemic responses observed after grain feeding. Plasma glucose area under the curve (AUC) was 63 in corn, 63 in oat groats and 57% in barley-fed horses, when compared with that of horses administered glucose ($P = 0.13$). Serum immunoreactive insulin concentration peaked between 2 and 3 h after feeding or glucose administration, and barley-fed horses had lower serum IRI concentration than corn-fed horses or after glucose administration 3 to 4 h ($P < 0.05$). We conclude that oat groats, corn and barley have similar plasma glucose AUC in horses, and compared with the glycemic index of 100 as the glucose reference, corn, oat groats and barley were approximately 60.

4.2. INTRODUCTION

The glycemic index concept was developed in human nutrition in an attempt to characterize foods according to their postprandial glycemic response rather than their chemical composition [1]. The glycemic impact of a food in humans is influenced by the nature of the starch granules, the type of carbohydrates, the physical form of the food, and processing [2].

Energy intake of horses is primarily from carbohydrates in forages and grains. The majority of starch and sugars ingested are digested in the small intestine of hindgut fermenters and absorbed as monosaccharides (e.g. glucose, fructose). Grains commonly fed to horses (oats, corn and barley) contain on average 45 to 65% of non-structural carbohydrate (NSC) as fed (50 to 73% on a DM basis) [3]. Corn has a greater content of starch than oats or barley [4,5]. It has been shown in horses that oat starch is more digestible than corn starch in some studies [6,7], but not in others [8].

Digestion of starch and sugar in feedstuffs provides most of the substrate required for muscle glycogen synthesis after exercise. The rate of muscle glycogen replenishment after exercise in humans and horses depends on glucose availability [9,10]. Because of inherent differences in grains commonly fed to horses, the availability of glucose after ingestion of a grain meal may differ, which may impact muscle glycogen synthesis and athletic performance.

The present study was undertaken to determine the glycemic and insulinemic response of horses to ingestion of equal amounts of hydrolyzable carbohydrates in the form one of three grain meals (cracked corn, steamed oat groats or rolled barley), or intragastric administration of a glucose solution. We hypothesized that oat groats would have higher glucose availability than corn and barley in horses, when assessed as the glycemic index compared with oral glucose.

4.3. MATERIALS AND METHODS

Animals

Thoroughbred horses, 4 to 11 yr old (3 geldings and 4 mares) of 484 ± 17 kg average(± SE) BW, and body condition scores of 4 to 5 out of 9 were used in this study [11]. The horses had been trained 3 d per wk for 6 wk and were part of an exercise physiology study. Exercise consisted of 30 min of trotting at 4 to 4.5 m/sec with the treadmill set at 4º incline. Horses were fed mixed hay and cracked corn (Table 4.1) to meet energy requirements described by the NRC guidelines for horses in moderate intensity exercise prior to the study [3]. The diet consisted of 11 kg of mixed hay and 2.8 kg of corn daily, fed in 2 equal portions. Horses were kept in stalls and had access to water and a trace

mineralized salt block at all times. Experiments were performed in compliance with the guidelines and recommendations of the Institutional Laboratory Animal Care and Use Committee of The Ohio State University.

Experimental protocol

Horses were used in a randomized crossover design. Each of 7 horses were studied on 4 occasions and assignment to treatment (glucose, corn, oat groats and barley) was randomized. The study was conducted during a 2 wk period and between trials, each horse had a minimum of 2 d of a washout period, during which they were fed their regular diet (mixed hay and corn). Two weeks prior to the beginning of the study, the horses were adjusted to feed containing oat groats and rolled barley in order to familiarize them with all grains used during the study.

In each trial, following an 18-h feed withdrawal, horses were offered a meal of cracked corn, steamed oat groats, or rolled barley, or were administered a 50% glucose solution by nasogastric intubation. All four treatments were designed to provide 2 g of available carbohydrate per kg of BW. Available carbohydrate was considered to be 100% for glucose solution, and the summation of starch and sugar for grains (Table 4.1). The amounts of corn, oat groats and barley offered to these horses were 1.5 ± 0.1, 1.9 ± 0.1 and 1.9 ± 0.1 kg (mean ± SE), respectively. Blood samples for determination of plasma glucose concentration were obtained via an indwelling jugular catheter before feeding, every 30 min for the first 4 h after feed was offered, and every 1 h for another 4 h; whereas blood samples for determination of serum immunoreactive insulin (IRI) concentration were only obtained before feeding, every 60 min for the first 4 h, and at 6 and 8 h after feeding. Horses were kept in a straw-bedded stall and left undisturbed with

the exception of blood collection. Horses were allowed to consume grain for a maximum of 3 h and remained muzzled until completion of blood sample collection (8 h). Grain not consumed was removed and weight recorded. Horses had access to water but no access to roughage during the entire 8 h trial.

Data from horses that did not consume more than 75% of the meal were not considered in the statistical analysis. One horse consumed only 62 and 28% of the oat groats and barley meals, respectively, and another horse consumed only 58% of the oat groats meal.

Analytical methods

Blood samples were collected into tubes containing potassium oxalate and sodium fluoride for analysis of plasma glucose, and into tubes without additives for analysis of serum immunoreactive insulin (IRI). Within 10 min of collection, samples were centrifuged (1500 x g) in a refrigerated centrifuge and plasma and serum were collected and stored at -80ºC until analysis. Glucose analysis was performed using a spectrophotometric method based on glucose hexokinase (Glucose hexokinase kit, Sigma Diagnostics, St. Louis, MO). Analyses were performed in duplicate, and all samples obtained from one horse were analyzed in a single session to avoid interassay variation. Intra- and inter-assay coefficients of variation were 5 and 6.5%, respectively. Serum IRI was determined in duplicate by use of a commercially available RIA (insulin kit, Coat-a-Count Diagnostics, Los Angeles, CA) validated for horse blood [12]. Intra- and interassay coefficients of variation were <10%, and the limit of detection was 5.08 μIU/mL.

Mixed grass/alfalfa hay and grains were analyzed in a commercial laboratory for nutrient composition, starch and sugar content (Cumberland Valley Analytical

Services, Inc., Maugansville, MD). Cracked corn, steamed oat groats and rolled barley were analyzed enzymatically for starch [13] and for sugar using a solvent extraction and enzymatic analysis [14] (Table 4.1). The total amount of starch and sugar was considered as the total amount of carbohydrate available to the equine small intestine for absorption as mono- and disaccharides and eventual transformation to glucose. Digestible energy was calculated from Non-Structural Carbohydrate, (NSC), available protein (AP; CP minus ADF-CP), ether extract (EE), protein-free NDF (NDFpf), and lignin, using an equation based on Weiss' [15] ruminant equation and adapted by Cupp [16] to equine diets, as follows:

DE Mcal/kg = 0.04409*{0.9*(NSC)+AP+0.94*(2.25*(EE-1))+0.35*(NDFpf-Lig)*[1-(lig/NDFpf)$^{.667}$]}

Statistical analysis

Data are presented as means and standard errors of the mean. Plasma glucose, serum IRI concentrations, and glucose:insulin ratios were analyzed using a mixed model with repeated measures with treatment, time and the interaction of treatment and time as fixed effects and horse as random effect [17]. Treatment effects and means at specific time points were compared by the Dunn-Sidák multiple comparisons procedure with adjusted $P < 0.05$ [18]. Areas under the concentration-time curve (AUC) were calculated by the trapezoidal method [19] for plasma glucose and serum IRI concentrations. The AUC obtained for the glucose treatment was considered to correspond to a Glycemic Index (GI) of 100 and the 3 grains were given a GI based on their AUC expressed relative to the reference AUC of the glucose treatment. Areas under the curve, time of consumption of grain meals, and amount of starch and/or sugar ingested were compared by one-way ANOVA with repeated measures, with significance level set at $P < 0.05$. The Sigmastat

2.0 (Jandel Scientific, San Rafael, CA) and SPSS 12.0 (SPSS Inc., Chicago, IL) software packages were used for statistical computations.

4.4. RESULTS

Average meal consumption was 97 ± 2, 95 ± 3 and 93 ± 4% for corn, oat groats and barley, respectively. The amounts of hydrolyzable carbohydrates (starch plus sugars) ingested from corn, oat groats and barley were 921 ± 37, 946 ± 65 and 869 ± 56 g, respectively. The amount of glucose administered intragastrically was 939 ± 33 g. There was no difference among the four treatments in the combined amount of starch and sugar ingested ($P = 0.7$). The amount of digestible energy (DE) provided was 4.8 ± 0.2, 5.7 ± 0.4, and 5.4 ± 0.3 Mcal for corn, oat groats and barley, respectively. There were no difference among treatments in the amount of DE ingested ($P = 0.1$). The time needed to consume the entire meal or removal of the leftover grains was 50 ± 14, 126 ± 19 and 78 ± 25 min for corn, oat groats and barley, respectively. The time needed to consume each grain meal was significantly longer for oat groats than for corn ($P = 0.013$).

Glycemic response

Mean plasma glucose concentrations prior to treatments ranged from 4.1 to 4.5 mM. Plasma glucose concentrations peaked with all 4 treatments at 1.5 to 2 h after offering the meal, or administering glucose (Fig. 4.1). In horses fed corn or administered glucose solution, plasma glucose concentrations at 5 to 6 h after feeding were similar to that prior to feeding. In contrast, horses fed oat groats or barley continued to have elevated plasma glucose concentrations 6 h after feeding (Fig 4.1). Plasma glucose concentrations were higher in horses given glucose when compared with corn-fed and

oat-fed horses ($P < 0.001$) and tended to be higher in horses given glucose when compared with barley-fed horses ($P = 0.09$). Specifically, plasma glucose concentrations were higher at 90, 120 and 150 min in horses given glucose when compared with oat-fed horses ($P < 0.028$) and at 240 min in horses given glucose when compared with corn-fed horses ($P = 0.032$).

Area under the glucose concentration-time curve (AUC) for horses given glucose, corn, oat groats and barley were 818 ± 170, 519 ± 106, 514 ± 43 and 468 ± 42 mM·min, respectively, and were not statistically different ($P = 0.22$). Glucose AUC adjusted for hydrolyzable carbohydrate ingestion in horses given glucose, corn, oats and barley were 818 ± 170, 529 ± 108, 510 ± 38 and 505 ± 40 mM·min, respectively. When the glycemic index of the glucose reference treatment was set to 100, the corresponding glycemic indices for corn, oat groats and barley were 63, 63 and 57, respectively. The glycemic indices adjusted for hydrolyzable carbohydrate ingestion for corn, oat groats and barley were 65, 62, 62, respectively.

Insulinemic response

Serum immunoreactive insulin (IRI) concentrations prior to feeding or glucose administration were below the limit of detection (< 5.08 μIU/mL). Serum IRI concentrations peaked in horses receiving all 4 treatments at 2 to 3 h after offering the meal, or administering glucose (Fig. 4.2). Serum IRI concentrations declined gradually from 4 to 8 h in all 4 treatments. Serum IRI concentrations were lower in horses fed barley when compared with horses fed oat groats ($P = 0.006$) and horses given glucose ($P = 0.009$). Specifically, serum IRI concentrations were lower at 180 min in barley-fed horses when compared with corn-fed horses ($P = 0.03$) and after glucose administration

($P = 0.001$) and at 240 min in barley-fed horses when compared with glucose administration ($P = 0.001$).

Area under the serum IRI concentration-time curves (AUC) for horses receiving glucose, corn, oats and barley treatments were 7860 ± 2342, 5637 ± 1430, 5314 ± 1379 and 3030 ± 502 $\mu IU \cdot min \cdot mL^{-1}$, respectively. Barley-fed horses tended to have lower serum IRI AUC when compared with other treatments ($P = 0.06$). Serum IRI AUC adjusted for hydrolyzable carbohydrate ingestion in horses given glucose, corn, oats and barley were 7860 ± 2032, 5747 ± 1315, 5275 ± 1526 and 3274 ± 513 $\mu IU \cdot min \cdot mL^{-1}$, respectively.

The plasma glucose to serum IRI ratios decreased from values greater than 0.7 to 0.9 before feeding or glucose administration to ~0.25 to 0.4 during the following 4 h (Fig 4.3). Glucose:IRI ratios were higher in barley-fed horses than corn-fed horses ($P = 0.03$), oat-fed horses ($P = 0.001$) and after administration of glucose ($P = 0.005$). Specifically, glucose:IRI ratios were higher at 120 min in barley-fed horses when compared with oat-fed horses ($P = 0.04$) and at 180 min in barley-fed horses when compared with corn-fed horses ($P = 0.03$) and after glucose administration ($P = 0.04$).

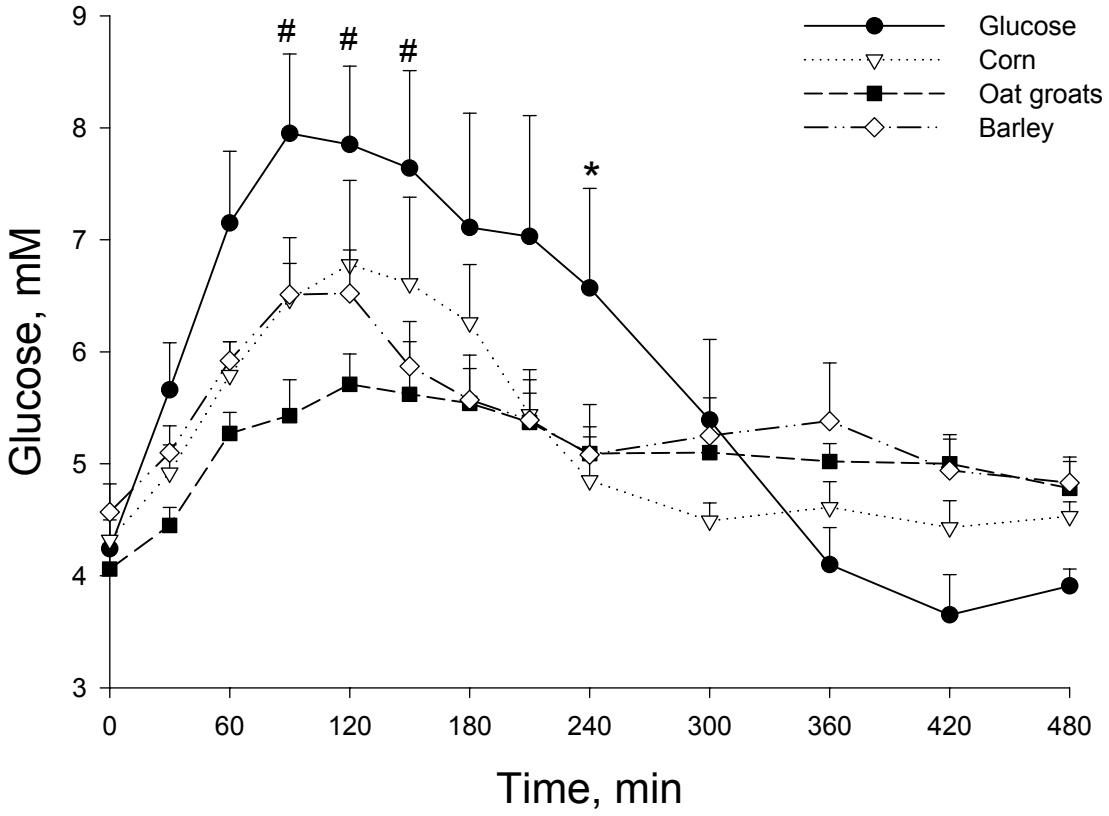

Figure 4.1. Plasma glucose concentration (mM) immediately before and during 8 h after ingestion of 2 g of starch and sugar in the form of cracked corn, oat groats, rolled barley or administration of glucose. Horses were fed right after 0 min and muzzled after finishing each meal or at 180 min if not finished. Values are means ± SE for 7 horses (except Oats n = 5, and Barley n = 6). Time points different among treatments: * glucose vs. corn, $P = 0.03$; # glucose vs. oat groats, $P < 0.03$; Treatment effect ($P = 0.001$): glucose vs. corn, $P < 0.001$; glucose vs. oats, $P < 0.001$; glucose vs. barley, $P = 0.09$. Time effect: $P < 0.001$; Time by treatment interaction: $P = 0.004$.

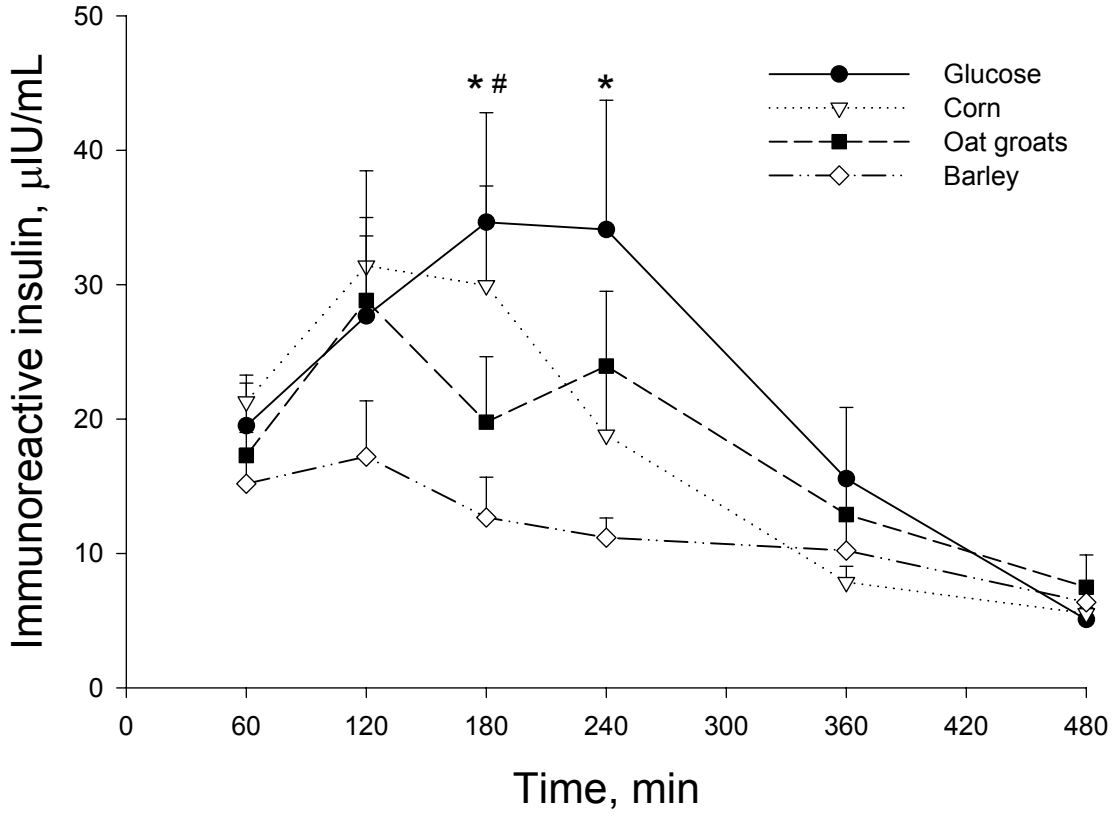

Figure 4.2. Serum immunoreactive insulin (IRI) concentration immediately before and during 8 h after ingestion of 2 g of starch and sugar in the form of cracked corn, oat groats, rolled barley or administration of glucose. Values are means ± SE for 7 horses (except Oats n = 5, and Barley n = 6). Values not reported at 0 min since insulin concentrations were below the limit of detection (<5.08 µIU/mL). Time points different among treatments: * glucose vs. barley, $P = 0.001$; # corn vs. barley, $P = 0.03$; Treatment effect ($P < 0.001$): glucose vs. barley, $P = 0.009$; oats vs. barley, $P = 0.006$. Time effect: $P < 0.001$; Time by treatment interaction: $P = 0.02$.

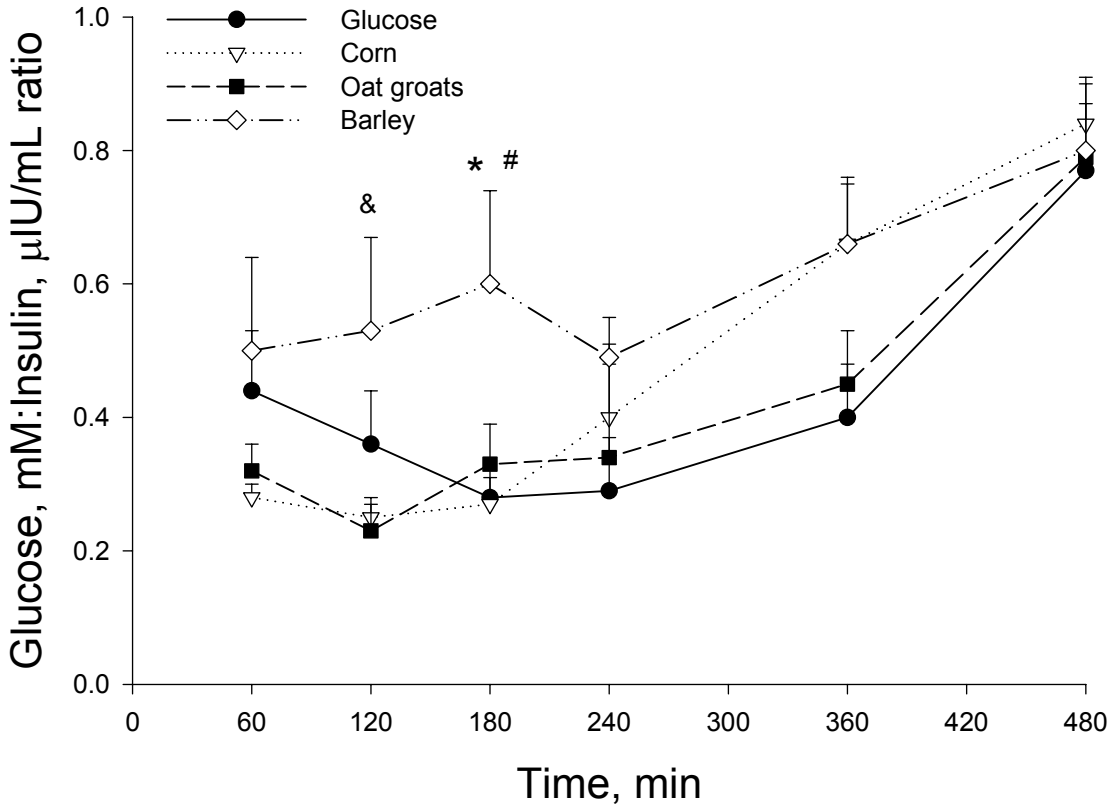

Figure 4.3. Glucose:insulin ratios after ingestion of 2 g of starch and sugar in the form of cracked corn, oats groats, rolled barley or administration of glucose. Values are means ± SE for 7 horses (except Oats n = 5, and Barley n = 6). Values not reported at 0 min since insulin concentrations were below the limit of detection (<5.08 µIU/mL). Time points different among treatments: * glucose vs. barley, $P = 0.04$; $^{\#}$ corn vs. barley, $P = 0.03$; $^{\&}$ oats vs. barley, $P = 0.04$; Treatment effect ($P < 0.001$): glucose vs. barley, $P = 0.005$, oats vs. barley, $P = 0.001$, corn vs. barley, $P = 0.03$. Time effect: $P < 0.001$; Time by treatment interaction: $P = 0.3$.

Item, %	Cracked corn	Oat groats	Rolled barley	Mixed hay
DM	85.9	87.1	88.0	87.7
CP	7.2 (8.3)	13.9 (15.9)	10.1 (11.5)	15.5 (17.7)
ADF	5.0 (5.9)	6.5 (7.4)	6.8 (7.8)	27.9 (31.8)
NDF	9.6 (11.2)	9.7 (11.1)	18.9 (21.5)	39.3 (44.8)
Lignin	0.4 (0.5)	1.8 (2.0)	1.9 (2.1)	3.9 (4.4)
Starch	60.5 (70.3)	46.8 (53.7)	46.7 (53.0)	3.8 (4.3)*
Sugar	1.1 (1.3)	1.0 (1.2)	1.1 (1.3)	-
NSC[a]	64.0 (74.5)	55.3 (63.5)	54.1 (61.5)	25.0 (28.5)
Crude fat	3.7 (4.3)	5.9 (6.8)	2.5 (2.8)	2.4 (2.7)
Ash	1.5 (1.7)	2.4 (2.7)	2.5 (2.8)	7.9 (9.0)
TDN	75.9 (88.3)	77.2 (88.7)	69.6 (79.1)	48.3 (55.0)
DE, Mcal/kg	3.23 (3.76)	3.35 (3.85)	2.94 (3.49)	2.13 (2.42)

Table 4.1. Feed analysis of mixed hay and grains. Dry matter (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), total digestible nutrients (TDN), digestible energy (DE). Data are as sampled and, in parentheses, on a 100% dry matter basis. Analyzed by Cumberland Valley Analytical Services, Inc. (Maugansville, MD).
[a]Non-structral carbohydrate (NSC = 100 – crude protein – NDF – crude fat – ash)

4.5. DISCUSSION

The present study examined the glycemic and insulinemic response to a meal of cracked corn, steamed oat groats and rolled barley that provided approximately equal amounts of starch and sugars, and compared these to intragastric administration of an equal amount of glucose. The main findings were: 1) plasma glucose concentration peaked in all four interventions by 1.5 to 2 h after the onset of grain ingestion or glucose administration; 2) when compared with plasma glucose AUC of horses administered glucose, plasma glucose AUC were ~57 to 63% in grain fed horses, however these differences did not reach statistical significance ($P = 0.22$); and 3) of the 3 grains studied, ingestion of corn resulted in the largest fluctuations in plasma glucose.

To our knowledge, this is the first study performed in horses to describe the glycemic and insulinemic response of feeding different grain meals that provide an approximately equal starch plus sugar content, and to compare these to intragastric administration of an equivalent amount of glucose. This approach is similar to studies performed in human nutrition, in which the glycemic index (GI) of foods is determined by serial measurement of plasma glucose concentration after ingestion of equal amounts of digestible carbohydrate in a food compared with a reference of glucose solution or white bread [1,2].

Previous studies performed in horses have compared the effect of ingestion of varying amounts of grains or concentrates on plasma glucose and serum insulin concentrations [20-23]. These studies showed that ingestion of grains, concentrates and/or roughage meals result in moderate to marked hyperglycemia and hyperinsulinemia between 1 and 3 h after eating. However, these studies compared ingestion of different

grains or concentrates as equal weight or isocaloric meals, which did not result in ingestion of equal amounts of starch and sugar. In addition, these studies did not perform a reference intervention such as administration of an equal amount of completely available carbohydrate.

The glycemic index (GI) provides a way to classify carbohydrate rich foods according to the magnitude of the glycemic response following their intake [1]. Using a more physiologically based classification of foods based on the GI, dietetic strategies have been developed for human athletes and non-insulin dependent diabetics. In human athletes ingestion of high-GI foods after exercise results in greater synthesis of muscle glycogen [24] when compared with ingestion of equal amounts of carbohydrate as low-GI foods. In contrast, ingestion of low-GI foods before exercise in endurance athletes may attenuate the insulin-mediated metabolic disturbances and may lead to more sustained carbohydrate availability [25]. Similarly, low-GI diets have been shown to improve glycemic control in non-insulin dependent diabetics, and reduce serum lipids in hyperlipidemic subjects [26]. Lower insulin sensitivity has been demonstrated in inactive and obese horses [27,28] and it has been proposed to play a role in the undefined syndrome referred to as Equine Metabolic Syndrome or Equine Peripheral Cushingoid Syndrome [29]. Description of GI of horse feeds is a necessary prerequisite to define dietetic strategies useful for athletic horses and those afflicted with clinical conditions characterized by reduced insulin sensitivity.

The magnitude of the glycemic response to ingestion of a carbohydrate-rich meal reflects its rate of digestion and absorption. In the case of grains commonly fed to horses, starch makes up most of the carbohydrate, therefore, inherent differences in starch

digestibility among grains may be reflected as different GI. Several studies using horses or ponies with ileal or cecal fistulae have determined that small intestinal digestibility of starch depends on its botanical origin and prior physical or thermal treatment. However, there is conflicting evidence for the difference in small intestinal digestibility of starch between grains commonly fed to horses. Several studies have shown that corn has a lower small intestinal digestibility of starch (29 to 45% depending on prior processing), than oat starch (80 to 99%) or barley starch (81%) [6,7,30]. However, other studies indicated that starch digestibility was not different between corn (80%), oats (81%) and barley (95%) [8] and between corn (71%) and oats (98%) [31]. In addition, alteration in the structure of corn starch granules by grinding or thermal treatment significantly increases small intestinal digestibility [6,32,33]. Despite possible differences in starch digestibility between different grains observed in previous studies, the glycemic response assessed as the glucose AUC was not different between corn, oat groats and barley in the present study.

One limitation of this study is the fact that the rate of ingestion was unequal among the grains. Despite familiarization with ingestion of all 3 grains for 2 weeks prior to the study, it took twice as long for horses to eat an oat groats meal than cracked corn. The shape of the glycemic responses after glucose administration and corn ingestion were similar and appeared to have larger fluctuations in plasma glucose when compared with oats and barley. This effect may be related to a slower rate of ingestion of oat groats and barley when compared with corn. We suggest that some of the differences related to the rate of ingestion should be accounted for by calculation of glucose AUC and the prolonged period of blood collection.

The upper limit of small intestinal starch digestibility in horses has previously

been reported to be 0.2 to 0.4% of BW per feeding [6,8]. The starch and sugar ingestion in this study was 0.19 ± 0.01% of BW, and no adverse effects were observed.

In summary, horses are fed grains after exercise in order to meet energy requirements and provide substrate for muscle glycogen replenishment. This study shows that ingestion of 3 different grains (cracked corn, steamed oat groats and rolled barley) at approximately equal amounts of starch plus sugar intake, results in similar magnitude of glycemic response (assessed as glucose area under the curve over time). In addition, the magnitude of the glycemic responses after ingestion of grains are approximately 60% of that after administration of an equivalent amount of glucose in the stomach. Characterization of grains commonly fed to horses according to their glycemic response may help to develop nutritional strategies intended to maximize glucose availability (in athletic horses for muscle glycogen synthesis) or when intending to minimize fluctuations in plasma glucose (as in clinical conditions suspected to be associated with reduceds insulin sensitivity in horses).

4.6. REFERENCES

1. Jenkins DJ, Wolever TM, Taylor RH, et al. Glycemic index of foods: a physiological basis for carbohydrate exchange. *Am J Clin Nutr* 1981;34:362-366.

2. Wolever TM. The glycemic index. *World Rev Nutr Diet* 1990;62:120-185.

3. National Research Council (U.S.). Subcommittee on Horse Nutrition. *Nutrient requirements of horses*. 5th rev. ed. Washington, D.C.: National Academy Press, 1989.

4. Murray SM, Flickinger EA, Patil AR, et al. In vitro fermentation characteristics of native and processed cereal grains and potato starch using ileal chyme from dogs. *J Anim Sci* 2001;79:435-444.

5. Borggreve GJ, van Kempen GJ, Cornelissen JP, et al. The net energy content of pig feeds according to the rostock formula the value of starch in the feed. *Z Tierphysiol Tierernahr Futtermittelkd* 1975;34:199-204.

6. Meyer H, Radicke S, Kienzle E, et al. Investigations on preileal digestion of starch from grain, potato and manioc in horses. *Zentralbl Veterinarmed A* 1995;42:371-381.

7. Meyer H, Radicke S, Kienzle E, et al. Investigations on preileal digestion of oats, corn, and barley starch in relation to grain processing. 13th Equine Nutrition and Physiology Symposium 1993;92-97.

8. Potter GD, Arnold FF, Householder DD, et al. Digestion of starch in the small or large intestine of the equine. *Pferdeheilkunde, Proceedings of the 1st European Conference of Horse Nutrition* 1992:107-111.

9. Lacombe VA, Hinchcliff KW, Geor RJ, et al. Muscle glycogen depletion and subsequent replenishment affect anaerobic capacity of horses. *J Appl Physiol* 2001;91:1782-1790.

10. Ivy JL, Katz AL, Cutler CL, et al. Muscle glycogen synthesis after exercise: effect of time of carbohydrate ingestion. *J Appl Physiol* 1988;64:1480-1485.

11. Henneke DR, Potter GD, Kreider JL, et al. Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet J* 1983;15:371-372.

12. Freestone JF, Shoemaker K, Bessin R, et al. Insulin and glucose response following oral glucose administration in well-conditioned ponies. *Equine Vet J Suppl* 1992:13-17.

13. Holm J, Björck I, Drews A, et al. A rapid method for the analysis of starch. *Starch/die*

starke 1986;7:224-226.

14. Dubois M, Gilles KA, Hamilton JK, et al. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;28:350-356.

15. Weiss WP. Symposium: prevailing concepts in energy utilization by ruminants. Predicting energy values of feeds. *J Dairy Sci* 1993;76:1802-1811.

16. Cupp C. How to determine energy in horse diets. *Feed Management* 2000;51:17-18.

17. Milliken GA, Johnson DE. *Analysis of messy data*. New York: Van Nostrand Reinhold, 1992.

18. Ludbrook J. On making multiple comparisons in clinical and experimental pharmacology and physiology. *Clin Exp Pharmacol Physiol* 1991;18:379-392.

19. Gibaldi M, Perrier D. *Pharmacokinetics*. 2nd rev. and expand ed. New York: M. Dekker, 1982.

20. Stull CL, Rodiek AV. Responses of blood glucose, insulin and cortisol concentrations to common equine diets. *J Nutr* 1988;118:206-213.

21. Ralston SL. Effect of soluble carbohydrate content of pelleted diets on postprandial glucose and insulin profiles in horses. *Pferdeheilkunde* 1992;8:112-115.

22. Pagan JD, Harris PA, Kennedy MAP, et al. Feed type and intake affects glycemic response in thoroughbred horses. 16th Equine Nutrition and Physiology Symposium 1999;149-150.

23. Williams CA, Kronfeld DS, Staniar WB, et al. Plasma glucose and insulin responses of Thoroughbred mares fed a meal high in starch and sugar or fat and fiber. *J Anim Sci* 2001;79:2196-2201.

24. Burke LM, Collier GR, Hargreaves M. Muscle glycogen storage after prolonged exercise: effect of the glycemic index of carbohydrate feedings. *J Appl Physiol* 1993;75:1019-1023.

25. Thomas DE, Brotherhood JR, Brand JC. Carbohydrate Feeding before Exercise - Effect of Glycemic Index. *International Journal of Sports Medicine* 1991;12:180-186.

26. Jenkins DJ, Kendall CW, Augustin LS, et al. Glycemic index: overview of implications in health and disease. *Am J Clin Nutr* 2002;76:266S-273S.

27. Hoffman RM, Boston RC, Stefanovski D, et al. Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. *J Anim Sci* 2003;81:2333-

2342.

28. Powell DM, Reedy SE, Sessions DR, et al. Effect of short-term exercise training on insulin sensitivity in obese and lean mares. *Equine Vet J Suppl* 2002:81-84.

29. Johnson PJ. Peripheral Cushingoid Syndrome ('Equine Metabolic Syndrome') In: Robinson NE, ed. *Current therapy in equine medicine*. 5th ed. Philadelphia, PA: W. B. Saunders, 2003;812-816.

30. De Fombelle A, Frumholtz P, Poillion D, et al. Effect of the botanical origin of starch on its prececal digestibility measured with the mobile bag technique. 17th Equine Nutrition and Physiology Symposium 2001;153-155.

31. Radicke S, Kienzle E, Meyer H. Preileal apparent digestibility of oats and corn starch and consequences for cecal metabolism. 12th Equine Nutrition and Physiology Symposium 1991;43-48.

32. Keinzle E, Pohlenz J, Radicke S. Morphology of starch digestion in the horse. *Zentralbl Veterinarmed A* 1997;44:207-221.

33. Hoekstra KE, Newman K, Kennedy MAP, et al. Effect of corn processing on glycemic response in horses. 16th Equine Nutrition and Physiology Symposium 1999;144-148.

CHAPTER 5

DOES ORAL GLUCOSE AVAILABILITY AFFECT GLUCOSE KINETICS AND MUSCLE GLYCOGEN REPLENISHMENT AFTER EXERCISE IN HORSES?

5.1 SUMMARY

Fatiguing exercise substantially decreases muscle glycogen concentration in horses, impairing athletic performance in subsequent exercise bouts. Feeding soluble carbohydrate rich meals may increase glucose uptake by skeletal muscle and hasten muscle glycogen replenishment, and as a consequence improve athletic performance. In this study, we hypothesized that ingestion of starch rich meals during the hours following exercise would result in enhanced whole body glucose availability to, and utilization by, peripheral tissues; and that increased glucose uptake by skeletal muscle would result in greater muscle glycogen replenishment after exercise. In a randomized, cross-over study, 7 trained adult Thoroughbred horses with exercise-induced muscle glycogen depletion were either not fed for 8 h (NF), fed half of the daily energy requirements (~15 Mcal DE) as mixed alfalfa and grass hay (H), or fed an isocaloric amount (~15 Mcal DE) of corn (C) immediately and 4 h after exercise. Plasma glucose, serum insulin and muscle

glycogen concentrations, as well as whole-body rates of glucose appearance and disappearance from blood were determined after exercise. Soluble carbohydrate-rich meals fed after exercise (C), when compared to feed withholding, resulted in mild to moderate hyperglycemia and hyperinsulinemia, 3-fold greater whole body availability and utilization of glucose, but these only minimally enhanced muscle glycogen replenishment. We conclude that after substantial exercise-induced muscle glycogen depletion, feeding status only minimally affects net muscle glycogen concentrations after exercise, despite marked differences in soluble carbohydrate ingestion and availability of glucose to skeletal muscle.

5.2. INTRODUCTION

High muscle glycogen concentrations prior to exercise enhance endurance exercise performance in humans and low muscle glycogen concentrations impair athletic performance [1-3]. Low muscle glycogen concentrations prior to exercise in horses decrease exercise performance at moderate and high-intensity exercise [4,5]. Feeding meals rich in soluble carbohydrate enhances muscle glycogen replenishment compared to isocaloric meals of low soluble carbohydrate content after exercise, but the magnitude of muscle glycogen replenishment is much less relative to that in humans or rodents fed high glycemic meals after exercise [6]. Whether this limited effect of feeding on muscle glycogen replenishment in horses is due to low systemic availability of glucose is unknown.

We hypothesized that ingestion of starch-rich meals during the hours following exercise would result in enhanced whole body glucose appearance and uptake, and

associated greater deposition as muscle glycogen. Therefore, the purpose of the present study was to determine the effect of glucose supply (postexercise ingestion of starch-rich meals vs. fiber-rich meals vs. withholding feed for 8 hours) on stable-isotope tracer-determined whole-body glucose rates of appearance and disappearance, and on muscle glycogen replenishment during the 8 hours following exercise. A further objective of this study was to determine the effects of different caloric intakes on muscle glycogen replenishment over the 24 hours after exercise.

5.3 MATERIALS AND METHODS

All animal experiments were conducted after approval by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University and were performed in compliance with their guidelines and recommendations.

Experimental Design. The effects of feeding isocaloric meals of varying starch and fiber content, or feed withholding, on whole body glucose kinetics, and muscle glycogen replenishment after exercise were examined in a semi-balanced, randomized, three-way crossover study. Seven horses were subjected to 3 consecutive days of strenuous treadmill exercise and feed restriction intended to lower muscle glycogen concentrations by at least 55% of the initial values (Fig 5.1) [4,7]. In each of three experimental conditions during the 8 hours after the 3rd consecutive day of exercise, horses were either: *1)* offered meals of cracked corn (C) (2.2 ± 0.2 kg/meal [average ± SD], ~7.4 Mcal DE/meal; grain trial); *2)* offered isocaloric meals of grass and alfalfa hay (H) (3.4 ± 0.4 kg/meal, ~7.4 Mcal DE/meal; hay trial); or *3)* not fed (NF). Whole body glucose kinetics were studied during and for 8 hours after exercise, and muscle glycogen concentrations were

determined over 24 hours after exercise. Trials were separated by 2 weeks, and the order of trials was randomized for individual horses. Due to musculoskeletal injuries, 3 out of 7 horses did not complete all 3 trials. Horses 1-6 completed the glycogen-depleting trials followed by corn feeding or feed withholding, and horses 1-4 and 7 completed the glycogen-depleting trials followed by hay feeding.

Horses. Seven Thoroughbred horses (5 mares and 2 geldings), 4–12 yr [7.3 ± 2.8 (SD) yr] and 405–513 kg [453 ± 38 (SD) kg], were housed indoors during the experimental period, and had unlimited access to a salt-mineral block and water. Horses were conditioned by regular treadmill exercise for at least 6 wk before the study. Horses were not exercised for 2 days prior to beginning glycogen-depleting exercise trials.

Preliminary Testing. For each horse, $V_{O_{2max}}$ and the relationship between rate of O_2 consumption (V_{O_2}) and speed were determined during an incremental exercise test 1 week before the first experiment. The incremental exercise test consisted of the horse running on a high-speed treadmill (Sato, Uppsala, Sweden) inclined at 4º for 90 s at 4 m/s; the treadmill speed was then increased by 1 m/s every 90 s until the horse was no longer able to maintain its position on the treadmill. V_{O_2} was measured every 10 s during the exercise test using an open-circuit calorimeter (Oxymax-XL, Columbus Instrument, Columbus, OH) [8]. The overall accuracy of the system for determination of V_{O_2} was verified for each run by the nitrogen-dilution method [9]. $V_{O_{2max}}$ was defined as the value at which V_{O_2} reached a plateau, despite further increases in speed. A plateau was defined as a change in V_{O_2} of <4 $ml \cdot kg^{-1} \cdot min^{-1}$ with an increase in speed. The running speed that elicited 70%, 90% and 100% of $V_{O_{2max}}$ were calculated for each horse using linear regression analysis of speeds below $\dot{V}_{O_{2max}}$.

Experimental Protocol. Before the third consecutive day of glycogen-depleting exercise, food was withheld for 18 hours. All experiments began at 0700 when horses were moved to stockades in a temperature-controlled building containing the high-speed treadmill. Catheters (14 gauge, 5.25 in.; Angiocath, Becton Dickinson) were inserted into the right and left jugular veins for isotope infusion and blood sample collection, respectively. A blood sample was obtained for subsequent determination of background isotopic enrichment. A primed (17.5 mmol/kg), continuous intravenous infusion of [6,6-^{2}H]glucose (99% enriched; Cambridge Isotopes, Cambridge, MA) in 0.9% saline was administered before, during and for 8 hours after exercise to allow determination of whole-body rates of glucose appearance and disappearance. Based on the results of pilot data and in order to minimize changes in plasma isotopic enrichment, the infusion rates of [6,6-^{2}H]glucose were as follows: after priming dose and during 90 min prior to exercise at 0.25 ± 0.01 mmol·kg^{-1}·min^{-1}, during exercise at 0.75 ± 0.01 mmol·kg^{-1}·min^{-1}, immediately after to 2 hours after exercise at 0.5 ± 0.01 mmol·kg^{-1}·min^{-1}, from 2 to 4 hours after exercise at 0.38 ± 0.01 mmol·kg^{-1}·min^{-1}, and from 4 to 8 hours after exercise at 0.25 ± 0.01 mmol·kg^{-1}·min^{-1}. After collection of blood for final baseline (pre-exercise) hormone, substrate, and glucose kinetic determinations, the horses were positioned on an inclined treadmill (4° incline) and performed 25 min of high-intensity exercise and, after 30-min rest, a series six 1-min sprints with 5 min of walking between each sprint [7]. Immediately after the final sprint, horses were placed in stockades and a sample of middle gluteal muscle was obtained by percutaneous biopsy. Thereafter, horses were moved to a stall where they had free access to water and received the glucose tracer infusion. At 15 min and 4 hours after exercise, horses were offered either a meal of

cracked corn (2.2 ± 0.2 kg [average ± SD] each meal, ~7.4 Mcal DE/meal), or an isocaloric meal of mixed grass and alfalfa hay (3.4 ± 0.4 kg each meal), or had feed withheld. At 8 hours after exercise all horses were given another half of the daily energy requirements in the form of mixed grass and alfalfa hay (6.9 ± 0.8 kg; ~14.8 Mcal DE). Experimental trials concluded 24 hours after the 3rd bout of glycogen-depleting exercise, when the last blood collection and percutaneous muscle biopsy were obtained.

Glycogen-Depleting Exercise Protocol. Horses were subjected to 3 consecutive days of exercise intended to deplete muscle glycogen stores by at least 55% of initial values, as previously described [7] (see Fig 5.1).

Diet Composition. Horses were fed a basal diet of mixed alfalfa and grass hay and cracked corn to maintain ideal body weight (condition score 5–6 out of 9). The diet consisted of 8.5 kg of mixed hay and 2.8 kg of corn daily for a 450-kg horse, fed in 2 equal portions. The amounts of hay and grain were given to meet energy requirements described by the NRC guidelines for horses in moderate to heavy intensity exercise [10]. The equation used for estimation of daily requirements of digestible energy (Mcal of DE/day) was:

$$DE = 1.75 \cdot (1.4 + 0.03 \cdot BW)$$

where DE is the daily requirement of digestible energy (Mcal/ day) and BW is the body weight (kg). Mixed hay and cracked corn were analyzed by a commercial laboratory (Holmes Laboratory, Millersburg, OH) (Table 5.1). During the three days of glycogen-depleting exercise, the horses were fed 8.5 kg of mixed hay per day, with estimated digestible energy of 17.5 Mcal/day in a 450-kg horse.

Sampling procedures. Time point 0 min was defined as the time immediately before starting the primed constant intravenous infusion of [6,6-^{2}H]glucose (before exercise on day 3). Blood samples were obtained in all trials immediately before the 1st of 3 consecutive days of glycogen-depleting exercise, before the 3rd consecutive bout of exercise (0, 75 and 90 min), while running at 90% of $V_{O_{2max}}$ (~120 min), at the end of 30 min of rest (150 min), at the end of six 1 min sprints at 100% of $V_{O_{2max}}$ (180 min), for 8 hours after exercise (195, 210, 240, 270, 300, 360, 420, 480, 540, 600, 660 min), and at 24 hours after the 3rd bout of exercise (Fig 5.1). Muscle biopsy samples were collected percutaneously at a depth of 6 cm from the middle gluteal muscle by needle biopsy immediately before the 1st of 3 consecutive days of exercise, within 10 min of the last sprint, and at 4 hours, 8 hours, and 24 hours after exercise. Muscle samples were immediately placed in liquid nitrogen and stored at −80°C until analysis.

Biochemical analyses.

Plasma glucose and lactate concentrations were measured as previously described [11]. Intra- and interassay coefficients of variation for measurement of plasma glucose and lactate were 1.0 and 2.5%, respectively. Serum immunoreactive insulin (IRI) was determined in duplicate by use of a commercially available RIA (Insulin kit, Coat-a-Count Diagnostics, Los Angeles, CA) that has been validated for horse blood [12]. Intra- and interassay coefficients of variation were ~6 and ~8%. To control for between-day variability, all samples for a given horse (feed-withholding, hay, and grain trials) were analyzed during the same analytic session. Muscle glycogen concentration was determined as previously described [13]. Intra- and interassay coefficients of variation were 11 and 15%, respectively.

Plasma isotopic enrichment and glucose kinetics calculations.

Plasma [6,6-^{2}H]glucose enrichment was determined as previously described [11]. The intra- and interassay coefficients of variation were 1.7 ± 0.6% and 2.4 ± 1.0%, respectively. Glucose rate of appearance (R_a) and rate of disappearance (R_d) at rest were calculated by using the steady-state tracer dilution equation [14]:

$$R_a = R_d = F \cdot \left[\left(IE_i / IE_p\right) - 1\right]$$

where F is the infusion rate of the isotope ($\mu mol \cdot kg^{-1} \cdot min^{-1}$), IE_i and IE_p are the stable isotopic enrichment of the infusate and plasma, respectively, and – 1 accounts for the tracer's contribution to the turnover rate of the substrate[14]. The rate of infusion was calculated by multiplying the infusion pump rate by the concentration of glucose in the infusate. During exercise, glucose R_a and R_d were calculated using the non-steady-state equation developed by Steele and modified for use with stable isotopes[15]. This equation is modified for use with stable isotopes as the amount of tracer infused is no longer negligible

$$R_a = \frac{F - V_d \dfrac{C_m}{1+E}\dfrac{dE}{dt}}{E}$$

and

$$R_d = R_a - V_d \frac{\dfrac{dC_m}{dt}\left(1+E\right) - C_m \dfrac{dE}{dt}}{\left(1+E\right)^2}$$

where V_d is the effective volume of distribution, E is the plasma isotopic enrichment, C_m is the measured plasma concentration of the tracee, and dE/dt and dC_m/dt are maximum rates of change in enrichment and glucose concentration, respectively, as a function of

time. With use of this fixed, one-compartment model of Steele, it is assumed that 1) the apparent glucose space is 25% of body weight and 2) 65% of this space represents the rapidly mixing portion of the glucose pool. Therefore, the effective V_d for glucose was assumed to be 162 ml/kg. Glucose metabolic clearance rate (MCR) was calculated by dividing glucose R_d by the plasma glucose concentration. Glucose R_a was assumed to represent glucose absorption from the gastrointestinal tract plus hepatic glucose production, although a small contribution from renal glycogenolysis and gluconeogenesis is possible [11,14-16].

Statistical analyses.

Values are means ± SE. The data for plasma glucose, serum IRI, plasma lactate and muscle glycogen concentrations as well as plasma isotopic enrichments and glucose R_a and R_d were analyzed using an ANOVA mixed model with repeated measures, with treatment (feed withholding, hay, and grain), time and the interaction of treatment and time as fixed effects. The null hypothesis was rejected at a $P < 0.05$ for the main effects (treatment and time) and a $P < 0.10$ for the interaction. Significant differences identified by ANOVA were isolated using the Dunn-Sidak post hoc test. The data for relative feed consumption, digestible energy and starch intake, and body weights were analyzed with one-way ANOVA for repeated measures. The null hypothesis was rejected at a $P < 0.05$ and significant differences identified by ANOVA were isolated using the Tukey post hoc test. The Sigmastat 2.0[1] and SPSS 12.0[2] software packages were used for statistical computations.

5.4 RESULTS

Individual values for $V_{O_{2max}}$ ranged from 128 to 186 ml·kg^{-1}·min^{-1} (mean 150 ± 7 ml·kg^{-1}·min^{-1}). Mean running speeds were: 7.4 ± 0.1 m/s (range 7.0-7.8 m/s) at 70% $V_{O_{2max}}$, 9.6 ± 0.2 m/s (range 9.0-10.5 m/s) at 90% $V_{O_{2max}}$, and 10.8 ± 0.1 m/s (range 10.0-11.0 m/s) at 100% $V_{O_{2max}}$. Body weights prior to glycogen-depleting exercise, mean body weight loss over the 3 consecutive days of exercise, ambient temperature and humidity, and rectal temperatures after exercise were not significantly different among the three trials (data not shown).

Feed consumption. The amount of digestible energy (DE) provided was not different between hay and corn-fed horses (7.2 ± 0.4 and 7.3 ± 0.2 Mcal DE, respectively, P = 0.8). There was no significant statistical difference between hay-fed and corn-fed horses in the amount of DE ingested (9.9 ± 1.0 and 13.3 ± 0.8 Mcal DE total over 8 h, respectively, P = 0.13). At 8 h following exercise all horses were fed half of the daily energy requirements as mixed hay, therefore in feed-withholding trials caloric intake over the 24 h following exercise was approximately half that in feeding trials (14.7 ± 0.4, 24.3 ± 1.6 and 28.0 ± 0.8 Mcal DE total over 24 h for NF, H and C trials, respectively, P < 0.001). The estimated amount of starch ingested over 8 h following exercise in corn-fed horses was 14-fold higher than that in hay-fed horses (5.3 ± 0.4 and 0.38 ± 0.03 g starch/kg body weight, respectively, P = 0.002).

Plasma glucose concentration. Feeding type significantly affected plasma glucose concentrations during the 8 h following exercise (grain different from feed withholding, P < 0.001; hay different from feed withholding, P = 0.03; and corn had a tendency to be different from hay, P = 0.07) (Fig 5.2A). During the 8 h period following exercise, when

the horses were allowed to eat two grain meals equivalent to one-quarter of the daily energy requirements per meal, plasma glucose concentration steadily increased from 4.8 ± 0.2 mM at 15-min after exercise to 5.7 ± 0.3 mM at 3 h after exercise in corn-fed horses and remained elevated for the following 5 hours. In contrast, plasma glucose concentration remained essentially unchanged during the 8 hours following exercise in feed-withholding trials. Plasma glucose concentrations were higher (P<0.01) in corn-fed horses than in feed-withholding trials during the period from 2 to 8 hours following exercise.

Serum immunoreactive insulin concentration. Feeding status significantly altered serum immunoreactive insulin (IRI) concentrations after exercise (corn different from feed withholding, $P < 0.001$; hay different from feed withholding $P = 0.05$) (Fig 5.2B). Serum IRI concentrations increased after exercise in corn-fed horses to reach a peak of 79.9 ± 9.3 pM by 2 hours after exercise and remained elevated during the following 6 hours. In contrast, serum IRI concentrations in feed-withholding trials remained essentially unchanged from prior to exercise, over the 8 hours following exercise. Serum IRI concentrations at 2 hours following exercise were higher in corn-fed when compared to unfed horses ($P = 0.001$) and to hay-fed horses ($P = 0.03$).

Glucose kinetics. Feeding type significantly altered plasma glucose isotopic enrichment, glucose R_a and glucose R_d following exercise (all 3 treatments different from each other, P <0.01). Before exercise, plasma isotopic enrichment (Fig 5.3) was similar among all 3 treatments (0.024 to 0.027 ± 0.001). Despite tripling the [6,6-^{2}H]-glucose infusion rate at the onset of exercise, plasma isotopic enrichment decreased to 0.020 ± 0.001 at the end of running at 90% $V_{O_{2max}}$ in all 3 treatments. From 1 to 8 hours following exercise as the

[6,6-^{2}H]-glucose infusion rate was gradually decreased, plasma glucose isotopic enrichment also decreased from ~0.033 ± 0.002 at 1 h following exercise to 0.017 ± 0.001 and 0.025 ± 0.001 at 8 hours following exercise in corn-fed and hay-fed horses, respectively. In contrast, during withholding trials plasma isotopic enrichment steadily increased from 0.036 ± 0.001 at 1 hours to 0.053 ± 0.002 at 4 h after exercise, and remained approximately stable for the next 4 hours. Plasma glucose isotopic enrichment was higher in feed-withholding trials than that in corn-fed and hay fed trials from 1.5 to 8 hours following exercise ($P < 0.01$).

At rest, glucose R_a and R_d (Fig 5.4) were similar among all 3 treatments. While running at 90% V_{O2max} glucose R_a and R_d were similar in all trials and sharply increased by ~5 fold when compared to those at rest ($P < 0.001$). During the first 4 hours following exercise, glucose R_a and R_d remained stable or increased in corn-fed and hay-fed horses; in contrast, glucose R_a and R_d in feed withholding trials gradually decreased and were lower than those in other trials (Fig 5.4). From 4 to 8 hours following exercise, glucose R_a and R_d continued to decrease gradually in hay-fed horses and feed withholding trials whereas glucose R_a and R_d gradually increased in corn-fed horses ($P < 0.05$).

Muscle glycogen concentration. Three days of strenuous treadmill exercise resulted in marked muscle glycogen depletion ($P < 0.001$) in all trials (Table 5.2). Muscle glycogen concentration at 24 hours was higher than that at 0 h in grain-fed horses ($P = 0.04$) whereas no differences were observed in the other trials.

Plasma lactate. Feeding type did not alter plasma lactate concentration after exercise (data not shown).

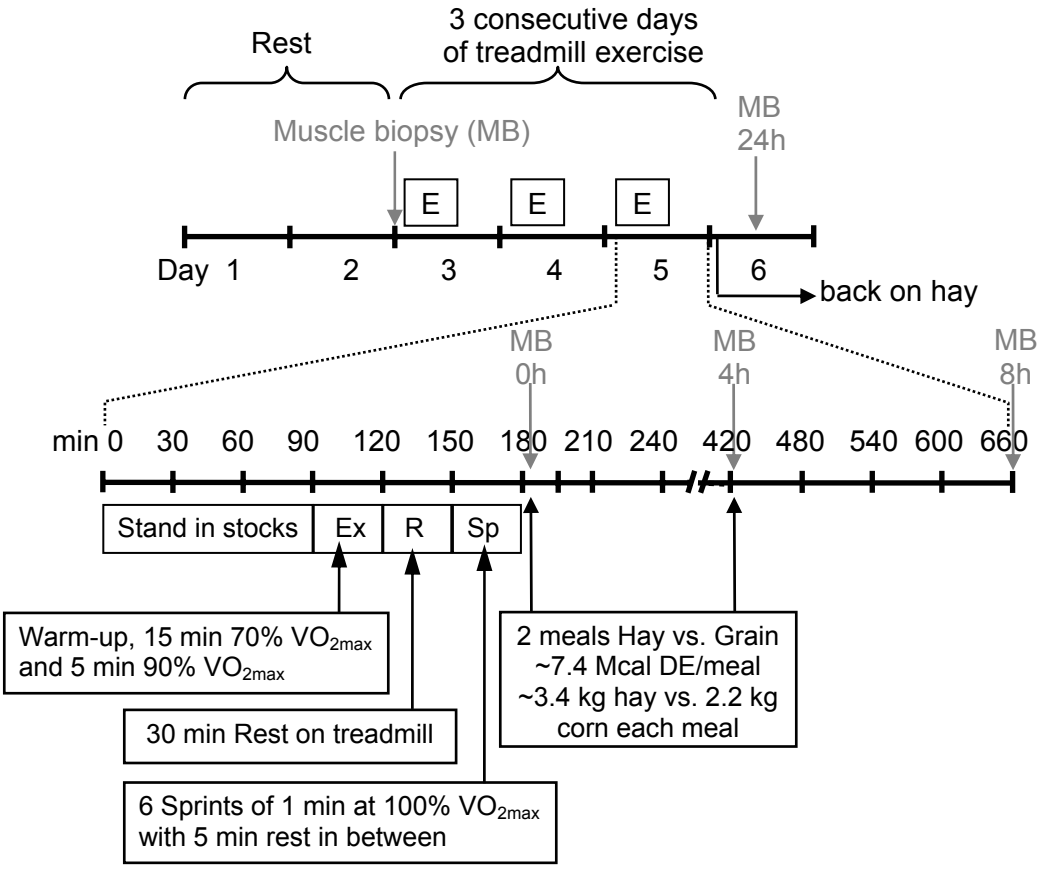

Figure 5.1. Timeline of the experimental protocol. MB, muscle biopsy.

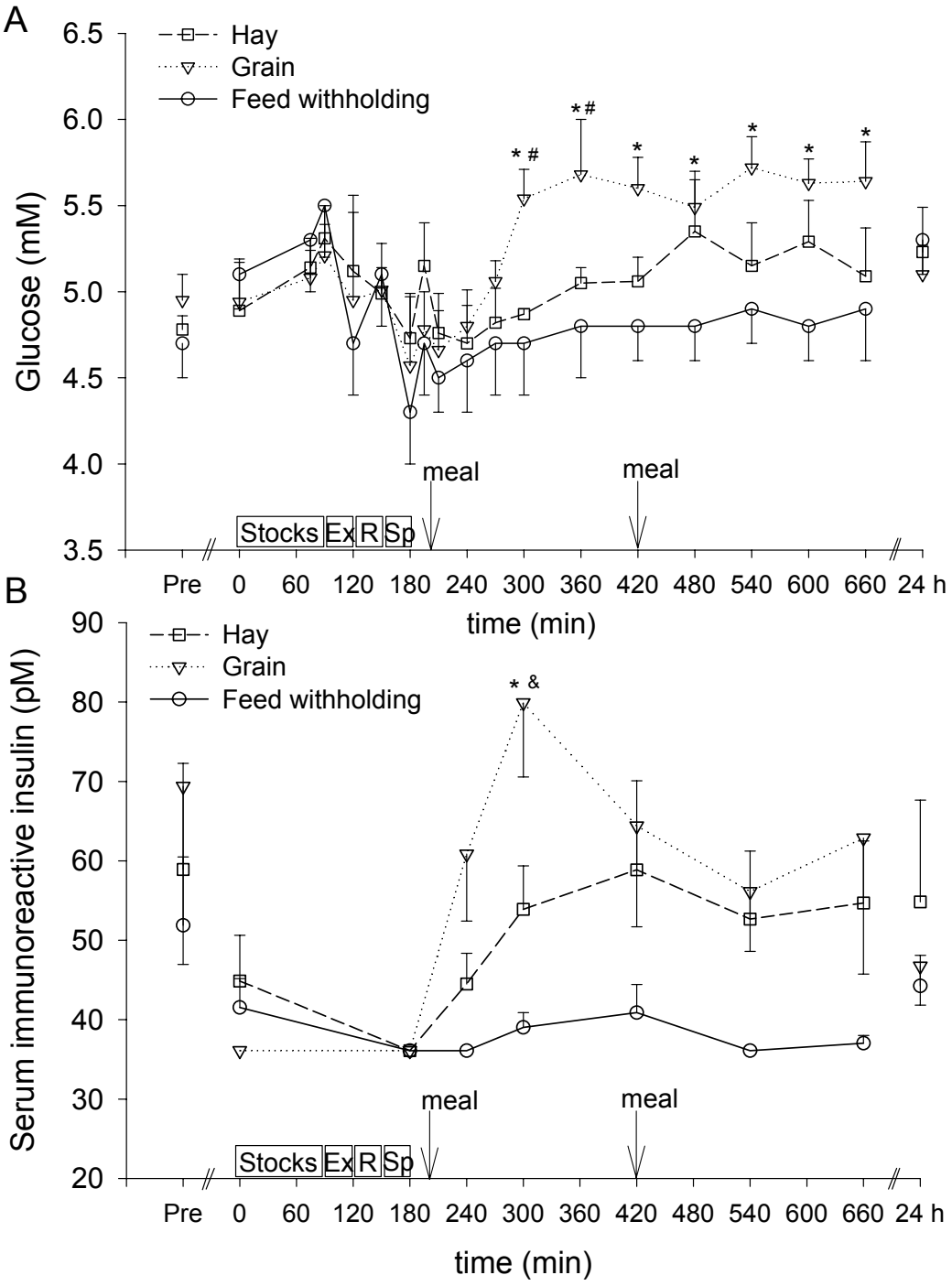

Figure 5.2. Plasma glucose (A) and serum immunoreactive insulin (B) at rest, during and after 3rd bout of glycogen-depleting exercise in horses that had feed withheld (n = 6), were fed hay (n = 5), or fed grain (n = 6) immediately and 4 h after exercise. Values are means ± SE. Ex; 5 min warm-up, 15 min at 70% $V_{O_{2max}}$ and 5 min at 90% $V_{O_{2max}}$; R, 30 min rest; Sp; six 1 min sprints. * Grain different than feed withholding ($P < 0.01$); # Hay different than feed withholding ($P < 0.05$), & Grain different than hay ($P < 0.05$).

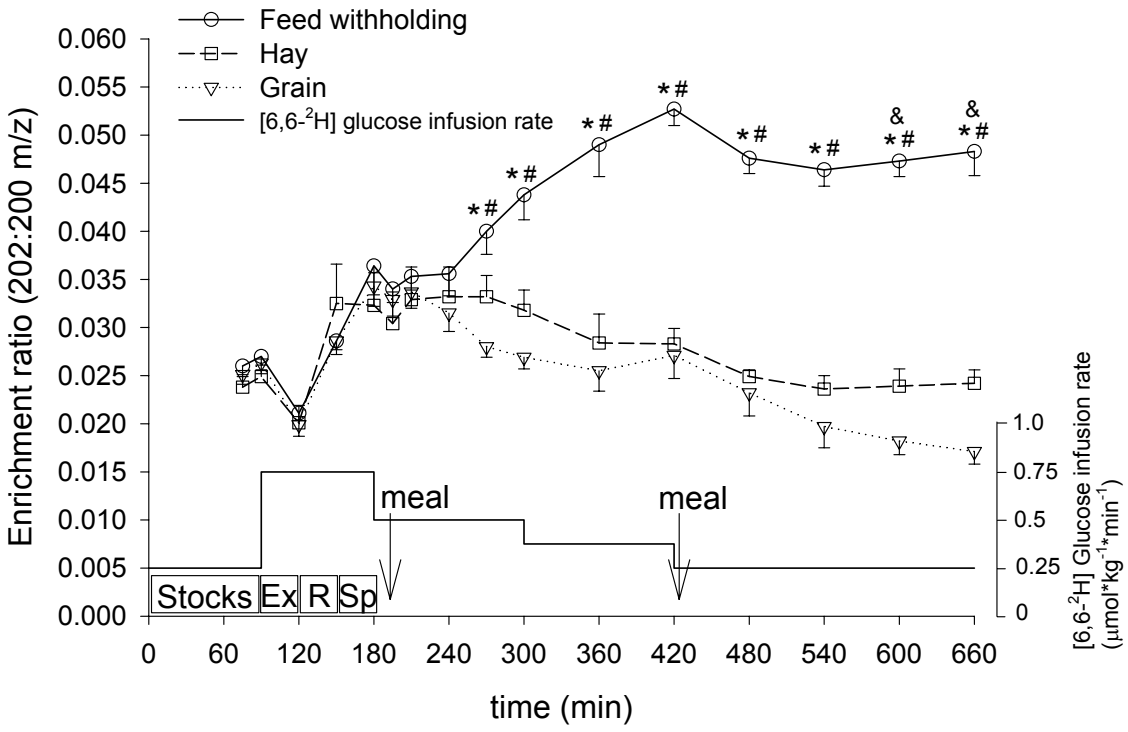

Figure 5.3. Plasma glucose isotopic enrichment. 202/200; Mass-to-charge ratio of labeled ([6,6-^{2}H]glucose) and unlabeled (glucose) ions, respectively. See Figure 2 legend for further details. * Grain different than feed withholding ($P < 0.001$); # Hay different than feed withholding ($P < 0.001$); & Grain different than hay ($P < 0.05$).

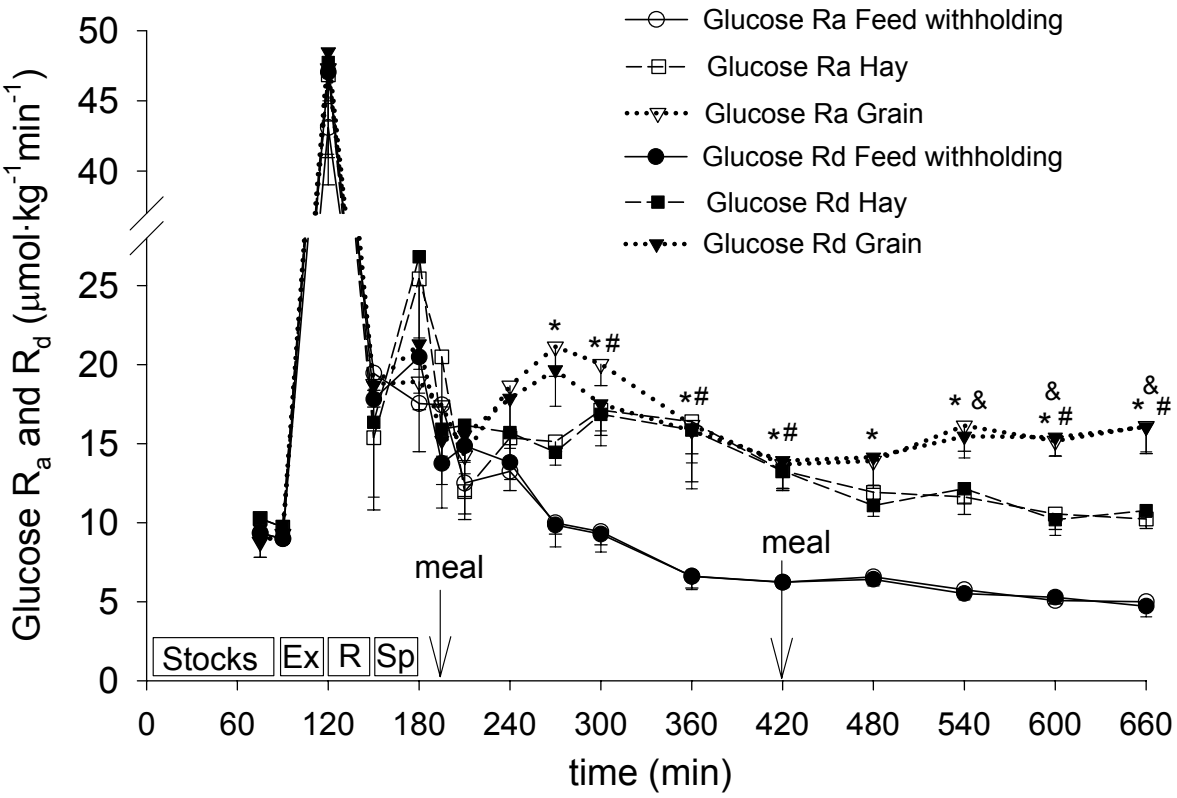

Figure 5.4. Glucose rate of appearance (R_a) and disappearance (R_d). See Figure 5.2 legend for further details. * Grain different than feed withholding ($P < 0.05$); # Hay different than feed withholding ($P < 0.05$); & Grain different than hay ($P < 0.05$).

	Mixed Hay	Cracked corn
Dry matter, %	87.3	85.9
Crude protein, %	16.2 (18.5)	7.2 (8.3)
ADF, %	34.4 (39.4)	5.0 (5.9)
NDF, %	40.3 (46.2)	9.6 (11.2)
Lignin, %	5.27 (6.04)	0.4 (0.5)
Starch, %	3.69 (4.23)*	60.5 (70.3)
Crude fat, %	2.36 (2.70)	3.7 (4.3)
Ash, %	5.74 (6.58)	1.5 (1.7)
Calcium, %	1.12 (1.28)	0.02 (0.02)
Phosphorus, %	0.23 (0.26)	0.21 (0.25)
DE, Mcal/kg feed	2.09 (2.40)	3.32 (3.87)

Table 5.1. Nutritional analysis of mixed grass and alfalfa hay and cracked corn. Values are as sampled and, in parentheses, on a dry matter basis. ADF, acid detergent fiber; NDF, neutral detergent fiber; DE, digestible energy. *Starch content of hay was estimated.

	Time				
	Before	0 h	4 h	8 h	24 h
Feed withholding (n=6)	499 ± 32†	171 ± 19	231 ± 48	222 ± 32	260 ± 45
Hay (n=5)	556 ± 35†	205 ± 37	265 ± 41	237 ± 28	231 ± 21
Grain (n=6)	563 ± 53†	170 ± 56	226 ± 60	247 ± 23	294 ± 29‡

Table 5.2. Muscle glycogen concentrations ($mmol \cdot kg^{-1}$ dw) before the glycogen-depleting exercise protocol, and after the 3rd consecutive day of exercise.
† $P<0.001$ muscle glycogen concentration before is higher than all other time points after exercise in all trials.
‡ $P=0.04$ muscle glycogen concentration at 24 h is higher than that at 0 h in grain-fed horses.

5.5 DISCUSSION

The main findings of this study were: 1) a ~20% increase in plasma glucose concentrations after exercise in corn-fed horses compared to unfed horses; 2) 2-3 fold higher glucose turnover rates after exercise in fed horses compared to unfed horses; 3) glucose turnover rates substantially lower than corresponding rates in humans or dogs after exercise and carbohydrate feeding; and 4) minimal effect of oral glucose availability on muscle glycogen concentrations after exercise.

Glycogen replenishment after exercise in humans is maximized when carbohydrate supplements are ingested in excess of 1 g/kg BW immediately after exercise and every 2 h for up to 6 h [17]. In contrast, oral administration of glucose or glucose polymers at 2-3 g/kg immediately after exercise in horses does not result in enhanced muscle glycogen replenishment [18,19]. In horses, only intravenous administration of large amounts of glucose (6 g/kg over 8-12 h) significantly enhances muscle glycogen replenishment (from depletion by ~2/3 of basal muscle glycogen concentrations after exercise to complete repletion in 24 h)[4,20]. Furthermore, when feeding conventional diets, replenishment of muscle glycogen stores requires up to 72 h after exercise in horses [6,21]. In the present study, ingestion of carbohydrate as starch (5.3 ± 0.4 g/kg over 8 h) in amounts similar to previous studies using intravenous glucose administration [4,20] resulted in muscle glycogen concentrations similar to those in unfed horses by 8 h after exercise, and only slightly higher by 24 h after exercise (Table 5.2). From the results of this and previous studies [4,6,20,21] one could speculate that an inherent lower ability to digest starch and other sources of glucose in the small intestine, relative to that in humans and rodents, may result in slower muscle glycogen replenishment due to lack of substrate availability.

This contention is supported by the following comparison between horses and humans. In humans, ingestion of glucose or sucrose solutions within 20-30 min after exercise (0.8-1 g/kg) results in an increase in glucose R_d from 22-30 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ immediately after exercise to 60-90 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ at 1-2 h after exercise [22,23]. In comparison, horses in this study ingested 2.7 g/kg of starch as cracked corn after exercise (0.9 g/kg of glucose if small intestinal corn starch digestibility is 30%) and glucose R_d only increased from 15 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ immediately after exercise to 20 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ by 1.5 h after exercise. Therefore, despite a 3-fold difference in systemic glucose delivery between unfed and corn-fed horses after exercise, the magnitude of increased systemic glucose availability after feeding was less than that in other species and the resulting deposition as muscle glycogen was small.

In the present study, feeding starch rich meals resulted in increased glucose availability but the magnitude of the increase was not sufficiently high to result in an accompanying substantial increase in muscle glycogen concentration. The cumulative glucose R_d over the 8 h following exercise were 3.7 ± 0.1, 6.4 ± 0.3 and 7.8 ± 0.3 mmol/kg ($P < 0.01$) in unfed, hay-fed and corn-fed horses, respectively (in a 450-kg horse, ~1.7, ~2.9 and ~3.5 mols of glucose in unfed, hay-fed and corn-fed horses, respectively). If all glucose R_d were directed to deposition as muscle glycogen and blood-borne glucose was the sole substrate for muscle glycogen synthesis, muscle glycogen concentrations would increase from ~170 mmol/kg dw immediately after exercise to ~201 and ~233 mmol/kg dw at 8 h after exercise in unfed and corn-fed horses, respectively, which is similar to measured glycogen concentrations (Table 5.2).

Estimations are based on skeletal muscle being 50% of body weight [24] and all muscle groups having similar exercise-induced muscle glycogen depletion and post-exercise repletion.

The above estimates of maximal amounts of glucose available for muscle glycogen synthesis are in accordance with estimates based on corn ingestion, starch content in corn and small intestinal corn starch digestibility in horses. As an example, a 450-kg horse fed corn in the present study would have absorbed a total of ~800 to 2130 g of glucose (4.4 to 11.8 mols) depending on corn starch digestibility (estimations based on 2 meals of 2.2 kg each; starch content of 60.5% in corn [Table 5.1]; and small intestinal digestibility of corn starch of 30 to 80% [25,26]). If we assume that glucose was preferentially used for liver glycogen synthesis after exercise, a 450-kg horse in the present study with near complete liver glycogen depletion after 3 consecutive days of exercise would have required ~130 g of glucose (~0.7 mols) to replenish liver glycogen to resting values (estimations based on resting liver glycogen concentration being 120 mmols/kg ww [27] and liver weight being 1.3% of body weight [28]). Therefore, even if glucose was initially used exclusively for liver glycogen repletion there would still be ~670 to 2000 g of glucose (3.7 to 11.1 mols) available for muscle glycogen synthesis. In comparison, the amount of glucose required to completely replenish muscle glycogen would be ~2080 to 2700 g (11.6 to 15 mols) in a 450-kg (estimations based on resting muscle glycogen concentration of 120 mmol/kg ww [27], overall muscle glycogen depletion in skeletal muscles of 60% and skeletal muscle weight being 50% of body weight [24]). These crude estimations underscore the fact that quantitatively the greatest storage of carbohydrate is in the form of muscle glycogen, and accurate estimation of the ultimate

fate of glucose (oxidation vs. storage as carbohydrate or fat) would require simultaneous administration of multiple metabolic tracers and sampling of plasma and respiratory gases.

Constant infusion rates of [6,6-^{2}H] glucose achieved the aim of obtaining adequate and approximately stable plasma enrichment ratios (Fig 5.3). Estimation of glucose kinetics when using a one-compartment fixed volume model is more accurate during non-steady state conditions if fluctuations in plasma isotopic enrichment are minimized by adjusting the tracer infusion rates [29]. The present [6,6-^{2}H]glucose tracer infusion protocol at least partially achieved the goal of minimizing fluctuations of plasma isotopic enrichment.

Glucose turnover rates prior to exercise were similar to those previously reported by our laboratory [11,16] using the same isotopically labeled glucose tracer ([6,6-^{2}H]glucose). During exercise at 90% $V_{O_{2max}}$, glucose Ra and Rd were ~5-fold higher than prior to exercise and similar to those observed at lower exercise intensities (50% $V_{O_{2max}}$) [11,16]. In this study, the increase in plasma glucose concentration induced by corn-feeding after exercise was accompanied by increments in glucose R_a and R_d, when compared to that in withholding feed. Glucose turnover rates after exercise in hay-fed horses were similar to those seen prior to exercise (Fig 5.4). In contrast, corn-fed horses had higher, and fasted horses had lower, glucose turnover rates than those observed prior to exercise.

Developing nutritional strategies that optimize muscle glycogen synthesis after exercise in horses is particularly relevant in horses because of their relatively slow rate of muscle glycogen synthesis [30]. Muscle glycogen replenishment after exercise in humans is hastened within several hours after exercise by carbohydrate supplementation as glucose

drinks [17] and ingestion of high-glycemic index meals [31]. In the present study muscle glycogen replenishment over 24 h following exercise was, from a functional standpoint, essentially the same in all 3 trials despite ingestion of markedly different amounts of starch over the 8 h following exercise, and markedly different caloric intake over the 24 h following exercise. The ultimate fate of glucose (oxidation vs. synthesis of liver or muscle glycogen vs. substrate for triglyceride synthesis) cannot be determined by infusion of [6,6-^{2}H] glucose as a metabolic tracer. Previous studies in horses have shown that one bout of prolonged low to moderate intensity exercise results in 40-80% liver glycogen depletion [27,32]. In the present study we did not measure the magnitude of liver glycogen depletion or the contribution of gut derived glucose R_a (intestinal absorption of glucose) to the total glucose R_a. However, it is reasonable to assume that 3 consecutive days of strenuous exercise and a starch-poor fiber-rich diet resulted in near complete liver glycogen depletion. In that case glucose R_a in feed withholding trials should be an adequate estimate of hepatic gluconeogenesis (~5 $\mu mol \cdot kg^{-1} \cdot min^{-1}$), and the difference between glucose R_a in corn-fed and unfed horses should be a crude estimate of the minimal contribution of gut derived glucose R_a (~11 $\mu mol \cdot kg^{-1} \cdot min^{-1}$).

In summary, this study has demonstrated that feeding soluble carbohydrate rich meals after glycogen-depleting exercise augments availability and utilization of blood-borne glucose in horses but not to an extent that substantially hastens muscle glycogen replenishment in contrast to parenteral administration of similar amounts of carbohydrate. Conversely, feeding of an isocaloric starch-poor fiber-rich meal of roughage after exercise or feed withholding for 8 h, compared with a starch-rich meal, does not significantly impair muscle glycogen replenishment despite lower availability of

substrate to skeletal muscle. Further investigations are warranted to better understand the underlying mechanisms responsible for the relatively slower muscle glycogen synthesis rate after exercise in horses when compared to other species.

5.6 REFERENCES

1. Hawley JA, Schabort EJ, Noakes TD, et al. Carbohydrate-loading and exercise performance. An update. *Sports Med* 1997;24:73-81.

2. Jacobs KA, Sherman WM. The efficacy of carbohydrate supplementation and chronic high- carbohydrate diets for improving endurance performance. *International Journal of Sport Nutrition* 1999;9:92-115.

3. Bergström J, Hermansen L, Hultman E, et al. Diet, muscle glycogen and physical performance. *Acta Physiol Scand* 1967;71:140-150.

4. Lacombe VA, Hinchcliff KW, Geor RJ, et al. Muscle glycogen depletion and subsequent replenishment affect anaerobic capacity of horses. *J Appl Physiol* 2001;91:1782-1790.

5. Topliff DR, Potter GD, Dutson TR, et al. Diet manipulation and muscle glycogen in the equine. 8th Equine Nutrition and Physiology Symposium 1983;119-124.

6. Lacombe VA, Hinchcliff KW, Kohn CW, et al. Post-exercise feeding of meals of varying glycemic index affects muscle glycogen resynthesis in horses. *Am J Vet Res* 2004;in press.

7. Lacombe V, Hinchcliff KW, Geor RJ, et al. Exercise that induces substantial muscle glycogen depletion impairs subsequent anaerobic capacity. *Equine Vet J Suppl* 1999;30:293-297.

8. Hinchcliff KW, McKeever KH, Muir WW, 3rd, et al. Effect of furosemide and weight carriage on energetic responses of horses to incremental exertion. *Am J Vet Res* 1993;54:1500-1504.

9. Fedak MA, Rome L, Seeherman HJ. One-Step N2-Dilution Technique for Calibrating Open-Circuit Vo2 Measuring Systems. *Journal of Applied Physiology* 1981;51:772-776.

10. National Research Council (U.S.). Subcommittee on Horse Nutrition. *Nutrient requirements of horses*. 5th rev. ed. Washington, D.C.: National Academy Press, 1989.

11. Geor RJ, Hinchcliff KW, Sams RA. Glucose infusion attenuates endogenous glucose production and enhances glucose use of horses during exercise. *J Appl Physiol* 2000;88:1765-1776.

12. Freestone JF, Shoemaker K, Bessin R, et al. Insulin and glucose response following oral glucose administration in well-conditioned ponies. *Equine Vet J Suppl* 1992:13-17.

13. Passonneau JV, Lauderdale VR. A comparison of three methods of glycogen

measurement in tissues. *Anal Biochem* 1974;60:405-412.

14. Wolfe RR. *Radioactive and stable isotope tracers in biomedicine : principles and practice of kinetic analysis*. New York: Wiley-Liss, 1992.

15. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci* 1959;82:420-430.

16. Jose-Cunilleras E, Hinchcliff KW, Sams RA, et al. Glycemic index of a meal fed before exercise alters substrate use and glucose flux in exercising horses. *J Appl Physiol* 2002;92:117-128.

17. Ivy JL. Glycogen resynthesis after exercise: effect of carbohydrate intake. *Int J Sports Med* 1998;19 Suppl 2:S142-145.

18. Nout YS, Hinchcliff KW, Jose-Cunilleras E, et al. Effect of moderate exercise immediately followed by induced hyperglycemia on gene expression and content of the glucose transporter-4 protein in skeletal muscles of horses. *Am J Vet Res* 2003;64:1401-1408.

19. Davie AJ, Evans DL, Hodgson DR, et al. The effects of an oral glucose polymer on muscle glycogen resynthesis in standardbred horses. *J Nutr* 1994;124:2740S-2741S.

20. Davie AJ, Evans DL, Hodgson DR, et al. Effects of intravenous dextrose infusion on muscle glycogen resynthesis after intense exercise. *Equine Veterinary Journal* 1995;18:195-198.

21. Snow DH, Harris RC, Harman JC, et al. Glycogen repletion following different diets. Equine Exercise Physiology 2 1987;701-706.

22. Bowtell JL, Gelly K, Jackman ML, et al. Effect of different carbohydrate drinks on whole body carbohydrate storage after exhaustive exercise. *J Appl Physiol* 2000;88:1529-1536.

23. Rose AJ, Howlett K, King DS, et al. Effect of prior exercise on glucose metabolism in trained men. *Am J Physiol Endocrinol Metab* 2001;281:E766-771.

24. Gunn HM. Muscle, bone and fat proportions and muscle distribution of thoroughbreds and other horses. Equine Exercise Physiology 2 1987;253-264.

25. Potter GD, Arnold FF, Householder DD, et al. Digestion of starch in the small or large intestine of the equine. *Pferdeheilkunde, Proceedings of the 1st European Conference of Horse Nutrition* 1992:107-111.

26. Meyer H, Radicke S, Kienzle E, et al. Investigations on preileal digestion of oats, corn, and barley starch in relation to grain processing. 13th Equine Nutrition and

Physiology Symposium 1993;92-97.

27. Lindholm A, Bjerneld H, Saltin B. Glycogen depletion pattern in muscle fibres of trotting horses. *Acta Physiol Scand* 1974;90:475-484.

28. Webb AI, Weaver BM. Body composition of the horse. *Equine Vet J* 1979;11:39-47.

29. Gastaldelli A, Coggan AR, Wolfe RR. Assessment of methods for improving tracer estimation of non-steady-state rate of appearance. *J Appl Physiol* 1999;87:1813-1822.

30. Lacombe VA, Hinchcliff KW, Taylor LE. Interactions of substrate availability, exercise performance, and nutrition with muscle glycogen metabolism in horses. *Journal of the American Veterinary Medical Association* 2003;223:1576-1585.

31. Burke LM, Collier GR, Hargreaves M. Muscle glycogen storage after prolonged exercise: effect of the glycemic index of carbohydrate feedings. *J Appl Physiol* 1993;75:1019-1023.

32. Pagan JD, Essén-Gustavsson B, Lindholm A, et al. The effect of dietary energy source on exercise performance in Standardbred horses. Equine Exercise Physiology 2 1987;686-700.

CHAPTER 6

EXPRESSION OF EQUINE GLUCOSE TRANSPORTER TYPE-4 IN SKELETAL MUSCLE INCREASES AFTER GLYCOGEN DEPLETING EXERCISE INDEPENDENTLY OF MEAL TYPE AFTER EXERCISE.

6.1 SUMMARY

The aim of this study was to clone and sequence the cDNA for equine insulin-responsive glucose transporter (glucose transporter type-4 [GLUT-4]); and determine the effects of glycogen-depleting exercise and of meal type after exercise on GLUT4 gene expression in skeletal muscle biopsies from 7 healthy adult Thoroughbred horses. Total RNA was extracted from skeletal muscle biopsies, and GLUT-4 cDNA was synthesized and sequenced. Horses were exercised on 3 consecutive days in order to decrease muscle glycogen concentration by ~66%. In the 3rd day of exercise, during 8 hours following exercise in a randomized cross-over design, horses were either not fed (NF), fed half of the daily energy requirements as hay (H) or fed an isocaloric amount of corn (C). GLUT4 mRNA was determined using real-time RT-PCR in muscle biopsies obtained before 3 consecutive days of exercise and within 15 min, 4 h, 8 h, and 24 h after the 3rd exercise

bout. A total of 1629 bp were sequenced, of which 1530 bp corresponded to the coding region, encoding a protein of 509 amino acids (NCBI GenBank no. AF531753). GLUT 4 gene expression in muscle increased by ~2.3, ~4.3, ~3.3, and ~2.6 fold 10 min, 4 h, 8 h and 24 h after exercise, respectively, when compared to that prior to exercise. No differences were observed in the level of GLUT4 gene expression in muscle when comparing NF, C or H feeding during 8 h post-exercise. We conclude that lack of increased of GLUT4 gene expression after grain feeding and exercise may at least partly explain the apparently slower rate of glycogen synthesis after exercise in horses relative to that of other species.

6.2. INTRODUCTION

During exercise, the two primary substrates available for energy production in working skeletal muscle of mammals are carbohydrates (muscle glycogen, and blood glucose) and fats (fatty acids from adipose tissue and intramuscular triglycerides)[1]. Restoration of skeletal muscle glycogen stores is critical for exercise performance in human athletes who undertake multiple exercise bouts on the same or successive days[2]. Similarly, low muscle glycogen concentrations prior to exercise in horses lead to decreased exercise performance[3]. Muscle glycogen synthesis is catalyzed by the enzyme glycogen synthase. Availability of the substrate (glucose) rather than glycogen synthase activity appears to be the rate-limiting factor for muscle glycogen synthesis[4,5]. Stimulation of glucose transport into cells by insulin and/or muscular contraction is mediated by translocation of the insulin-regulated glucose transporter (glucose transporter type 4 [GLUT-4]) from intracellular sites into the plasma membrane[5]. GLUT-

4 is of particular importance in glucose homeostasis because it mediates insulin-mediated glucose uptake in skeletal muscle, the main site of glucose disposal, and in adipose tissue[6]. GLUT-4 is expressed in skeletal and cardiac muscle and white and brown adipose tissues[7]. Gene expression of GLUT-4 increases in skeletal muscle after exercise as an early adaptation that facilitates replenishment of the muscle glycogen stores (Ren 1994). Therefore, GLUT-4 gene expression, GLUT-4 protein content and translocation of GLUT-4 protein to the cellular membrane are key events in glucose homeostasis at rest and during exercise.

The complementary DNA (cDNA) nucleotide sequence of GLUT-4 messenger RNA (mRNA) has been reported in man[8], mice[9], rats[10], cattle[11], rabbits[12] and partially in dogs[13], pigs[14], sheep and goats[15], and water buffalo[16]. Recent studies performed in horses have demonstrated that exercise bouts and training results in increased GLUT-4 protein content in skeletal muscle[17,18]; however, to date, the effect of exercise and diet on equine GLUT-4 mRNA expression has been difficult to quantify in part because the nucleotide sequence was unknown.

The purpose of the present study was to determine the effect of exercise that substantially depletes muscle glycogen and of type of meal after exercise on GLUT-4 gene expression in equine skeletal muscle. As a necessary preliminary step for mRNA quantitation by real-time RT-PCR, the equine GLUT-4 gene was cloned and sequenced. We hypothesized that exercise would increase GLUT-4 gene expression in equine skeletal muscle, and starch-rich meals would further enhance GLUT-4 gene expression when compared to isocaloric meals of roughage or to feed withholding.

6.3. MATERIALS AND METHODS

Animals.

In the first portion of the study samples of semitendinosus or semimembranosus muscles were obtained by cut-down incisions from 4 horses, and spleen samples from one of them. Horses had been donated to The Ohio State University and were euthanized due to chronic musculoskeletal disease. Samples were collected within 20 minutes after euthanasia, immediately frozen in liquid nitrogen and stored at −80ºC until subsequent analysis. Skeletal muscle from hindlegs of mice also was harvested similarly within 10 min of euthanasia, as a positive control during the PCR assays intended to clone and sequence equine GLUT-4. In the second portion of the study, 7 horses were used to determine the effects of exercise and feed withholding or isocaloric corn or hay meals on GLUT-4 mRNA quantitation in skeletal muscle. All animal experiments were conducted after approval by the Institutional Laboratory Animal Care and Use Committee of The Ohio State University and were performed in compliance with their guidelines and recommendations.

Extraction of total RNA

Total RNA was isolated from tissues using the acid guanidium thiocyanate-phenol-chloroform extraction method[19] (RNAwiz, Ambion, Austin, TX), and reconstituted in nuclease-free water. Any possible contaminating DNA was removed at this point by treating the sample with DNase I (DNAfree, DNase treatment and removal reagents, Ambion, Austin, TX). RNA was quantitated by UV spectrophotometry and evaluated by formaldehyde gel electrophoresis to confirm RNA integrity. The intra- and inter-assay coefficients of variation for RNA quantitation were <6%.

Cloning and sequencing of equine GLUT-4.

Cloning and sequencing were performed using standard reverse transcriptase-polymerase chain reaction (RT-PCR) techniques, initially using primers designed by comparing published sequences of GLUT4 cDNA in human, rat, mouse and cow, and choosing oligonucleotides in areas with the highest homology between species (Table 6.1). Primers were designed, using a software package (Vector NTI Suite 5.5, Informax Inc., Frederick, MD), so as to obtain in separate reactions DNA fragments of 700-800 bp that overlapped a minimum of 100 bp and spanned the entire coding region of the equine GLUT-4 gene. Two-step RT-PCR reactions involved cDNA synthesis (Thermoscript RT-PCR system for first strand cDNA synthesis, Invitrogen Corp, Carlsbad, CA) using oligo(dT) primer and incubation at 50ºC for 1 h, and PCR amplification of a 2 µl aliquot of the cDNA synthesis reaction. PCR conditions involved denaturation at 94ºC for 2 min and 40 cycles of denaturing (30 sec at 92ºC), annealing (45 sec; see temperatures in Table 6.1), and extension (1 min at 72ºC), with a final extension step at 72ºC for 5 min, using a commercial PCR kit (Platinum *Taq* DNA Polymerase, Invitrogen Corp, Carlsbad, CA). All RT-PCR reactions for the cloning experiment were performed using total RNA from mouse skeletal muscle as positive control, with nuclease free water and total RNA from equine spleen as negative controls (because in other species GLUT-4 is not expressed in spleen tissues), in addition to total RNA from equine skeletal muscle. Ten microliters of PCR product were resolved on a 1% agarose gel. DNA fragments of predicted size were ligated into TA cloning vectors (pCR2.1 TA cloning kit, and DH5α chemically competent *E. coli*, Invitrogen Corp., Carlsbad, CA). Candidate clones were screened by restriction enzyme digestion with EcoRI (EcoRI, Invitrogen Corp, Carlsbad, CA) and

positive clones were sequenced at the institutional sequencing facility using universal M13 forward and M13 reverse primers and an automated sequencing analyzer (ABI PRISM 3700 DNA Analyzer, Applied Biosystems, Foster City, CA). Sequence results were compared, using a software package (Align Plus 4.0, Sci-ed Software, Durham, NC), with those of other species within the GenBank database at the National Center for Biotechnology Information.

Real-time reverse transcriptase PCR for measurement of equine GLUT-4 gene expression in skeletal muscle biopsies.

Species specific primers for real-time RT-PCR of the equine GLUT-4 gene were designed by adapting those used in rats[20], and primers for the equine β-actin gene were adapted from those used in humans[21] (Table 6.1). To compensate for variations in input RNA amounts, and efficiency of reverse transcription, β-actin mRNA was quantified and the GLUT-4 mRNA results were normalized to these values. β-actin mRNA levels have been reported not to change during training in rat skeletal muscle[22] and therefore β-actin was considered an adequate "housekeeping" gene. In contrast, another "housekeeping" gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been commonly used, but it has been shown that insulin induces GAPDH gene expression in cell cultures[23]. Therefore, we considered GAPDH not to be an adequate "housekeeping" gene since feeding after exercise was anticipated to increase plasma insulin concentrations and possibly induce GAPDH gene expression.

Real-time RT-PCR using SYBR Green was employed to quantify mRNA expression, the principles of which have been described in detail elsewhere[24]. In this method, amplification is monitored as it occurs by collection of fluorescence data during each

cycle of amplification, which allows for quantification in the log-linear range of amplification. Fluorescence occurs due to intercalation of SYBR Green into the double stranded DNA being generated in the PCR. Levels of mRNA were quantitated using the fit points method of the LightCycler software (Lightcycler system, Roche Diagnostics Corp, Mannheim, Germany). Real-time RT-PCR reactions were performed in duplicate using a quantitative, real-time, one-step RT-PCR with SYBR Green kit (QuantiTect SYBR Green RT-PCR kit, Qiagen Inc., Valencia, CA) and Lightcycler detection system (Lightcycler instrument, Roche Diagnostics Corp, Mannheim, Germany). Each 20 μl reaction contained 1X QuantiTect SYBR Green RT-PCR master mix, forward and reverse primers (1 μM each) (Table 6.1), and total RNA (100 ng). Real-time RT-PCR conditions involved reverse transcription at 50ºC for 20 min, denaturation at 95ºC for 15 min, and 45 cycles of denaturing (95ºC for 15 sec), annealing (55 and 58ºC for GLUT-4 and β-actin, respectively, for 20 sec), extension (72ºC for 10 sec), and fluorescence data acquisition. Specific amplification of equine GLUT-4 and equine β-actin were confirmed in all samples by melting temperature analysis of the product of amplification and by resolving on a 1% agarose gel. During the preliminary studies, specific amplification of equine GLUT-4 and equine β-actin also were confirmed by sequencing. In order to quantify mRNA copy number, a standard curve was generated using equine GLUT-4 and equine β-actin RNA previously synthesized by in vitro transcription. RNA standards were transcribed from the linearized cDNA cloned into pCR2.1 plasmid (pCR2.1 TA cloning kit, Invitrogen Corp., Carlsbad, CA) using T7 RNA polymerase (MaxiScript In Vitro Transcription Kit, Ambion, Austin, TX). Purified RNA transcripts were verified by formaldehyde gel electrophoresis for size and integrity, quantified by UV

spectrophotometry and tested for amplification with the gene-specific primers with and without reverse-transcriptase (to rule out DNA contamination). Aliquots of 10^9 copies/μl were stored at –80°C and a fresh aliquot was diluted in nuclease-free water prior to use, in order to obtain standard curves ranging from 10^7 to 10^2 transcripts/μl.

Effect of exercise and meal type after exercise on GLUT-4 gene expression.

The effects of feeding isocaloric meals of varying starch and fiber content (or food withholding) in combination with exercise were examined in a semi-balanced, randomized, three-way crossover study. Seven horses undertook 3 consecutive days of strenuous treadmill exercise intended to lower muscle glycogen concentrations by at least 55% of the initial values[3,25]. Exercise intensity and duration of the glycogen depleting exercise protocol used in this study are described in greater detail elsewhere[25]. In each of three trials during the 8h after the 3rd consecutive day of exercise, horses were either: *1)* offered meals of cracked corn (2.2 ± 0.2 kg/meal [average ± SD], ~7.4 Mcal DE/meal; grain trial); *2)* offered isocaloric meals of grass and alfalfa hay (3.4 ± 0.4 kg/meal, ~7.4 Mcal DE/meal; hay trial); or *3)* had food withheld. Trials were separated by 2 weeks, and the order of trials was randomized for individual horses. Due to musculoskeletal injuries 3 out of 7 horses did not complete all 3 trials. Horses 1-6 completed the glycogen-depleting trials followed by corn feeding or feed withholding, and horses 1-4 and 7 completed the glycogen-depleting trials followed by hay feeding. Other results of this study are reported elsewhere[26].

Collection of blood and muscle samples and biochemical analyses.

Blood samples were obtained before and hourly for 8 h after exercise using catheters (14 gauge, 5.25 in.; Angiocath, Becton Dickinson) inserted into the left jugular

vein. Plasma and serum samples were harvested by centrifugation and stored at -80ºC until subsequent analysis. Plasma glucose concentration and serum immunoreactive insulin (IRI) were measured in duplicate as described elsewhere[27,28]. Muscle biopsy samples were obtained percutaneously at a depth of 6 cm from the middle gluteal muscle by needle biopsy, before the 3 consecutive days of glycogen-depleting exercise and 10 min after, 4h, 8h and 24 h after the last bout of exercise. Muscle samples were placed immediately in liquid nitrogen and stored at −80°C until analysis. Muscle glycogen concentration was determined as previously described[27].

Statistical analyses.

Data are presented as means ± SE. Plasma glucose, serum IRI, and muscle glycogen concentrations, as well as GLUT-4 mRNA, β-actin mRNA and GLUT-4 mRNA normalized to β-actin expression were analyzed using an ANOVA mixed model with repeated measures, with treatment (feed withholding, hay, and grain), time and the interaction of treatment and time as fixed effects. The null hypothesis was rejected at $P < 0.05$ for the main effects (treatment and time) and $P < 0.10$ for the interaction. Significant differences identified by ANOVA were isolated using the Dunn-Sidak post hoc test. Statistical computations were performed with a software package (SPSS 12.0, SPSS Inc., Chicago, IL).

6.4. RESULTS

Characterization of equine GLUT-4 cDNA.

Equine GLUT-4 nucleotide and deduced amino acid sequences are presented in Fig. 6.1. The total 1623 bp of cDNA sequenced contained a 7-bp of 5’ untranslated

region (UTR), 1530-bp of open reading frame (from 8 to 1537 inclusive), and a 86-bp 3' UTR. Similarly to other species the equine GLUT-4 cDNA encoded 509 amino acids. The calculated molecular weight of the resulting protein was 54.8 kd, and the isoelectric point was 7.17. The nucleotide and deduced amino acid sequences of equine GLUT-4 were compared with other mammals for which complete mRNA sequences are available (Fig. 6.2). Analysis of the amino acid sequence deduced from equine GLUT-4 cDNA suggested a secondary structure consisting of 12 transmembrane helices, which is identical to other species. A phylogeny dendrogram for known complete GLUT-4 amino acid sequences was constructed (Fig. 6.3), which showed that equine GLUT-4 is closely related to that of other mammals. Nucleotide and deduced amino acid sequences of equine GLUT-4 are >85% identical to that of other mammalian species with known GLUT-4 cDNA sequences.

Plasma glucose, serum immunoreactive insulin and muscle glycogen.

Results of plasma substrates and hormones, muscle glycogen and whole body glucose turnover rates in this study are reported in greater detail elsewhere[26]. In brief, feeding type after exercise significantly affected plasma glucose and serum insulin concentrations after exercise (comparison of main effects: corn vs. unfed $P < 0.001$, hay vs. unfed $P = 0.03$, corn vs. hay $P = 0.07$) (Table 6.3). Feeding 2 meals of cracked corn that provided approximately half of the daily energy requirements immediately and 4 h after exercise resulted in a ~20% increase in plasma glucose concentrations when compared to unfed horses (corn feeding different than feed withholding from 2 to 8 h after exercise, $P < 0.01$). Peak serum insulin concentration was higher in corn fed horses when compared to feed withholding (at 2 h corn vs. unfed, $P < 0.01$). Three consecutive

days of exercise lowered muscle glycogen concentrations by 67 ± 3% of the initial values (prior to exercise vs. other time points after exercise, $P < 0.001$). Muscle glycogen concentration at 24 h was higher than at 0-h in grain-fed horses ($P = 0.04$) whereas non-significant increases were observed in the other trials.

Muscle GLUT-4 mRNA.

Muscle glycogen depleting exercise, but not feeding type, significantly altered GLUT-4 mRNA levels in skeletal muscle (main effects: time $P < 0.001$, treatment $P = 0.4$, interaction $P = 0.9$; Fig. 6.4). All horses had a higher number of GLUT-4 transcripts in skeletal muscle at 4h after exercise when compared to prior to exercise (both with and without normalization for β-actin expression). GLUT-4 gene expression gradually decreased and by 24 h after exercise was similar to that prior to exercise ($P = 0.3$). It is of interest to note that β-actin mRNA levels in skeletal muscle had an inverse relationship to GLUT-4 mRNA levels. β-actin gene expression was lower at 4 h post-exercise when compared with prior to and 24 h after exercise (pre vs. 4 h, $P = 0.002$; 4 h vs. 24 h, $P = 0.03$). For this reason, GLUT-4 mRNA levels are reported with and without normalization for β-actin gene expression.

```
   1  AAACAAGATGCCGTCGGGTTTTCAACAGATCGGCTCAGAAGATGGGGAACCGCCTCAGCA
              M  P  S  G  F  Q  Q  I  G  S  E  D  G  E  P  P  Q
  61  GCGAGTAACTGGGACCCTGGTCCTCGCAGTATTTTCTGCTGTGCTTGGCTCCCTGCAGTT
      Q  R  V  T  G  T  L  V  L  A  V  F  S  A  V  L  G  S  L  Q
 121  TGGCTACAACATTGGGGTCATCAATGCCCCACAGAAGGTGATTGAACAGAGCTACAATGA
      F  G  Y  N  I  G  V  I  N  A  P  Q  K  V  I  E  Q  S  Y  N
 181  GACATGGCTGGGGAGGCAGGGGCCTGAGGGGCCCAGCTCCATCCCACCAGGCACCCTCAC
      E  T  W  L  G  R  Q  G  P  E  G  P  S  S  I  P  P  G  T  L
 241  CACCCTCTGGGCTCTCTCCGTGGCCATCTTTTCTGTGGGCGGCATGATCTCCTCCTTCCT
      T  T  L  W  A  L  S  V  A  I  F  S  V  G  G  M  I  S  S  F
 301  CATTGGCATCATCTCTCAGTGGCTGGGAAGGAAAAGGGCAATGCTGGTCAACAATGCCCT
      L  I  G  I  I  S  Q  W  L  G  R  K  R  A  M  L  V  N  N  A
 361  GGCAGTGCTGGGGGGCAGCCTCATGGGCCTGGCTGACACTGCTGCCTCCTATGAGATGCT
      L  A  V  L  G  G  S  L  M  G  L  A  D  T  A  A  S  Y  E  M
 421  CATTCTTGGACGGTTCCTCATTGGCGCCTACTCAGGGCTGACGTCAGGGCTGGTGCCCAT
      L  I  L  G  R  F  L  I  G  A  Y  S  G  L  T  S  G  L  V  P
 481  GTATGTGGGGGAGATCGCCCCCACTCACCTGCGGGGTGCCTTGGGGACACTCAACCAACT
      M  Y  V  G  E  I  A  P  T  H  L  R  G  A  L  G  T  L  N  Q
 541  GGCCATCGTCATTGGCATTCTGATCGCCCAGGTGCTGGGCTTGGAGTCTATGCTGGGCAC
      L  A  I  V  I  G  I  L  I  A  Q  V  L  G  L  E  S  M  L  G
 601  TGCCACCCTATGGCCACTGCTCCTGGGCATCACAGTGCTGCCTGCCCTTCTGCAGTTGGT
      T  A  T  L  W  P  L  L  L  G  I  T  V  L  P  A  L  L  Q  L
 661  CCTACTGCCCTTCTGCCCAGAAAGCCCTCGCTACCTCTACATCAGCCGGAACCTGGAGGG
      V  L  L  P  F  C  P  E  S  P  R  Y  L  Y  I  S  R  N  L  E
 721  GCCCGCCAGAAAGAGTCTGAAGCGCCTGACAGGCTGGGCTGACGTGTCTGGAGTGCTGGC
      G  P  A  R  K  S  L  K  R  L  T  G  W  A  D  V  S  G  V  L
 781  TGAGCTAAAGGAAGAGAAGCGGAAGCTGGAGCGTGAGCGGCCACTGTCCCTGCTCCAGCT
      A  E  L  K  E  E  K  R  K  L  E  R  E  R  P  L  S  L  L  Q
 841  CCTGGGCAGCCGTGTCCACCGGCAGCCCCTAGTCATTGCAGTTGTGCTGCAGCTCAGCCA
      L  L  G  S  R  V  H  R  Q  P  L  V  I  A  V  V  L  Q  L  S
 901  GCAGCTATCGGGCATCAACGCTGTTTTCTATTATTCAACCAGCATCTTCGAGAAGGCAGG
      Q  Q  L  S  G  I  N  A  V  F  Y  Y  S  T  S  I  F  E  K  A
 961  AGTAGGGCAGCCAGCCTATGCCACCATAGGAGCTGGTGTGGTCAACACAGTCTTCACCTT
      G  V  G  Q  P  A  Y  A  T  I  G  A  G  V  V  N  T  V  F  T
1021  GGTCTCGGTGTTTTTGGTAGAACGAGCTGGGCGCCGGACACTCCATCTCCTGGGCCTGGC
      L  V  S  V  F  L  V  E  R  A  G  R  R  T  L  H  L  L  G  L
1081  GGGAATGTGTGGCTGTGCCATCTTGATGACTGTGGCCCTGCTTCTGCTGGAGCGAGTTCC
      A  G  M  C  G  C  A  I  L  M  T  V  A  L  L  L  L  E  R  V
1141  AGCCATGAGCTATGTCTCCATCGTGGCCATCTTTGGCTTTGTGGCATTCTTTGAGATTGG
      P  A  M  S  Y  V  S  I  V  A  I  F  G  F  V  A  F  F  E  I
1201  CCCTGGCCCCATCCCCTGGTTCATCGTGGCTGAGCTCTTCAGCCAGGGACCCCGCCCGGC
      G  P  G  P  I  P  W  F  I  V  A  E  L  F  S  Q  G  P  R  P
1261  AGCCATGGCTGTGGCTGGCTTCTCCAACTGGACGTGCAACTTCATCATTGGCATGGGCTT
      A  A  M  A  V  A  G  F  S  N  W  T  C  N  F  I  I  G  M  G
1321  CCAGTATGTCGCGGATGCTATGGGTCCCTACGTCTTCCTTCTATTTGCGGTCCTCCTGCT
      F  Q  Y  V  A  D  A  M  G  P  Y  V  F  L  L  F  A  V  L  L
1381  TGGCTTCTTCATCTTCACCTTCTTAAGAGTGCCTGAAACCCGAGGCCGGACGTTTGACCA
      L  G  F  F  I  F  T  F  L  R  V  P  E  T  R  G  R  T  F  D
1441  GATCTCAGCCGCTTTCCACCGGACACCCTCTCTTTTAGAGCAGGAGGTGAAGCCCAGCAC
      Q  I  S  A  A  F  H  R  T  P  S  L  L  E  Q  E  V  K  P  S
1501  GGAACTTGAGTATTTAGGGCCAGATGAGAATGACTGAGGGCAGGCAGGGGTGGGAGAGCC
      T  E  L  E  Y  L  G  P  D  E  N  D  *
1561  GGTGCTCTCTACCCCCCTCAGAGCCCCCTCCTTCTCTGCAGCACTTTAACCCTCTCTTCCCCA
```

Figure 6.1. Complementary DNA and deduced amino acid sequences of equine glucose transporter type-4 (GLUT-4; insulin-responsive glucose transporter). Numbers on the left indicate nucleotides, numbers on the right indicate amino acids from the start codon. Start (ATG) and stop (TGA) codons specifying the 1530-bp open reading frame are in bold.

```
Equine   1 MPSGFQQIGSEDGEPPQQRVTGTLVLAVFSAVLGSLQFGYNIGVINAPQKVIEQSYNETW
Human    1 ............................................................
Bovine   1 ................R.........G...............................
Rat      1 ............................................................A..
Mouse    1 ..........D.....R.....................................A..

Equine  61 LGRQGPEGPSSIPPGTLTTLWALSVAIFSVGGMISSFLIGIISQWLGRKRAMLVNNALAV
Human   61 ...........................................................V...
Bovine  61 .........G.....................................FS.....
Rat     61 ......G..D...Q.....................................A..V...
Mouse   61 ......G..D...Q.....................................A..V...

Equine 121 LGGSLMGLADTAASYEMLILGRFLIGAYSGLTSGLVPMYVGEIAPTHLRGALGTLNQLAI
Human  121 .........NA.....................................................
Bovine 121 ...T.....KA............F.....................................
Rat    121 ...A.....NA.....I.....................................
Mouse  121 ...A.....NA.....I.....................................

Equine 181 VIGILIAQVLGLESMLGTATLWPLLLGITVLPALLQLVLLPFCPESPRYLYISRNLEGPA
Human  181 ..............L....S.......L.....................IQ......
Bovine 181 .T.................................M....L.........I.......
Rat    181 .....V.....................A..........L.............I.......
Mouse  181 .....V.....................AL.........I.............I.......

Equine 241 RKSLKRLTGWADVSGVLAELKEEKRKLERERPLSLLQLLGSRVHRQPLVIAVVLQLSQQL
Human  241 ......................D.....................T.....I...........
Bovine 241 ..............E.................S.........HT........I........
Rat    241 ..............DA.....D.....................T.....I...........
Mouse  241 ..............DA.....D..........M.........T.....I...........

Equine 301 SGINAVFYYSTSIFEKAGVGQPAYATIGAGVVNTVFTLVSVFLVERAGRRTLHLLGLAGM
Human  301 ...............T.........................L...................
Bovine 301 ...............S...EK.........................H...........
Rat    301 ...............L...E.....................L...................
Mouse  301 ...............S.........................L...................

Equine 361 CGCAILMTVALLLLERVPAMSYVSIVAIFGFVAFFEIGPGPIPWFIVAELFSQGPRPAAM
Human  361 ............................................................
Bovine 361 .A.........................................C...............
Rat    361 ...................S....................................
Mouse  361 ............................................................

Equine 421 AVAGFSNWTCNFIIGMGFQYVADAMGPYVFLLFAVLLLGFFIFTFLRVPETRGRTFDQIS
Human  421 .........S............E.....................................
Bovine 421 ..............................................K.............
Rat    421 ............V.....................................................
Mouse  421 ............V.................................K.............

Equine 481 AAFHRTPSLLEQEVKPSTELEYLGPDEND
Human  481 ............................
Bovine 481 .V.......................H.
Rat    481 .T.R.........................
Mouse  481 ...R.........................
```

Figure 6.2. Alignment of the deduced amino acid sequence of equine GLUT-4 with known complete sequences of other mammals. Numbers on the left represent amino acids from the start codon. Dots indicate amino acids identical to those in equine GLUT-4.

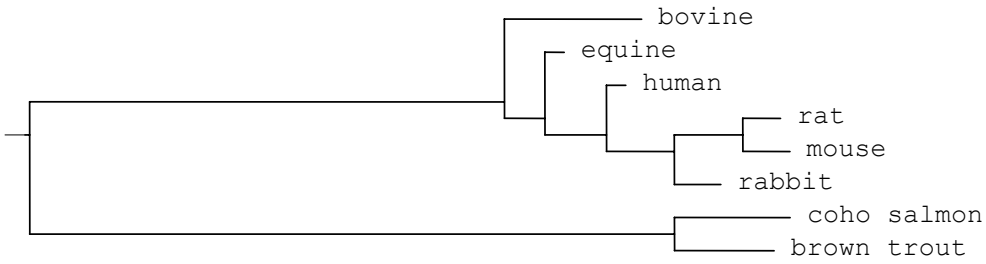

Figure 6.3. Phylogeny dendrogram calculated for known complete GLUT-4 amino acid sequences of various vertebrates. Two branches are evident; 1 represents mammalian GLUT-4, and the other represents lower vertebrates.

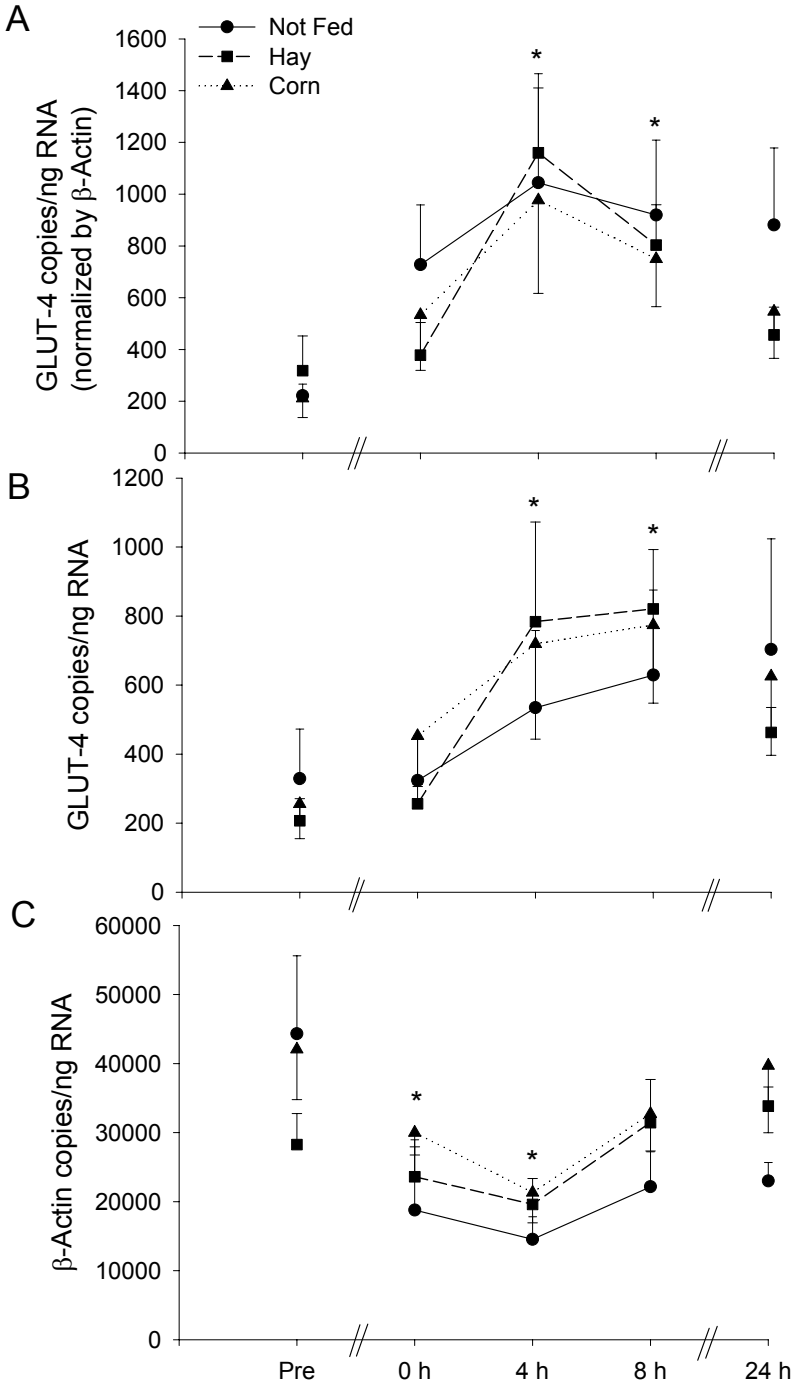

Figure 6.4. Mean (±SE) number of muscle GLUT-4 transcripts normalized for β-actin gene expression (A), number of muscle GLUT-4 transcripts without correction for β-actin gene expression (B), and number of muscle β-actin transcripts (C), before 3 consecutive days of exercise (Pre) and immediately (0 h), 4 h, 8 h, and 24 h after exercise. Treatments were grain feeding (n = 6) or isocaloric hay feeding (n = 5) or feed withholding (n=6). * Significantly different time points when compared to prior to exercise ($P < 0.05$).

Target	Amplicon size (bp)	Annealing temp. ºC	Primer sequence
GLUT-4 5' coding region (1-713)	713	50	Forward 5'-AAA CAA GAT GCC GTC GGG-3' Reverse 5'-GGT TCC GGA TGA TGT AGA GGT A-3'
GLUT-4 Mid-coding region (248-905)	658	66	Forward 5'-TGG GCT CTC TCC GTG GCC ATC TT-3' Reverse 5'-GCT GCT GGC TCA GCT GCA GCA-3'
GLUT-4 3' coding region (725-1527)	803	64	Forward 5'-GCC AGA AAG AGT CTR AAG CGC CTG A-3' Reverse 5'-CCT CAG TCR TKC TCA TCT GGC CCT A-3'
GLUT-4 3' coding region and UTR (725-1623)	899	64	Forward 5'-GCC AGA AAG AGT CTR AAG CGC CTG A-3' Reverse 5'-TGG GGA AGA GAG GGT TAA AGT GCT GC-3'
GLUT-4 Real-time RT-PCR	63	55	Forward 5'-TTT GTG GCA TTC TTT GAG ATT GG-3' Reverse 5'-CTG AAG AGC TCA GCC ACG A-3'
β-actin Real-time RT-PCR	142	58	Forward 5'-GAC AGG ATG CAG AAG GAG ATC ACA-3' Reverse 5'-TGA TCC ACA TCT GCT GGA AGG T-3'

Table 6.1. Primers used to determine the cDNA sequence of equine GLUT-4, real-time RT-PCR of equine GLUT-4 and β-actin, as well as length of the product of amplification and annealing temperature used with each primer pair

Species	NCBI accession No.	Identity	
		cDNA	aa
Human	NM_001042.1	92	96
Mouse	AB008453.1	88	94
Rat	X14771.1	88	94
Bovine	D63150	90	90
Rabbit	Y339876	91	94
Pig*	AF141956	91	93
Dog*	AJ388533	91	94
Sheep*	AB005283	86	94
Goat*	AB005284	85	94
Buffalo*	AF254423	92	96

Table 6.2. Nucleotide and deduced amino acid (aa) sequence identity (%) between equine GLUT-4 (National Center for Biotechnology Information [NCBI] accession no. AF531753) and the homologous sequences of several other mammals (* partial sequences, identity calculated using the corresponding partial equine GLUT-4)

6.5. DISCUSSION

The results reported here confirm that the equine GLUT-4 gene has a high degree of homology with that in other mammals. In agreement with our hypothesis, equine GLUT-4 gene expression in skeletal muscle increased in the hours following exercise. However, ingestion of carbohydrates as starch-rich meals did not enhance GLUT-4 gene expression in muscle, when compared to isocaloric fiber-rich meals or feed withholding.

Previous studies in humans and rodents have demonstrated that expression of GLUT-4 mRNA is subject to tissue-specific, hormonal and metabolic regulation. In general, exercise and carbohydrate supplementation result in increased GLUT-4 gene expression in skeletal muscle, measured as mRNA levels or total GLUT-4 protein content[29]. Conversely, diabetes and fasting decrease GLUT-4 mRNA levels in adipose tissue, and diabetes, but not fasting, decreases GLUT-4 mRNA in skeletal muscle[7]. To our knowledge, this is the first study that demonstrates an increase in GLUT-4 mRNA in equine skeletal muscle associated with glycogen depleting exercise. In contrast, a previous study conducted by our laboratory did not show an increase in GLUT-4 protein or mRNA in equine muscle after a single bout of exercise[30]. The apparent discrepancy may be due to differences in the exercise protocols and degree of muscle glycogen depletion (30% depletion in the previous study vs. more than 60% in the present study) and analytical techniques (Northern blot analysis using a human GLUT-4 cDNA probe in the previous study vs. real-time RT-PCR using equine specific primers in the present study).

Carbohydrate supplementation after exercise in rats results in enhanced muscle glycogen replenishment, increased muscle GLUT-4 protein content and blunted increase

in muscle GLUT-4 mRNA when compared to exercised fasted rats[31]. Therefore, it was suggested that carbohydrate supplementation following exercise downregulates GLUT-4 transcription while simultaneously increasing GLUT-4 translation, which was mediated possibly by changes in circulating insulin levels. In contrast, studies performed in sedentary rats infused with glucose and insulin demonstrated increased muscle GLUT-4 protein content and mRNA concentration[32]. In the present study, carbohydrate supplementation by ingestion of starch rich meals in horses resulted in higher insulin concentrations when compared to unfed horses, however muscle glycogen replenishment and GLUT-4 mRNA concentrations were not different regardless of the feeding state. In contrast to that seen in rodents, horses receiving carbohydrate supplementation by intravenous or oral glucose administration after exercise have decreased[18] or unchanged[30] muscle GLUT-4 protein content when compared with exercised, non-supplemented horses. Therefore, regulation of GLUT-4 transcription and translation in horses in relation to exercise and carbohydrate supplementation bears some similarities but cannot be directly extrapolated from that seen in other species.

Transport of glucose through the sarcolemma appears to be the primary rate-limiting step in glucose uptake by muscle and muscle glycogen synthesis after exercise[33,34]. In the absence of stimuli such as exercise or insulin, over 90% of GLUT-4 remains inactive, sequestered in intracellular vesicles. Insulin and exercise can independently stimulate translocation of GLUT-4 from intracellular storage pools to the sarcolemma and T-tubules to facilitate glucose uptake[35]. Therefore, determination of total GLUT-4 protein content does not account for changes in the fraction of membrane-bound GLUT-4 vs. inactive GLUT-4 sequestered intracellularly. One may speculate that

carbohydrate supplementation after exercise in horses may not increase total GLUT-4 protein content or GLUT-4 mRNA, but may increase the fraction of membrane-bound GLUT-4 allowing glucose to be transported into the sarcolemma. In order to determine translocation of GLUT-4 within the cell, separation of cellular components is required. This procedure was not performed in our study.

The nucleotide and deduced amino acid sequence information and methodology for quantitative determination of GLUT-4 mRNA described in the present study may serve in further studies of physiological adaptations and pathophysiological mechanisms involving glycogen metabolism, insulin resistance and muscle energetics in horses.

6.6. REFERENCES

1. Brooks GA, Mercier J. Balance of carbohydrate and lipid utilization during exercise: the "crossover" concept. *J Appl Physiol* 1994;76:2253-2261.

2. Burke LM, Collier GR, Hargreaves M. Muscle glycogen storage after prolonged exercise: effect of the glycemic index of carbohydrate feedings. *J Appl Physiol* 1993;75:1019-1023.

3. Lacombe VA, Hinchcliff KW, Geor RJ, et al. Muscle glycogen depletion and subsequent replenishment affect anaerobic capacity of horses. *J Appl Physiol* 2001;91:1782-1790.

4. Ren JM, Marshall BA, Gulve EA, et al. Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem* 1993;268:16113-16115.

5. Kuo CH, Browning KS, Ivy JL. Regulation of GLUT4 protein expression and glycogen storage after prolonged exercise. *Acta Physiol Scand* 1999;165:193-201.

6. Mueckler M. Family of glucose-transporter genes. Implications for glucose homeostasis and diabetes. *Diabetes* 1990;39:6-11.

7. Charron MJ, Katz EB, Olson AL. GLUT4 gene regulation and manipulation. *J Biol Chem* 1999;274:3253-3256.

8. Fukumoto H, Kayano T, Buse JB, et al. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *J Biol Chem* 1989;264:7776-7779.

9. Kaestner KH, Christy RJ, McLenithan JC, et al. Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A* 1989;86:3150-3154.

10. James DE, Strube M, Mueckler M. Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* 1989;338:83-87.

11. Abe H, Morimatsu M, Nikami H, et al. Molecular cloning and mRNA expression of the bovine insulin-responsive glucose transporter (GLUT4). *J Anim Sci* 1997;75:182-188.

12. Navarrete Santos A, Kirstein M, Fischer B. Two insulin-responsive glucose transporter isoforms and the insulin receptor are developmentally expressed in rabbit preimplantation embryos: NCBI GenBank, 2003.

13. Pichon B, Mercan D, Pouillon V, et al. Canis familiaris mRNA for partial Insulin-responsive glucose transporter, glut4 gene.: NCBI GenBank, 2001.

14. McNeel RL, Ding ST, Smith EO, et al. Effect of feed restriction on adipose tissue transcript concentrations in genetically lean and obese pigs. *J Anim Sci* 2000;78:934-942.

15. Abe H, Kawakita Y, Miyashige T, et al. Comparison of amino acid sequence of the C-terminal domain of insulin-responsive glucose transporter (GLUT4) in livestock mammals. *J Vet Med Sci* 1998;60:769-771.

16. Patil SSB, Totey SSM. Partial cDNA sequence of glucose transporter type four of Bubalus bubalis: NCBI GenBank, 2000.

17. McCutcheon LJ, Geor RJ, Hinchcliff KW. Changes in skeletal muscle GLUT4 content and muscle membrane glucose transport following 6 weeks of exercise training. *Equine Vet J Suppl* 2002:199-204.

18. Lacombe VA, Hinchcliff KW, Devor ST. Effects of exercise and glucose administration on content of insulin-sensitive glucose transporter in equine skeletal muscle. *Am J Vet Res* 2003;64:1500-1506.

19. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-159.

20. Lee JS, Bruce CR, Tunstall RJ, et al. Interaction of exercise and diet on GLUT-4 protein and gene expression in Type I and Type II rat skeletal muscle. *Acta Physiol Scand* 2002;175:37-44.

21. Wadley GD, Tunstall RJ, Sanigorski A, et al. Differential effects of exercise on insulin-signaling gene expression in human skeletal muscle. *J Appl Physiol* 2001;90:436-440.

22. Murakami T, Shimomura Y, Fujitsuka N, et al. Enzymatic and genetic adaptation of soleus muscle mitochondria to physical training in rats. *Am J Physiol* 1994;267:E388-395.

23. Barroso I, Benito B, Garci-Jimenez C, et al. Norepinephrine, tri-iodothyronine and insulin upregulate glyceraldehyde-3-phosphate dehydrogenase mRNA during Brown adipocyte differentiation. *Eur J Endocrinol* 1999;141:169-179.

24. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25:169-193.

25. Lacombe V, Hinchcliff KW, Geor RJ, et al. Exercise that induces substantial muscle glycogen depletion impairs subsequent anaerobic capacity. *Equine Vet J Suppl* 1999;30:293-297.

26. Jose-Cunilleras E, Hinchcliff KW, Lacombe VA, et al. Ingestion of high-glycemic meals after exercise increases glucose flux but fails to enhance muscle glycogen synthesis in horses. *Equine Vet J* 2004;submitted.

27. Jose-Cunilleras E, Hinchcliff KW, Sams RA, et al. Glycemic index of a meal fed before exercise alters substrate use and glucose flux in exercising horses. *J Appl Physiol* 2002;92:117-128.

28. Freestone JF, Shoemaker K, Bessin R, et al. Insulin and glucose response following oral glucose administration in well-conditioned ponies. *Equine Vet J Suppl* 1992:13-17.

29. MacLean PS, Zheng D, Dohm GL. Muscle glucose transporter (GLUT 4) gene expression during exercise. *Exerc Sport Sci Rev* 2000;28:148-152.

30. Nout YS, Hinchcliff KW, Jose-Cunilleras E, et al. Effect of moderate exercise immediately followed by induced hyperglycemia on gene expression and content of the glucose transporter-4 protein in skeletal muscles of horses. *Am J Vet Res* 2003;64:1401-1408.

31. Kuo CH, Hunt DG, Ding Z, et al. Effect of carbohydrate supplementation on postexercise GLUT-4 protein expression in skeletal muscle. *J Appl Physiol* 1999;87:2290-2295.

32. Jones JP, Dohm GL. Regulation of glucose transporter GLUT-4 and hexokinase II gene transcription by insulin and epinephrine. *Am J Physiol* 1997;273:E682-687.

33. Ren JM, Semenkovich CF, Gulve EA, et al. Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin-stimulated glycogen storage in muscle. *J Biol Chem* 1994;269:14396-14401.

34. Fink RI, Wallace P, Brechtel G, et al. Evidence that glucose transport is rate-limiting for in vivo glucose uptake. *Metabolism* 1992;41:897-902.

35. Shepherd PR, Kahn BB. Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *N Engl J Med* 1999;341:248-257.

CHAPTER 7

GENERAL DISCUSSION

7.1. WHY ARE CARBOHYDRATE METABOLISM AND THE IMPACT OF NUTRITION ON SUBSTRATE METABOLISM IMPORTANT IN ATHLETIC HORSES?

Biological processes and cellular function requires use of chemical energy provided in the form of adenosine triphosphate (ATP). Energy homeostasis and recycling of ATP is ultimately due to utilization of oxygen and molecules containing hydrogenated carbon structures. The energy in hydrogen-carbon bonds is released by oxidation and transported by reduced coenzymes (NADH and $FADH_2$) to the electron transport chain in the mitochondria where these are oxidized to allow recycling of adenosine diphosphate into ATP by the process of oxidative phosphorilation. The substrates utilized as energy sources are carbohydrates, lipids and proteins. However, proteins are a relatively minor source of energy because these are preserved for their functional and structural properties, and only in cases of starvation can protein catabolism become an important source of energy. That leaves carbohydrates and lipids as the quantitatively important sources of energy necessary to sustain mechanical muscular work during exercise. Carbohydrate supply and oxidation during exercise have received greater attention given that: (1) carbohydrates stored within the body are in short supply relative to lipids (e.g. 4

kg and 18 Mcal as carbohydrate versus 42 kg and 380 Mcal as lipid in a 450-kg horse; see Chapter 1), (2) oxidation of fatty acids requires provision of sufficient intermediates of the tricarboxylic acid cycle ("fats burn in the flame of carbohydrates"), (3) increasing exercise intensities result in increasing energy expenditures that are met by increasing relative and absolute contributions from carbohydrate oxidation (predominantly muscle glycogen), and (4) muscle glycogen concentrations prior to exercise are directly associated with indices of athletic performance such as time-to-exhaustion at a fixed exercise intensity. Due to the aforementioned reasons carbohydrate metabolism is of prime importance in athletic animals.

In trained subjects, nutrition is the most practical strategy to lawfully maintain and enhance athletic performance. That is why the effect of long-term diets as well as meal or beverage consumption prior to, during and after exercise on metabolism and athletic performance have been extensively studied in athletic species. Previous studies in our laboratory established that: (1) intravenous glucose administration during moderate intensity exercise in horses prolonged time to exhaustion by ~14-19% [1,2]; (2) intravenous and oral administration of glucose leads to greater relative contribution from carbohydrate oxidation to energy expenditure during moderate intensity exercise [3,4]. Based on that previous work, in the first study (Chapter 3) we set out to determine the effect of ingestion of isocaloric meals of varying carbohydrate composition and of feed withholding on substrate use and glucose flux during exercise as determined by combination of isotopic tracer and indirect calorimetry methods (see Chapter 3). Our hypothesis was that that ingestion of a soluble carbohydrate-rich meal (cracked corn)

prior to exercise would result in enhanced carbohydrate oxidation, attenuated lipid oxidation, greater relative contributions to energy expenditure from blood-borne glucose, and attenuated muscle glycogenolysis ("muscle glycogen sparing effect").

Another situation in which diet may have an impact on carbohydrate metabolism and athletic performance is after marked muscle glycogen depletion. Based on the observations that decreased muscle glycogen concentrations prior to exercise lead to decreased athletic performance in humans and horses [5,6], and that ingestion of meals rich in soluble carbohydrates (high-glycemic index meals) after exercise enhance muscle glycogen replenishment in humans and horses [7,8], in the second study we set out to determine the effect of ingestion of isocaloric meals of varying carbohydrate composition and of feed withholding on glucose flux and muscle glycogen replenishment after exercise (see Chapter 5). We hypothesized that ingestion of soluble carbohydrate-rich meals during the hours following exercise would result in enhanced whole body glucose appearance and uptake, and associated greater deposition as muscle glycogen. As a preliminary study, we set out to refine the assessment of glycemic index of grains in horses since previous studies performed in horses had compared starch rich meals on a weight basis (e.g. 1 kg corn vs. 2 kg corn) or without an appropriate comparison group (e.g. ingestion of equal amounts of carbohydrate in the form of glucose solution and compare the area under the concentration-time curve after ingestion of grains to that after intragastric administration of glucose solution; see Chapter 4).

Finally, given that muscle glycogen replenishment after exercise in horses is slower relative to that seen in humans and rodents we became interested in describing the effect of exercise and ingestion of meals of varying starch and fiber content on gene expression

of glucose transporter type-4 (GLUT-4) in skeletal muscle after exercise (see Chapter 6, and 7.5 below). GLUT-4 is the predominant glucose transporter in muscle, which is required for uptake of glucose into the muscle fibers and subsequent oxidation or deposition as glycogen. We hypothesized that exercise induced muscle glycogen depletion would increase GLUT-4 gene expression in equine skeletal muscle, and starch-rich meals would further enhance GLUT-4 gene expression when compared to isocaloric meals of roughage or feed withholding.

7.2. CONCLUSIONS

The following conclusions can be made based on the data presented in this dissertation:

1. Feeding a soluble carbohydrate-rich meal prior to exercise (~1/4 daily energy requirements) results in modest changes in substrate use during exercise characterized by increased muscle utilization of blood-borne glucose and whole-body carbohydrate oxidation, and decreased lipid oxidation when compared to an isocaloric fiber-rich meal (alfalfa cubes) or not feeding. Carbohydrate feedings did not produce a sparing of muscle glycogen utilization during exercise when compared to not feeding.
2. Feeding equal amounts of soluble carbohydrates (starch plus sugars) in the form of 3 different grains (cracked corn, steamed oat groats and rolled barley) to resting horses at approximately equal levels of starch plus sugar intake results in similar magnitude of glycemic response (assessed as glucose area under the curve over time). In

addition, the magnitude of the glycemic responses observed after ingestion of grains are approximately 60% of that after administration of an equivalent amount of glucose in the stomach.

3. Feeding of soluble carbohydrate-rich meals (~1/2 daily energy requirements as cracked corn) over 8 h following glycogen-depleting exercise augments whole-body rates of glucose availability and utilization, when compared to not feeding. However, the magnitude of increase is relatively low when compared to that observed in humans and dogs undergoing similar exercise and ingestion of sugar solutions after exercise. In addition, the greater rates of glucose availability and utilization in grain fed horses does not lead to substantial hastening of muscle glycogen replenishment, when compared to hay feeding or feed withholding after exercise.
4. Exercise induced muscle glycogen depletion in horses results in increased insulin responsive glucose transporter (GLUT-4) gene expression in skeletal muscle during the hours following exercise; however, ingestion of soluble carbohydrate-rich meals after exercise and associated greater serum insulin concentrations do not further enhance GLUT-4 gene expression.

7.3. REFERENCES

1. Farris JW, Hinchcliff KW, McKeever KH, et al. Glucose infusion increases maximal duration of prolonged treadmill exercise in Standardbred horses. *Equine Vet J Suppl* 1995:357-361.

2. Farris JW, Hinchcliff KW, McKeever KH, et al. Effect of tryptophan and of glucose on exercise capacity of horses. *J Appl Physiol* 1998;85:807-816.

3. Geor RJ, Hinchcliff KW, Sams RA. Glucose infusion attenuates endogenous glucose production and enhances glucose use of horses during exercise. *J Appl Physiol* 2000;88:1765-1776.

4. Geor RJ, Hinchcliff KW, McCutcheon LJ, et al. Epinephrine inhibits exogenous glucose utilization in exercising horses. *J Appl Physiol* 2000;88:1777-1790.

5. Bergström J, Hermansen L, Hultman E, et al. Diet, muscle glycogen and physical performance. *Acta Physiol Scand* 1967;71:140-150.

6. Lacombe VA, Hinchcliff KW, Geor RJ, et al. Muscle glycogen depletion and subsequent replenishment affect anaerobic capacity of horses. *J Appl Physiol* 2001;91:1782-1790.

7. Burke LM, Collier GR, Hargreaves M. Muscle glycogen storage after prolonged exercise: effect of the glycemic index of carbohydrate feedings. *J Appl Physiol* 1993;75:1019-1023.

8. Lacombe VA, Hinchcliff KW, Kohn CW, et al. Post-exercise feeding of meals of varying glycemic index affects muscle glycogen resynthesis in horses. *Am J Vet Res* 2004;in press.

LIST OF REFERENCES

1. Abe H, Morimatsu M, Nikami H, et al. Molecular cloning and mRNA expression of the bovine insulin-responsive glucose transporter (GLUT4). *J Anim Sci* 1997;75:182-188.

2. Abe H, Kawakita Y, Miyashige T, et al. Comparison of amino acid sequence of the C-terminal domain of insulin-responsive glucose transporter (GLUT4) in livestock mammals. *J Vet Med Sci* 1998;60:769-771.

3. Alexander F, Chowdhury AK. Enzymes in the ileal juice of the horse. *Nature* 1958;181:190.

4. Barroso I, Benito B, Garci-Jimenez C, et al. Norepinephrine, tri-iodothyronine and insulin upregulate glyceraldehyde-3-phosphate dehydrogenase mRNA during Brown adipocyte differentiation. *Eur J Endocrinol* 1999;141:169-179.

5. Bergh U, Thorstensson A, Sjodin B, et al. Maximal oxygen uptake and muscle fiber types in trained and untrained humans. *Med Sci Sports* 1978;10:151-154.

6. Bergström J, Hermansen L, Hultman E, et al. Diet, muscle glycogen and physical performance. *Acta Physiol Scand* 1967;71:140-150.

7. Boileau RA, Horswill CA. Body composition in Sports: Measurement and Applications for Weight Loss and Gain In: Garrett WE,Kirkendall DT, eds. *Exercise and sport science*. Philadelphia: Lippincott Williams & Wilkins, 2000;319-338.

8. Borggreve GJ, van Kempen GJ, Cornelissen JP, et al. The net energy content of pig feeds according to the rostock formula the value of starch in the feed. *Z Tierphysiol Tierernahr Futtermittelkd* 1975;34:199-204.

9. Bowtell JL, Gelly K, Jackman ML, et al. Effect of different carbohydrate drinks on whole body carbohydrate storage after exhaustive exercise. *J Appl Physiol* 2000;88:1529-1536.

10. Brooks GA, Mercier J. Balance of carbohydrate and lipid utilization during exercise: the "crossover" concept. *J Appl Physiol* 1994;76:2253-2261.

11. Burke LM, Collier GR, Hargreaves M. Muscle glycogen storage after prolonged exercise: effect of the glycemic index of carbohydrate feedings. *J Appl Physiol* 1993;75:1019-1023.

12. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 2000;25:169-193.

13. Charron MJ, Katz EB, Olson AL. GLUT4 gene regulation and manipulation. *J Biol Chem* 1999;274:3253-3256.

14. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-159.

15. Coggan AR, Coyle EF. Reversal of Fatigue During Prolonged Exercise by Carbohydrate Infusion or Ingestion. *Journal of Applied Physiology* 1987;63:2388-2395.

16. Coggan AR, Coyle EF. Effect of Carbohydrate Feedings During High-Intensity Exercise. *Journal of Applied Physiology* 1988;65:1703-1709.

17. Coggan AR, Kohrt WM, Spina RJ, et al. Endurance training decreases plasma glucose turnover and oxidation during moderate-intensity exercise in men. *J Appl Physiol* 1990;68:990-996.

18. Comline RS, Hall LW, Hickson JCD, et al. Pancreatic secretion in the horse. *Journal of Physiology-London* 1969:10P-11P.

19. Costill DL, Sherman WM, Fink WJ, et al. The Role of Dietary Carbohydrates in Muscle Glycogen Resynthesis after Strenuous Running. *American Journal of Clinical Nutrition* 1981;34:1831-1836.

20. Coyle EF, Hagberg JM, Hurley BF, et al. Carbohydrate Feeding During Prolonged Strenuous Exercise Can Delay Fatigue. *Journal of Applied Physiology* 1983;55:230-235.

21. Coyle EF, Coggan AR, Hemmert MK, et al. Muscle Glycogen Utilization During Prolonged Strenuous Exercise When Fed Carbohydrate. *Journal of Applied Physiology* 1986;61:165-172.

22. Coyle EF, Jeukendrup AE, Wagenmakers AJM, et al. Fatty acid oxidation is

directly regulated by carbohydrate metabolism during exercise. *American Journal of Physiology-Endocrinology and Metabolism* 1997;36:E268-E275.

23. Cupp C. How to determine energy in horse diets. *Feed Management* 2000;51:17-18.

24. Davie AJ, Evans DL, Hodgson DR, et al. The effects of an oral glucose polymer on muscle glycogen resynthesis in standardbred horses. *J Nutr* 1994;124:2740S-2741S.

25. Davie AJ, Evans DL, Hodgson DR, et al. Effects of intravenous dextrose infusion on muscle glycogen resynthesis after intense exercise. *Equine Veterinary Journal* 1995;18:195-198.

26. Davie AJ, Evans DL, Hodgson DR, et al. Effects of muscle glycogen depletion on some metabolic and physiological responses to submaximal treadmill exercise. *Can J Vet Res* 1999;63:241-247.

27. De Fombelle A, Frumholtz P, Poillion D, et al. Effect of the botanical origin of starch on its prececal digestibility measured with the mobile bag technique. 17th Equine Nutrition and Physiology Symposium 2001;153-155.

28. Dubois M, Gilles KA, Hamilton JK, et al. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956;28:350-356.

29. Eaton MD, Evans DL, Hodgson DR, et al. Effect of treadmill incline and speed on metabolic rate during exercise in thoroughbred horses. *J Appl Physiol* 1995;79:951-957.

30. Essén -Gustavsson B, Karlstrom K, Lindholm A. Fibre types, enzyme activities and substrate utilisation in skeletal muscles of horses competing in endurance rides. *Equine Vet J* 1984;16:197-202.

31. Essén -Gustavsson B, McMiken D, Karlstrom K, et al. Muscular adaptation of horses during intensive training and detraining. *Equine Vet J* 1989;21:27-33.

32. Essén-Gustavsson B, Blomstrand E, Karlström K, et al. Influence of diet on substrate metabolism during exercise. Equine Exercise Physiology 3 1991;288-298.

33. Farris JW, Hinchcliff KW, McKeever KH, et al. Glucose infusion increases maximal duration of prolonged treadmill exercise in Standardbred horses. *Equine Vet J Suppl* 1995:357-361.

34. Farris JW, Hinchcliff KW, McKeever KH, et al. Effect of tryptophan and of glucose on exercise capacity of horses. *J Appl Physiol* 1998;85:807-816.

35. Febbraio MA, Stewart KL. CHO feeding before prolonged exercise: Effect of glycemic index on muscle glycogenolysis and exercise performance. *Journal of Applied Physiology* 1996;81:1115-1120.

36. Febbraio MA, Keenan J, Angus DJ, et al. Preexercise carbohydrate ingestion, glucose kinetics, and muscle glycogen use: effect of the glycemic index. *Journal of Applied Physiology* 2000;89:1845-1851.

37. Fedak MA, Rome L, Seeherman HJ. One-Step N2-Dilution Technique for Calibrating Open-Circuit Vo2 Measuring Systems. *Journal of Applied Physiology* 1981;51:772-776.

38. Ferrannini E. The Theoretical Bases of Indirect Calorimetry - a Review. *Metabolism-Clinical and Experimental* 1988;37:287-301.

39. Fink RI, Wallace P, Brechtel G, et al. Evidence that glucose transport is rate-limiting for in vivo glucose uptake. *Metabolism* 1992;41:897-902.

40. Frayn KN. Calculation of Substrate Oxidation Rates In vivo from Gaseous Exchange. *Journal of Applied Physiology* 1983;55:628-634.

41. Freestone JF, Shoemaker K, Bessin R, et al. Insulin and glucose response following oral glucose administration in well-conditioned ponies. *Equine Vet J Suppl* 1992:13-17.

42. Fukumoto H, Kayano T, Buse JB, et al. Cloning and characterization of the major insulin-responsive glucose transporter expressed in human skeletal muscle and other insulin-responsive tissues. *J Biol Chem* 1989;264:7776-7779.

43. Gastaldelli A, Coggan AR, Wolfe RR. Assessment of methods for improving tracer estimation of non-steady-state rate of appearance. *J Appl Physiol* 1999;87:1813-1822.

44. Geor RJ, Hinchcliff KW, Sams RA. β-adrenergic blockade augments glucose utilization in horses during graded exercise. *J Appl Physiol* 2000;89:1086-1098.

45. Geor RJ, Hinchcliff KW, Sams RA. Glucose infusion attenuates endogenous glucose production and enhances glucose use of horses during exercise. *J Appl Physiol* 2000;88:1765-1776.

46. Geor RJ, Hinchcliff KW, McCutcheon LJ, et al. Epinephrine inhibits exogenous glucose utilization in exercising horses. *J Appl Physiol* 2000;88:1777-1790.

47. Geor RJ, McCutcheon LJ, Hinchcliff KW, et al. Training-induced alterations in

glucose metabolism during moderate-intensity exercise. *Equine Vet J Suppl* 2002:22-28.

48. Gibaldi M, Perrier D. *Pharmacokinetics*. 2nd rev. and expand ed. New York: M. Dekker, 1982.

49. Gleeson M, Maughan RJ, Greenhaff PL. Comparison of the Effects of Pre-Exercise Feeding of Glucose, Glycerol and Placebo on Endurance and Fuel Homeostasis in Man. *European Journal of Applied Physiology and Occupational Physiology* 1986;55:645-653.

50. Goodpaster BH, Costill DL, Fink WJ, et al. The effects of pre-exercise starch ingestion on endurance performance. *International Journal of Sports Medicine* 1996;17:366-372.

51. Gunn HM. Muscle, bone and fat proportions and muscle distribution of thoroughbreds and other horses. Equine Exercise Physiology 2 1987;253-264.

52. Hargreaves M, Costill DL, Fink WJ, et al. Effect of Pre-Exercise Carbohydrate Feedings on Endurance Cycling Performance. *Medicine and Science in Sports and Exercise* 1987;19:33-36.

53. Hargreaves M, Costill DL, Katz A, et al. Effect of Fructose Ingestion on Muscle Glycogen Usage During Exercise. *Medicine and Science in Sports and Exercise* 1985;17:360-363.

54. Harris RC, Marlin DJ, Snow DH. Metabolic response to maximal exercise of 800 and 2,000 m in the thoroughbred horse. *J Appl Physiol* 1987;63:12-19.

55. Harris P. Energy sources and requirements of the exercising horse. *Annu Rev Nutr* 1997;17:185-210.

56. Hawley JA, Schabort EJ, Noakes TD, et al. Carbohydrate-loading and exercise performance. An update. *Sports Med* 1997;24:73-81.

57. Henneke DR, Potter GD, Kreider JL, et al. Relationship between condition score, physical measurements and body fat percentage in mares. *Equine Vet J* 1983;15:371-372.

58. Hinchcliff KW, McKeever KH, Muir WW, 3rd, et al. Effect of furosemide and weight carriage on energetic responses of horses to incremental exertion. *Am J Vet Res* 1993;54:1500-1504.

59. Hodgson DR, Rose RJ, Allen JR. Muscle glycogen depletion and repletion patterns

in horses performing various distances of endurance exercise. Equine Exercise Physiology 1983;229-236.

60. Hodgson DR, Rose RJ, Allen JR, et al. Glycogen depletion patterns in horses performing maximal exercise. *Res Vet Sci* 1984;36:169-173.

61. Hoekstra KE, Newman K, Kennedy MAP, et al. Effect of corn processing on glycemic response in horses. 16th Equine Nutrition and Physiology Symposium 1999;144-148.

62. Hoffman RM, Boston RC, Stefanovski D, et al. Obesity and diet affect glucose dynamics and insulin sensitivity in Thoroughbred geldings. *J Anim Sci* 2003;81:2333-2342.

63. Holm J, Björck I, Drews A, et al. A rapid method for the analysis of starch. *Starch/die starke* 1986;7:224-226.

64. Hoppeler H. Muscle aerobic potential in the animal kingdom. In: Saltin B, ed. *Biochemistry of Exercise VI, Intenational Series on Sport Sciences*. Champaign, IL: Human Kinetics Publishers, 1985;417-434.

65. Hoppeler H, Jones JH, Lindstedt SL, et al. Relating maximal oxygen consumption to skeletal muscle mitochondria in horses. Equine Exercise Physiology 2 1987;278-289.

66. Horowitz JF, MoraRodriguez R, Byerley LO, et al. Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. *American Journal of Physiology-Endocrinology and Metabolism* 1997;36:E768-E775.

67. Hyyppä S, Rasanen LA, Pösö AR. Resynthesis of glycogen in skeletal muscle from standardbred trotters after repeated bouts of exercise. *Am J Vet Res* 1997;58:162-166.

68. Hyyppä S, Saastamoinen M, Pösö AR. Effect of a post exercise fat-supplemented diet on muscle glycogen repletion. *Equine Veterinary Journal Suppl* 1999;30:493-498.

69. Ivy JL, Katz AL, Cutler CL, et al. Muscle glycogen synthesis after exercise: effect of time of carbohydrate ingestion. *J Appl Physiol* 1988;64:1480-1485.

70. Ivy JL. Glycogen resynthesis after exercise: effect of carbohydrate intake. *Int J Sports Med* 1998;19 Suppl 2:S142-145.

71. Jacobs KA, Sherman WM. The efficacy of carbohydrate supplementation and

chronic high- carbohydrate diets for improving endurance performance. *International Journal of Sport Nutrition* 1999;9:92-115.

72. James DE, Strube M, Mueckler M. Molecular cloning and characterization of an insulin-regulatable glucose transporter. *Nature* 1989;338:83-87.

73. Jenkins DJ, Wolever TM, Taylor RH, et al. Glycemic index of foods: a physiological basis for carbohydrate exchange. *Am J Clin Nutr* 1981;34:362-366.

74. Jenkins DJ, Kendall CW, Augustin LS, et al. Glycemic index: overview of implications in health and disease. *Am J Clin Nutr* 2002;76:266S-273S.

75. Jeukendrup AE, Raben A, Gijsen A, et al. Glucose kinetics during prolonged exercise in highly trained human subjects: effect of glucose ingestion. *Journal of Physiology-London* 1999;515:579-589.

76. Johnson PJ. Peripheral Cushingoid Syndrome ('Equine Metabolic Syndrome') In: Robinson NE, ed. *Current therapy in equine medicine*. 5th ed. Philadelphia, PA: W. B. Saunders, 2003;812-816.

77. Jones JP, Dohm GL. Regulation of glucose transporter GLUT-4 and hexokinase II gene transcription by insulin and epinephrine. *Am J Physiol* 1997;273:E682-687.

78. Jose-Cunilleras E, Hinchcliff KW, Sams RA, et al. Glycemic index of a meal fed before exercise alters substrate use and glucose flux in exercising horses. *J Appl Physiol* 2002;92:117-128.

79. Jose-Cunilleras E, Hinchcliff KW, Lacombe VA, et al. Ingestion of high-glycemic meals after exercise increases glucose flux but fails to enhance muscle glycogen synthesis in horses. *J Appl Physiol* 2004;submitted.

80. Kaestner KH, Christy RJ, McLenithan JC, et al. Sequence, tissue distribution, and differential expression of mRNA for a putative insulin-responsive glucose transporter in mouse 3T3-L1 adipocytes. *Proc Natl Acad Sci U S A* 1989;86:3150-3154.

81. Keinzle E, Pohlenz J, Radicke S. Morphology of starch digestion in the horse. *Zentralbl Veterinarmed A* 1997;44:207-221.

82. Kirwan JP, O'Gorman D, Evans WJ. A moderate glycemic meal before endurance exercise can enhance performance. *Journal of Applied Physiology* 1998;84:53-59.

83. Komi PV, Karlsson J. Skeletal muscle fibre types, enzyme activities and physical performance in young males and females. *Acta Physiol Scand* 1978;103:210-218.

84. Kuo CH, Browning KS, Ivy JL. Regulation of GLUT4 protein expression and glycogen storage after prolonged exercise. *Acta Physiol Scand* 1999;165:193-201.

85. Kuo CH, Hunt DG, Ding Z, et al. Effect of carbohydrate supplementation on postexercise GLUT-4 protein expression in skeletal muscle. *J Appl Physiol* 1999;87:2290-2295.

86. Lacombe V, Hinchcliff KW, Geor RJ, et al. Exercise that induces substantial muscle glycogen depletion impairs subsequent anaerobic capacity. *Equine Vet J Suppl* 1999;30:293-297.

87. Lacombe VA, Hinchcliff KW, Geor RJ, et al. Muscle glycogen depletion and subsequent replenishment affect anaerobic capacity of horses. *J Appl Physiol* 2001;91:1782-1790.

88. Lacombe VA, Hinchcliff KW, Devor ST. Effects of exercise and glucose administration on content of insulin-sensitive glucose transporter in equine skeletal muscle. *Am J Vet Res* 2003;64:1500-1506.

89. Lacombe VA, Hinchcliff KW, Taylor LE. Interactions of substrate availability, exercise performance, and nutrition with muscle glycogen metabolism in horses. *Journal of the American Veterinary Medical Association* 2003;223:1576-1585.

90. Lacombe VA, Hinchcliff KW, Kohn CW, et al. Post-exercise feeding of meals of varying glycemic index affects muscle glycogen resynthesis in horses. *Am J Vet Res* 2004;in press.

91. Lawrence L, Soderholm LV, Roberts A, et al. Feeding Status Affects Glucose-Metabolism in Exercising Horses. *Journal of Nutrition* 1993;123:2152-2157.

92. Lawrence LM, Williams J, Soderholm LV, et al. Effect of feeding state on the response of horses to repeated bouts of intense exercise. *Equine Vet J* 1995;27:27-30.

93. Lee JS, Bruce CR, Tunstall RJ, et al. Interaction of exercise and diet on GLUT-4 protein and gene expression in Type I and Type II rat skeletal muscle. *Acta Physiol Scand* 2002;175:37-44.

94. Lewis LD. Water, energy, protein, carbohydrates, and fats for horses. *Equine Clinical Nutrition: feeding and care*. Baltimore, USA: Williams and Wilkins, 1995;3-24.

95. Lindholm A, Piehl K. Fibre composition, enzyme activity and concentrations of

metabolites and electrolytes in muscles of standardbred horses. *Acta Vet Scand* 1974;15:287-309.

96. Lindstedt SL, Hokanson JF, Wells DJ, et al. Running energetics in the pronghorn antelope. *Nature* 1991;353:748-750.

97. Ludbrook J. On making multiple comparisons in clinical and experimental pharmacology and physiology. *Clin Exp Pharmacol Physiol* 1991;18:379-392.

98. MacLean PS, Zheng D, Dohm GL. Muscle glucose transporter (GLUT 4) gene expression during exercise. *Exerc Sport Sci Rev* 2000;28:148-152.

99. Marchand I, Chorneyko K, Tarnopolsky M, et al. Quantification of subcellular glycogen in resting human muscle: granule size, number, and location. *J Appl Physiol* 2002;93:1598-1607.

100. Marmy-Conus N, Fabris S, Proietto J, et al. Preexercise glucose ingestion and glucose kinetics during exercise. *Journal of Applied Physiology* 1996;81:853-857.

101. McCutcheon LJ, Geor RJ, Hinchcliff KW. Changes in skeletal muscle GLUT4 content and muscle membrane glucose transport following 6 weeks of exercise training. *Equine Vet J Suppl* 2002:199-204.

102. McNeel RL, Ding ST, Smith EO, et al. Effect of feed restriction on adipose tissue transcript concentrations in genetically lean and obese pigs. *J Anim Sci* 2000;78:934-942.

103. Meyer H, Radicke S, Kienzle E, et al. Investigations on preileal digestion of starch from grain, potato and manioc in horses. *Zentralbl Veterinarmed A* 1995;42:371-381.

104. Meyer H, Radicke S, Kienzle E, et al. Investigations on preileal digestion of oats, corn, and barley starch in relation to grain processing. 13th Equine Nutrition and Physiology Symposium 1993;92-97.

105. Milliken GA, Johnson DE. *Analysis of messy data*. New York: Van Nostrand Reinhold, 1992.

106. Mueckler M. Family of glucose-transporter genes. Implications for glucose homeostasis and diabetes. *Diabetes* 1990;39:6-11.

107. Murakami T, Shimomura Y, Fujitsuka N, et al. Enzymatic and genetic adaptation of soleus muscle mitochondria to physical training in rats. *Am J Physiol* 1994;267:E388-395.

108. Murray SM, Flickinger EA, Patil AR, et al. In vitro fermentation characteristics of native and processed cereal grains and potato starch using ileal chyme from dogs. *J Anim Sci* 2001;79:435-444.

109. National Research Council (U.S.). Subcommittee on Horse Nutrition. *Nutrient requirements of horses*. 5th rev. ed. Washington, D.C.: National Academy Press, 1989.

110. Navarrete Santos A, Kirstein M, Fischer B. Two insulin-responsive glucose transporter isoforms and the insulin receptor are developmentally expressed in rabbit preimplantation embryos: NCBI GenBank, 2003.

111. Nimmo MA, Snow SH. Changes in muscle glycogen, lactate and pyruvate concentrations in the thoroughbred horse following maximal exercise. Equine Exercise Physiology 1983;237-244.

112. Nout YS, Hinchcliff KW, Jose-Cunilleras E, et al. Effect of moderate exercise immediately followed by induced hyperglycemia on gene expression and content of the glucose transporter-4 protein in skeletal muscles of horses. *Am J Vet Res* 2003;64:1401-1408.

113. Oldham Sl, Potter GD, Evans JW, et al. Storage and mobilization of muscle glycogen in exercising horses fed a fat-supplemented diet. 11th Equine Nutrition and Physiology Symposium 1989;57-62.

114. Pagan JD, Hintz HF. Equine Energetics. II. Energy-Expenditure in Horses During Submaximal Exercise. *Journal of Animal Science* 1986;63:822-830.

115. Pagan JD, Essén-Gustavsson B, Lindholm A, et al. The effect of dietary energy source on exercise performance in Standardbred horses. Equine Exercise Physiology 2 1987;686-700.

116. Pagan JD, Burger I, Jackson SG. The influence of time of feeding on exercise response in Thoroughbreds fed a fat supplemented or high carbohydrate diet. 14th Equine Nutrition and Physiology Symposium 1995;92-93.

117. Pagan JD, Harris PA. The effects of timing and amount of forage and grain on exercise response in thoroughbred horses. *Equine Vet J Suppl* 1999;30:451-457.

118. Pagan JD, Harris PA, Kennedy MAP, et al. Feed type and intake affects glycemic response in thoroughbred horses. 16th Equine Nutrition and Physiology Symposium 1999;149-150.

119. Passonneau JV, Lauderdale VR. A comparison of three methods of glycogen measurement in tissues. *Anal Biochem* 1974;60:405-412.

120. Patil SSB, Totey SSM. Partial cDNA sequence of glucose transporter type four of Bubalus bubalis: NCBI GenBank, 2000.

121. Pichon B, Mercan D, Pouillon V, et al. Canis familiaris mRNA for partial Insulin-responsive glucose transporter, glut4 gene.: NCBI GenBank, 2001.

122. Potter GD, Arnold FF, Householder DD, et al. Digestion of starch in the small or large intestine of the equine. *Pferdeheilkunde, Proceedings of the 1st European Conference of Horse Nutrition* 1992:107-111.

123. Powell DM, Reedy SE, Sessions DR, et al. Effect of short-term exercise training on insulin sensitivity in obese and lean mares. *Equine Vet J Suppl* 2002:81-84.

124. Quiroz-Rothe E, Rivero JL. Co-ordinated expression of contractile and non-contractile features of control equine muscle fibre types characterised by immunostaining of myosin heavy chains. *Histochem Cell Biol* 2001;116:299-312.

125. Radicke S, Landes E, Kienzle E, et al. Aktivitat der amylase im Dunndarmchymus in Abhangigkeit von der Futterart [in German, English summary]. *Pferdeheilkunde, Proceedings of the 1st European Conference of Horse Nutrition* 1992:99.

126. Radicke S, Kienzle E, Meyer H. Preileal apparent digestibility of oats and corn starch and consequences for cecal metabolism. 12th Equine Nutrition and Physiology Symposium 1991;43-48.

127. Ralston SL. Effect of soluble carbohydrate content of pelleted diets on postprandial glucose and insulin profiles in horses. *Pferdeheilkunde* 1992;8:112-115.

128. Ren JM, Marshall BA, Gulve EA, et al. Evidence from transgenic mice that glucose transport is rate-limiting for glycogen deposition and glycolysis in skeletal muscle. *J Biol Chem* 1993;268:16113-16115.

129. Ren JM, Semenkovich CF, Gulve EA, et al. Exercise induces rapid increases in GLUT4 expression, glucose transport capacity, and insulin-stimulated glycogen storage in muscle. *J Biol Chem* 1994;269:14396-14401.

130. Richter EA, Ruderman NB, Gavras H, et al. Muscle glycogenolysis during exercise: dual control by epinephrine and contractions. *Am J Physiol* 1982;242:E25-32.

131. Rivero JL, Talmadge RJ, Edgerton VR. Correlation between myofibrillar ATPase activity and myosin heavy chain composition in equine skeletal muscle and the

influence of training. *Anat Rec* 1996;246:195-207.

132. Roberts TJ, Weber JM, Hoppeler H, et al. Design of the oxygen and substrate pathways. II. Defining the upper limits of carbohydrate and fat oxidation. *J Exp Biol* 1996;199 (Pt 8):1651-1658.

133. Rodiek A, Bonvicin S, Stull C, et al. Glycemic and endocrine responses to corn or alfalfa fed prior to exercise. Equine Exercise Physiology 3 1991;323-330.

134. Romijn JA, Coyle EF, Hibbert J, et al. Comparison of indirect calorimetry and a new breath 13C/12C ratio method during strenuous exercise. *Am J Physiol* 1992;263:E64-71

135. Romijn JA, Coyle EF, Sidossis LS, et al. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am J Physiol* 1993;265:E380-391.

136. Romijn JA, Klein S, Coyle EF, et al. Strenuous endurance training increases lipolysis and triglyceride-fatty acid cycling at rest. *J Appl Physiol* 1993;75:108-113.

137. Romijn JA, Coyle EF, Sidossis LS, et al. Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. *J Appl Physiol* 1995;79:1939-1945.

138. Romijn JA, Coyle EF, Sidossis LS, et al. Substrate metabolism during different exercise intensities in endurance-trained women. *J Appl Physiol* 2000;88:1707-1714.

139. Rose RJ, Hodgson DR, Kelso TB, et al. Maximum O2 uptake, O2 debt and deficit, and muscle metabolites in Thoroughbred horses. *J Appl Physiol* 1988;64:781-788.

140. Rose RJ, Knight PK, Bryden WL. Energy use and cardiorespiratory responses to prolonged submaximal exercise. Equine Exercise Physiology 3 1991;281-287.

141. Rose AJ, Howlett K, King DS, et al. Effect of prior exercise on glucose metabolism in trained men. *Am J Physiol Endocrinol Metab* 2001;281:E766-771.

142. Saltin B, Astrand PO. Maximal oxygen uptake in athletes. *J Appl Physiol* 1967;23:353-358.

143. Seeherman HJ, Morris EA. Methodology and repeatability of a standardised treadmill exercise test for clinical evaluation of fitness in horses. *Equine Vet J Suppl* 1990:20-25.

144. Shepherd PR, Kahn BB. Glucose transporters and insulin action--implications for insulin resistance and diabetes mellitus. *N Engl J Med* 1999;341:248-257.

145. Sherman WM, Brodowicz G, Wright DA, et al. Effects of 4 h Preexercise Carbohydrate Feedings on Cycling Performance. *Medicine and Science in Sports and Exercise* 1989;21:598-604.

146. Sherman WM, Peden MC, Wright DA. Carbohydrate Feedings 1-H before Exercise Improves Cycling Performance. *American Journal of Clinical Nutrition* 1991;54:866-870.

147. Simmons HA, Ford EJH. Gluconeogenesis from Propionate Produced in the Colon of the Horse. *British Veterinary Journal* 1991;147:340-345.

148. Snow DH, Baxter P, Rose RJ. Muscle fibre composition and glycogen depletion in horses competing in an endurance ride. *Vet Rec* 1981;108:374-378.

149. Snow DH, Guy PS. Fibre type and enzyme activities of the gluteus medius in different breeds of horse. In: Poortmans J,Niset G, eds. *Biochemistry of Exercise IV-B*. Baltimore,: University Park Press, 1981;275-282.

150. Snow DH, Kerr MG, Nimmo MA, et al. Alterations in blood, sweat, urine and muscle composition during prolonged exercise in the horse. *Vet Rec* 1982;110:377-384.

151. Snow DH, Harris RC, Harman JC, et al. Glycogen repletion following different diets. Equine Exercise Physiology 2 1987;701-706.

152. Snow DH, Harris RC. Effects of daily exercise on muscle glycogen in the Thoroughbred horse. Equine Exercise Physiology 3 1991;299-304.

153. Sparks MJ, Selig SS, Febbraio MA. Pre-exercise carbohydrate ingestion: effect of the glycemic index on endurance exercise performance. *Medicine and Science in Sports and Exercise* 1998;30:844-849.

154. Starritt EC, Howlett RA, Heigenhauser GJ, et al. Sensitivity of CPT I to malonyl-CoA in trained and untrained human skeletal muscle. *Am J Physiol Endocrinol Metab* 2000;278:E462-468.

155. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci* 1959;82:420-430.

156. Stull CL, Rodiek AV. Responses of blood glucose, insulin and cortisol concentrations to common equine diets. *J Nutr* 1988;118:206-213.

157. Stull C, Rodiek A. Effects of postprandial interval and feed type on substrate availability during exercise. *Equine Vet J Suppl* 1995;18:362-366.

158. Thomas DE, Brotherhood JR, Brand JC. Carbohydrate Feeding before Exercise - Effect of Glycemic Index. *International Journal of Sports Medicine* 1991;12:180-186.

159. Topliff DR, Potter GD, Dutson TR, et al. Diet manipulation and muscle glycogen in the equine. 8th Equine Nutrition and Physiology Symposium 1983;119-124.

160. Topliff DR, Potter GD, Kreider JL, et al. Diet manipulation, muscle glycogen metabolism and anaerobic work performance in the equine. 9th Equine Nutrition and Physiology Symposium 1985;224-229.

161. Tsintzas OK, Williams C, Wilson W, et al. Influence of carbohydrate supplementation early in exercise on endurance running capacity. *Medicine and Science in Sports and Exercise* 1996;28:1373-1379.

162. Valberg S. Glycogen depletion patterns in the muscle of standardbred trotters after exercise of varying intensities and durations. *Equine Vet J* 1986;18:479-484.

163. Wadley GD, Tunstall RJ, Sanigorski A, et al. Differential effects of exercise on insulin-signaling gene expression in human skeletal muscle. *J Appl Physiol* 2001;90:436-440.

164. Wagner PD. Determinants of VO_{2max}: man vs. horse. *Journal of Equine Veterinary Science* 1995;15:398-404.

165. Walker JA, Krehbiel CR, Harmon DL, et al. Effects of slaframine and 4-diphenylacetoxy-N-methylpiperidine methiodide (4DAMP) on pancreatic exocrine secretion in the bovine. *Can J Physiol Pharmacol* 1994;72:39-44.

166. Webb AI, Weaver BM. Body composition of the horse. *Equine Vet J* 1979;11:39-47.

167. Wee SL, Williams C, Gray S, et al. Influence of high and low glycemic index meals on endurance running capacity. *Medicine and Science in Sports and Exercise* 1999;31:393-399.

168. Weiss WP. Symposium: prevailing concepts in energy utilization by ruminants. Predicting energy values of feeds. *J Dairy Sci* 1993;76:1802-1811.

169. Williams CA, Kronfeld DS, Staniar WB, et al. Plasma glucose and insulin

responses of Thoroughbred mares fed a meal high in starch and sugar or fat and fiber. *J Anim Sci* 2001;79:2196-2201.

170. Wolever TM. The glycemic index. World Rev Nutr Diet 1990;62:120-185.

171. Wolfe RR. Radioactive and stable isotope tracers in biomedicine : principles and practice of kinetic analysis. New York: Wiley-Liss, 1992.

172. Wright DA, Sherman WM, Dernbach AR. Carbohydrate Feedings before, During, or in Combination Improve Cycling Endurance Performance. *Journal of Applied Physiology* 1991;71:1082-1088.