MUSCLE GLYCOGEN METABOLISM IN HORSES:
INTERACTIONS BETWEEN SUBSTRATE AVAILABILITY, EXERCISE
PERFORMANCE AND CARBOHYDRATE ADMINISTRATION

DISSERTATION

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ABSTRACT

Horses undertake recreational activities that decrease muscle glycogen stores. Similar to humans, muscle glycogen availability may influence exercise performance. In the first study, a model of glycogen depletion was developed and used to investigate the effect of muscle glycogen availability on the athletic capacity of horses. We demonstrated that exercise-induced muscle glycogen depletion was associated with a 60% decrease in anaerobic capacity during subsequent exercise, without effect on aerobic capacity. To rule out any confounding effects of other exercise-induced changes, a second study investigated the effects of muscle glycogen depletion and subsequent replenishment on athletic capacity. In a blinded crossover study, seven fit horses received either an intravenous glucose or saline infusion after exercise. Run time to fatigue, accumulated oxygen deficit and blood lactate concentration during a subsequent high-speed exercise test were greater for the horses with normal muscle glycogen concentration than for the horses with persistent muscle glycogen depletion. It was concluded that muscle glycogen availability influences anaerobic capacity and ability to perform high-intensity exercise in horses.
Given the critical role of adequate glycogen stores in enabling optimal performance, the factors and mechanisms affecting muscle glycogen replenishment in horses were studied. We first demonstrated that intravenous glucose infusion (6g/kg) hastened muscle glycogen resynthesis compared to saline infusion. The effect of 3 isoenergetic diets of varying glycemic indices on the rates of muscle glycogen synthesis after exercise was evaluated in a crossover study. Seven horses received either a high soluble carbohydrate diet (grain, HCO), or a low soluble carbohydrate diet (hay, LCO), or a mixed diet (M) every 8 hours for 72 hours after exercise. We demonstrated that muscle glycogen resynthesis is slower in horses than in humans. Feeding high-glycemic index (HCO) meals hastened muscle glycogen replenishment compared to LCO and MCO diets by increasing blood glucose and insulin availability to skeletal muscle. Finally, insulin-sensitive glucose transporters (GLUT-4) were characterized in equine muscles by Western blotting. We demonstrated that exercise increased GLUT-4 protein content by 27%. Replenishment of muscle glycogen stores after carbohydrate administration, either by glucose infusion or diet, attenuated the increase in GLUT-4 protein content of skeletal muscle.
To Claude,

The one who makes it all worthwhile
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INTRODUCTION

As a performance animal, the utility of the horse depends on its ability to perform muscular work. While in the early 20th century the major use of the horse was in agriculture, today the horse is used largely for recreation. Horses today frequently perform a broad range of sporting activities such as endurance rides (up to 160 km), show jumping, racing (up to 3200 m), dressage, three-days events, rodeo, barrel racing, polo and conventional warm-up exercise. In all these activities, the principal factor limiting performance is the availability of energy substrate, namely glycogen in the skeletal muscle and liver, and triglycerides in the various fat reserves. In particular, glycogen is an important energy source for the muscle during both vigorous and prolonged exercise (Lindholm 1974; Essén-Gustavsson et al. 1984). For instance, repeated intense exercise or endurance exercise depletes muscle glycogen, up to 50%, in the muscle of humans and horses (Ahlborg et al. 1967; Harris et al. 1987; Snow and Harris 1991). Reduced muscle glycogen stores have been associated with impairment of performance during brief, intense exercise, and earlier onset of fatigue during endurance exercise in humans (Ahlborg et al. 1967; Casey et al. 1996). However, the conclusions drawn from human exercise physiology could not be applied to horses because of the physiological differences, especially in regard to carbohydrate metabolism. Similar to humans, horses
rely heavily on carbohydrate metabolism during high-intensity exercise where glycogen and glucose are the obligate substrates to sustain anaerobic ATP production. However, horses have approximately two-fold greater muscle glycogen concentrations (Snow and Valberg 1994), and they have a greater capacity for energy production via aerobic and anaerobic metabolism compared to humans (Wagner 1995; Shuback and Essén-Gustavsson 1998). Furthermore, when compared to humans, the athletic capacity of horses may be limited to a greater extent by muscle glycogen stores because complete resynthesis of the muscle glycogen pool requires 48-72 hours after exercise (Snow and Harris 1991; Hyyppä et al. 1997). Because many horses undertake several competitions in a single day or on consecutive days, the interval between exercise bouts may be inadequate for complete or partial restoration of muscle glycogen stores. Therefore, it is possible that a reduction in muscle glycogen stores contributes to a decline in subsequent exercise performance in horses, although this important issue has received scarce attention. No study has conclusively demonstrated that manipulation of endogenous energy stores from carbohydrate affects either aerobic or anaerobic capacity of horses. While the observation from Farris et al. (1995) that provision of supplemental glucose to horses performing endurance exercise increases the duration of exercise is interesting and provides evidence that energy supplies limit performance in endurance exercise of horses, most athletic events involving horses are brief and require repeated bouts of exercise in shorter periods of time. Therefore, the first main question is whether decreased muscle glycogen availability is a contributing factor to fatigue during high-intensity exercise. To our knowledge, there are no studies that have conclusively demonstrated an
effect of manipulation of muscle glycogen stores on exercise performance of horses. Therefore, the effect of depletion and replenishment of muscle glycogen concentration on indices of anaerobic and aerobic capacities was examined by developing a glycogen-depleting exercise appropriate for horses (Chapters 2 and 3). The manipulations of muscle glycogen concentration are intended to maximize our ability to detect an effect of depletion and restoration of muscle glycogen on athletic capacity. The hypothesis was that manipulation of muscle glycogen concentration would affect exercise performance during high-intensity exercise in horses.

The equine industry uses an amount of feed each year equivalent to 40% of that of the broiler chick industry and 18% of that of the feedlot cattle industry (Stanton 1996). Horse owners in New Jersey, New York, Pennsylvania and Michigan spend between $35 and $91 million each year feeding horses undertaking various athletic events such as racing, endurance riding, and jumping (Stanton 1996). While most of these athletic activities decrease muscle glycogen stores, there are few scientific studies that investigate nutritional strategies to optimize muscle glycogen stores of horses in training and competition. In contrast, the effects of diet composition on energy availability and exercise capacity have been well investigated in human exercise physiology (Neufer et al. 1987). For instance, the rapid replenishment of body carbohydrate reserves immediately after exercise by carbohydrate supplementation is critical to the athlete undertaking multiple activities in a single day, or over several consecutive days, during intense training and for the daily restoration of these carbohydrate stores (Sherman 1995).
Given the number of horses involved (between 5-7 million in USA, Stanton 1996), and the fact that nutrition and feeding may markedly affect energy substrate, and the economic impact on the industry, we believe that studying the interaction between nutrition and muscle substrate availability, especially factors affecting replenishment of glycogen stores is clearly indicated in horses. In particular, if a detrimental effect of muscle glycogen depletion on exercise performance is demonstrated, then amelioration of replenishment of energy substrate stores after exercise might confer significant advantages in the welfare and performance of horses competing on successive days or in several activities during the same day. Therefore, the second question was whether **muscle glycogen replenishment could be enhanced by dietary manipulation**. The effect of a fat supplemented diet on muscle glycogen stores remains controversial in horses, and no study has clearly demonstrated that providing glycogen precursors, either glucose or dietary carbohydrate, affects muscle glycogen synthesis (Davie et al. 1994; Hyyppä et al.1999; Pöösö and Hyyppä 1999). Therefore, we investigated the effect of feeding isocaloric diets with different soluble carbohydrate content on muscle glycogen resynthesis after exercise (Chapter 5). The **hypothesis was that feeding a high soluble carbohydrate diet would enhance muscle glycogen replenishment as compared to feeding a moderate or low soluble carbohydrate diet**. Successful completion of this study will provide clear evidence of the effect, or lack thereof, of diet on muscle glycogen stores in horses, and provide an enhanced understanding of the mechanisms of glycogen metabolism in horses. To investigate the mechanism underlying muscle glycogen and glucose metabolism, we characterized the insulin-sensitive glucose-
transporter GLUT-4 in equine muscle, and we studied the effect of exercise and carbohydrate administration on total GLUT-4 protein content (Chapters 4 and 5). Our hypothesis was that exercise and/or carbohydrate administration would increase total GLUT-4 protein content. Understanding the factors controlling muscle glycogen synthesis is pertinent not only to the nutrition of horses, but also to an understanding of diseases associated with abnormal muscle glycogen metabolism, such as polysaccharide storage myopathy. Furthermore, these results may also be of interest in comparative exercise physiology. After providing an overview of the literature related to muscle glycogen metabolism (Chapter 1), the studies designed to answer the questions raised in this section are described in detail (Chapters 2-5).
CHAPTER 1

LITERATURE REVIEW

Fatigue is a complex chain of events, including both central and peripheral contributions, and its mechanisms are incompletely understood. While factors including training, age and thermoregulation may contribute to the onset of fatigue, the principal factor limiting muscular performance is substrate availability, mainly glycogen in the skeletal muscle and liver, and triglycerides in the various fat reserves. For brief and intense exercise, anaerobic energy supplied by adenosine triphosphate (ATP) and phosphocreatine provides sufficient fuel for about 30 seconds (Soderlund et al. 1992). The factor limiting muscular performance for exercise of greater than 30 seconds is the availability of glucose to enter glycolytic flux in the case of anaerobic metabolism, and to enter the oxidative phosphorylation pathway, in the case of aerobic metabolism. Carbohydrate in the form of muscle glycogen is the primary energy source for anaerobic glycolysis and oxidative phosphorylation during vigorous exercise in humans (Hargreaves 1995). While intramuscular glycogen, with triglycerides, is also an important fuel during prolonged exercise by horses (Lindholm 1979; Essén-Gustavsson et al. 1984), the role of muscle glycogen as a critical fuel for contracting skeletal muscle during brief
and intense exercise has not been clearly demonstrated in horses. The purpose of this chapter is to review the interactions between muscle glycogen availability, exercise performance and nutrition in comparative exercise physiology with a special emphasis on horses. This chapter will first review the role of decreased muscle glycogen stores as a limiting factor for exercise both in humans and in horses. Secondly, it will review the effect of nutrition on muscle glycogen replenishment, in the context of nutritional strategies to optimize performance in humans and in horses. Finally, the regulation of glucose uptake in relation to muscle glycogenesis will be reviewed.

1.1. IMPORTANCE OF MUSCLE GLYCOGEN AS A FUEL DURING EXERCISE

1.1.1. Importance of muscle glycogen depletion during exercise

1.1.1.1 In humans

Exercise decreases the muscle glycogen concentration in humans. In a classic study, Gollnick and coworkers (1974) demonstrated the pattern of muscle glycogen depletion of humans in response to cycling exercise of varying duration and intensity. Exercise of relatively low intensity (31% of maximal aerobic power, called VO₂ max) to fatigue induced only mild depletion of muscle glycogen concentration, whereas the greatest decreases occurred with fatiguing exertion of 80 to 150% VO₂ max. At an exercise intensity of 150% VO₂ max, which was maintained for approximately 6 minutes, vastus
lateralis muscle glycogen concentration declined from 62 mmol/kg to 13 mmol/kg (Gollnick et al. 1974). The importance of this study is that it demonstrated that muscle glycogen concentration could be significantly decreased by relatively brief, but intense exercise. Furthermore, twenty five percent of the pre-exercise muscle glycogen can be depleted following a single 30 s cycle sprint (Boosbis et al. 1982). In conclusion, exercise reduces muscle glycogen concentration of humans. The magnitude of the depletion and the types of fibers (I, IIA, IIB) depleted depend upon the duration and intensity of fatiguing exercise (Piehl 1974; Gollnick et al. 1973, 1974).

1.1.1.2 In horses

Athletic horses frequently perform endurance exercise and multiple bouts of moderate to intense fatiguing exercise during show jumping, racing, polo, but also during conventional warm-up exercise. Similar to humans, running reduces the muscle glycogen concentration of horses (Lindholm et al. 1974; Hodgson et al. 1983, 1984a,b; Essén-Gustavsson et al. 1984; Snow et al. 1985; Miller and Lawrence 1986; White and Snow 1987; Harris et al. 1987; Essén-Gustavsson et al. 1989; Foreman et al. 1990; Snow and Harris 1991; Davie et al. 1995). Decreases in muscle glycogen concentration induced by fatiguing exercise are similar for prolonged submaximal running or repeated bouts of galloping (White and Snow 1987; Harris et al. 1987; Farris et al. 1995). A single bout of galloping 800 to 2000 m by Thoroughbred racehorses, or trotting 1600 m by Standardbred racehorses depleted muscle glycogen concentration by approximately 30% (Lindholm et al. 1974; Harris et al. 1987; Snow and Harris 1991), whereas four bouts of
shorter (620 m) galloping depleted muscle glycogen by 40% (Snow et al. 1985).

Participation in Day 2 (roads and tracks, the endurance test of the event) of a Three-day event resulted in a 60% decline in muscle glycogen concentration (Hodgson et al. 1984b). Muscle glycogen stores were depleted by 50 to 75% after an 80- or 160-km ride (Snow et al. 1981, 1982). Partial or complete depletion of the different fibers types of the gluteal muscle was noted after a 50 to 100 km endurance ride (Snow et al. 1981; Hodgson et al. 1983b; Essén–Gustavsson et al. 1984). Furthermore, hypoglycemia was observed when liver glycogen became depleted during 72 km of low-intensity exercise (Lindholm et al. 1974), suggesting that full muscle and liver glycogen stores are necessary for optimum performance during endurance rides (Snow et al. 1981; Essén-Gustavsson et al. 1984).

More recently, laboratory studies have demonstrated substantial depletion of muscle glycogen (50% to 65% reduction) with incremental intensity exercise on a treadmill at speeds inducing 50%, 70%, 90% of VO$_2$ max followed after a 30 min rest period by six 1 min sprints at 100% VO$_2$ max (Davie 1995; Davie et al. 1996). In summary, exercise of a variety of durations and intensities can result in up to a 60% reduction of muscle glycogen concentration in horses.

Previous warm up and training also affect muscle glycogen stores. For instance, warm-up exercise decreased the rate of muscle glycogen degradation and the anaerobic contribution to energy production during subsequent sprint exercise (McCutcheon et al. 1999). Furthermore, training exercise decreased muscle glycogen stores by approximately 25%, and this decrease persisted for 24-48 hours after exercise despite consumption of a normal diet (Snow and Harris 1991). Similarly, low muscle glycogen
concentrations have been reported in Standardbreds during the first months of intense exercise training due to the slow rate of glycogen resynthesis (Essén-Gustavsson et al. 1989). In contrast, many authors have reported an increase in resting muscle glycogen concentration, which resulted from an increased oxidative capacity of the muscle and a progressive decrease in the rate of glycogen utilization during submaximal exercise, in response to several weeks of exercise conditioning (Guy and Snow 1977; Hodgson et al. 1985; Essén-Gustavsson et al. 1989; Foreman et al. 1990; Gansen et al. 1999).

Glycogen utilization rate is related to work intensity: when trotting speed is increased from 5 m/s to 12.5 m/s during an incremental exercise test, the rate of glycogenolysis increases from 0.3 to 14 mmol/kg/min wet weight (ww) (Lindholm and Saltin 1974; Hodgson 1984; Davie et al. 1999), and the rate of glycogen utilization decreased with a decrease in work intensity (Nimmo and Snow 1983). Similar glycogen depletion rates are measured after 1000 m and 1600 m gallops (Snow and Harris 1991), or four gallops of 620 m (Snow et al. 1985). For repeated, brief intense exercise (e.g., trotting six times 400 m at maximal speed, Lindholm and Saltin 1974) or for single bouts of exercise of varying duration (Hodgson et al. 1983a; Nimmo and Snow 1983; Harris et al. 1987) glycogen utilization was most pronounced during the initial stages, or during the first bouts of high-intensity exercise. Indeed, muscle glycogen utilization has been reported as high as 160 mmol/kg/min dry weight (dw), reflecting the intense anaerobiosis that occurs during maximal exercise (Harris et al. 1987). It should be noted that glycogen utilization could also be affected by previous feeding. When horses were fed a high soluble carbohydrate diet prior to exercise, glycogen utilization during an exercise test
was higher than when horses were fasted (Lawrence et al. 1993). However, these results are controversial (Lawrence et al. 1995).

As with humans, all equine muscle fibers at rest contain glycogen in higher concentrations in fast twitch (type IIA and IIB) fibers than in slow twitch (type I) fibers (Lindholm and Piehl 1974; Hodgson et al. 1984a; White and Snow 1987). The pattern of glycogen depletion of slow twitch and fast twitch fibers varies depending on the duration and intensity of exercise and reflects fiber type recruitment (Lindholm 1974; Hodgson et al. 1984; Snow et al. 1985; Snow and Valberg 1994). Prolonged submaximal exercise, such as competing in an endurance ride (40 to 160 km) depletes muscle glycogen concentration in slow twitch fibers to a greater extent than that of fast twitch fibers (Hodgson et al. 1983b; Essén-Gustavsson et al. 1984). As the exercise duration continues and slow twitch fibers become increasingly depleted of glycogen, depletion of fast twitch high oxidative fibers (type IIA) occurs (Lindholm 1974). Similar depletion patterns have been observed in Standardbreds pulling a weighted load at the walk with almost total depletion of glycogen in type I fibers (Gottlieb 1989). Conversely, galloping results in a greater depletion of muscle glycogen concentration of type IIB low oxidative fibers than of type I fibers (Lindholm 1974; Hodgson et al. 1984a; White and Snow 1987). Trotting at low speed depletes type I and a small portion of type IIA, while trotting at high speed (12.5 m/s) depletes all fiber types, with the least depletion seen in type II fibers (Valberg 1986). Glycogen utilization appears to be greater in type IIB fibers because the low oxidative capacity of these fibers makes them more dependent upon anaerobic glycolysis (Valberg et al. 1985). It should be noted that for both prolonged submaximal exercise and
brief, maximal exercise, muscle glycogen concentrations are reduced in all fiber types, it is just that some fiber types are depleted to a greater extent than others, depending on the type of exercise. The fact that at exhaustion during moderate to heavy workloads many muscle fibers are completely devoid of glycogen, indicates that endogenous carbohydrate stores make a significant contribution to total energy production (Hodgson et al. 1984a), and supports the theory that muscle fatigue could indeed be caused by a deficiency in this energy substrate (Snow et al. 1981). It should also be noted that total glycogen analysis from a biopsy sample after brief intense exercise might not reflect the true extent of glycogen depletion. Extensive glycogen use in some muscle fibers, as well as selective depletion of glycogen from specific cellular compartments may precipitate fatigue during brief intense exercise although the total depletion of glycogen from a mixture of muscle fibers may be quite modest (Rankin 2000).

As reviewed above, fatiguing exercise by horses or humans, whether of prolonged submaximal duration, repeated bouts of maximal intensity exertion, or a combination of prolonged running and repeated sprints, results in important declines in muscle glycogen concentration. Furthermore, many horses undertake several events in a single day or compete on successive days, and the interval between exercise bouts or training sessions may be inadequate for complete or partial restoration of the muscle glycogen pool (Snow et al. 1981; Essén-Gustavsson et al. 1989; Snow and Harris 1991; Hyyppä et al. 1997). Therefore, exercise causes muscle glycogen depletion that may persist at the time of subsequent exercise. It is possible that pre-exercise reductions in muscle glycogen stores contribute to a decline in performance during endurance and/or high-intensity exercise.
1.1.2 Effect of muscle glycogen availability on endurance performance

1.1.2.1 In humans

Decreases of pre-exercise muscle glycogen reserves by exercise manipulation have served to establish a close relationship between muscle glycogen availability and performance during endurance exercise in humans. Furthermore, the importance of carbohydrate for athletic performance has been recognized since the early part of this century. For instance, sixty years ago, Christensen and Hansen (1939) reported that glucose infusion to two men late in an exercise bout alleviated subjective symptoms of hypoglycemia, and allowed the men to continue exercise for an additional hour. Bergström et al. (1967) showed that time to exhaustion during cycling exercise at 75% of VO$_{2\text{max}}$ was increased when glycogen concentrations before exercise were elevated by a high carbohydrate diet (“carbohydrate loading”), compared with results obtained when glycogen concentrations were either normal or low as a result of eating a mixed or low carbohydrate diet, respectively. Moreover, Karlsson and Saltin (1971) reported a decreased time required by subjects to run 30 km when muscle glycogen levels were high as compared with when they were low. These studies demonstrated that pre-existing muscle glycogen depletion in humans decreases endurance time and increases the time required to cover a given distance during submaximal exercise. Consumption of a high carbohydrate diet before exercise can partially compensate for low pre-exercise muscle glycogen concentrations by enabling subjects to maintain an optimal pace during the later stages of prolonged self-paced exercise (Karlsson and Saltin 1971; Widrick et al. 1993;
Orestis-Konstantinos et al. 1996; Jacobs and Sherman 1999). Furthermore, consumption of carbohydrate during the later stages of endurance exercise appears to delay fatigue by 30 to 60 min and attenuate the fall in blood glucose by providing additional carbohydrate for oxidation (Coyle 1992). Additionally, ingestion of glucose polymer or intravenous infusion of glucose can delay fatigue (Coggan and Coyle 1987; Coyle et al. 1986). When studying carbohydrate ingestion and glycogen metabolism in single muscle fibers during prolonged running in man, Orestis-Konstantinos and colleagues (1996) concluded that higher glycogen content in type I fibers at the time of exhaustion in the carbohydrate group allowed the subjects to run for an additional 28 ± 8 min compared with the placebo trial. The temporal correlation between glycogen depletion in type I fibers and exhaustion in the placebo group (without carbohydrate ingestion) suggested that impairment of the contraction process during this type of exercise might be restricted to this population of fibers (Orestis-Konstantinos et al. 1996). From these studies, the concept of carbohydrate supplementation prior to, or during endurance exercise to optimize human performance became widely accepted by athletes, coaches and exercise scientists.

1.1.2.1 In horses

Similar to humans, depletion of intramuscular glycogen stores has been frequently associated with fatigue during endurance rides (Snow et al. 1981) and endurance horses are often fed a high carbohydrate diet (grain, which is rich in starch) at rest stops to increase carbohydrate availability. While the observation that provision of supplemental glucose to horses performing endurance exercise on the treadmill increases the duration
of exercise, provides evidence that glucose availability limits performance in endurance exercise of horses (Farris et al. 1995; Farris et al. 1996), there are few studies, to our knowledge, that have investigated the role of muscle glycogen stores in limiting endurance performance in horses. Muscle glycogen depletion in type I and II fibers (by 25%) had no effect on metabolic and hormonal responses during submaximal treadmill exercise (Davie et al. 1999), although the exercise test used induced severe hyperthermia (> 42°C) which could have contributed to fatigue to a greater extent than did glycogen availability. Therefore, further research needs to be performed in this field.

In conclusion, over 20 years of research in humans have led to a general agreement that the muscle glycogen availability is one of the most important limiting factors of performance during prolonged exercise, and some observations in equine exercise physiology suggest similar conclusions, raising the following question: Why is muscle glycogen availability a contributing factor to fatigue?

1.1.3 Muscle glycogen depletion and muscular fatigue: metabolic, biochemical and cellular mechanisms

The fact that at exhaustion during moderate to heavy workloads, many muscle fibers were completely devoid of glycogen and that the time to fatigue was directly proportional to the initial muscle glycogen concentration, supports the theory that muscle fatigue could indeed be induced by a deficiency in this substrate (Conlee 1987; Sahlin 1992). The mechanisms responsible for fatigue when muscle glycogen is low are
complex and remain uncertain (Sherman 1995). The most popular theory is that
decreased muscle glycogen availability results in a reduction in the rate of ATP
regeneration and an inability to maintain energy supply during excitation and contraction
of the muscle (Sahlin 1992).

1.1.3.1 Interaction between muscle glycogen depletion and adenine nucleotides

A common metabolic factor during fatigue is a decreased capacity to generate
ATP coupled to high ATP turnover, which is expressed in the cell as an increased
catabolism of the adenine nucleotide pool (Sahlin 1992). The observation that IMP
(inosine monophosphate) concentration is inversely related to glycogen level suggested a
relationship between muscle glycogen depletion and increased catabolism of the adenine
nucleotide pool, when fatigue occurs during prolonged exercise (Norman et al. 1988;
Sahlin 1992). For instance, glycogen-depleted fibers contain a greater IMP concentration
than glycogen-filled fibers, which may reflect a decrease ATP production when glycogen
concentration is low (Norman et al. 1988). It has been indeed suggested that the increase
in IMP associated with low muscle glycogen reflects the increases in ADP and AMP
concentrations at the enzymatic site, with secondary increased AMP deaminase activity
(Spencer et al. 1992). In support of this theory is the observation that low muscle
glycogen stores at the point of fatigue during prolonged exercise are associated with
increased AMP deamination, as evidenced by the greater IMP and NH3 concentrations
compared to exercise with normal muscle glycogen (Broberg and Sahlin 1989).
Furthermore, when carbohydrates are supplied, the duration of exercise can be prolonged
and the IMP formation is attenuated (Spencer et al. 1996). These studies demonstrated that exercise-induced depletion of muscle glycogen is associated with energetic deficiency at the adenine nucleotide level, which could contribute to muscular fatigue (Sahlin 1992). It has been speculated that a failure to maintain ADP homeostasis at the contractile site due to a relative impairment of ATP resynthesis may result in the decrease of power generation during prolonged exercise (Broberg and Sahlin 1989). Potential intracellular sites where ADP could limit the muscular contraction process are: 1) actin-myosin interaction; 2) the reuptake of calcium by the sarcoplasmic reticulum (SR), the maintenance of the Na⁺-K⁺ gradient possibility as a result of alteration in Na⁺-K⁺ ATPase pump activity; 3) the membrane potential over sarcolemma; and 4) the signal transduction between T-tubuli and SR (Sahlin 1992).

1.1.3.2 Low muscle glycogen stores and impairment of muscular contraction

Low muscle glycogen concentration, combined with prolonged exercise, decreased the force-generating capacity of the muscle: maximal voluntary and electrically evoked isometric forces of the triceps surae were reduced in the low muscle glycogen group, when performance was measured 24 and 48 hours after exercise (Young and Davies 1984). The force impairment observed in this study could be, in theory, energy related (Green 1991). As previously mentioned, transient increases in ADP, associated with muscle glycogen depletion, could interfere with muscular function at various intracellular sites.
However, Green (1991) emphasized on the lack of scientific evidence that linked muscle glycogen depletion with energetic deficiency at the contractile site (ATP), and considered some other non-metabolic factors associated with muscle glycogen depletion that could potentially cause fatigue during prolonged exercise. For instance, impairment of the SR function has been implicated during the contractile process with a failure to maintain cytosolic Ca\(^{2+}\) homeostasis (Green 1991). A fraction of muscle glycogen and glycogen phosphorylase appear also to be specifically associated with the SR in skeletal muscle. Glycogen and glycogen phosphorylase associated with the SR are reduced after fatiguing exercise (Lees et al. 2001). The tight association of glycogen with the SR suggests that glycogen depletion may lead to alterations in excitation-contraction coupling. Decreases in muscle glycogen concentration contribute to the reduction in force and Ca\(^{2+}\) concentration observed during fatigue. These reductions can be explained by impairment in Ca\(^{2+}\) release from the SR and inhibition of the myofibrillar proteins, associated with muscle glycogen depletion (Chin and Allen 1997).

1.1.3.3 Influence of reduced glycogen stores on metabolic regulation

Muscle glycogenolysis during exercise is regulated by a number of local and systemic factors, and some authors have demonstrated a direct relationship between muscle glycogen availability and the rate of glycogenolysis. It has been suggested that muscle glycogen level is an important regulator of phosphorylase activity in rat skeletal muscle (Hespel and Ritcher 1992). Reduced pre-exercise muscle glycogen availability results in lower rate of glycogenolysis during submaximal exercise and increases muscle
glycogen enhances glycogenolysis without affecting glucose uptake (Gollnick et al. 1972; Jansson and Kaijser 1982; Hargreaves et al. 1995). However, the rate of muscle glycogen breakdown is not affected by the initial glycogen level during short-term stimulation (Ren et al. 1990). Furthermore, muscle glycogen availability also influences glycolysis, as evidenced by the decreased phosphofructokinase and tricarboxylic acid cycle intermediates in humans with muscle glycogen depletion (Spencer et al. 1996).

Decreased muscle glycogen stores influence glycogenolysis and glycolysis, but also affect lipid and amino acid metabolism. Low muscle glycogen content results in a decrease in respiratory exchange ratio (RER), and an increase in free fatty acids and fat oxidation both at rest and during submaximal exercise (Weltan et al. 1998). There is also an inverse correlation between pre-exercise glycogen content and the activity of the branched-chain 2-oxoacid dehydrogenase complex, which is the rate limiting enzyme in the degradation of the branched-amino acids (Wagenmakers et al. 1991). Therefore, fatigue during prolonged exercise could be related to deficiencies in energy metabolism.

1.1.3.4 Other factors responsible for fatigue during high-intensity exercise

Similar to prolonged exercise, a mismatch between ATP production and synthesis occurs during high-intensity exercise with secondary increase in by-products of the catabolism of the adenine nucleotide pool (AMP, ADP and IMP) (Green 1997). Since glycogen is the major energy substrate to sustain glycolysis and oxidative phosphorylation during repeated bouts of high-intensity exercise, decreased glycogen stores have been proposed as a contributing factor to these metabolic disturbances (Green
However, Hargreaves and coworkers (1998) demonstrated that the decline in performance with repeated bouts of exercise does not appear to be related to a reduction in muscle glycogen. Rather, they concluded that fatigue might be caused by reduced creatine phosphate availability, impairment in SR function or some other fatigue-inducing agent. Alternatively, it has been speculated that intramuscular acidosis, as a consequence of the increased glycolytic flux and electrolyte shifts that occur during intense exercise, could impair high-intensity exercise performance (Greenhaff et al. 1987; Hargreaves et al. 1998). However, other studies in humans have questioned the inhibitory effects of increased muscle acidity on muscle force production and intense exercise performance (Bangsbo et al. 1992a).

In conclusion, the mechanisms underlying muscular fatigue during glycogen depletion are complex and not completely understood.

1.1.4 Effect of muscle glycogen availability on performance during short-term high-intensity exercise

1.1.4.1 In humans

During high-intensity exercise, the major pathways for ATP production are the breakdown of phosphocreatine and the degradation of muscle glycogen to lactic acid (Fig. 1.1, Hargreaves et al. 1998). It has been demonstrated that the contribution of glycogen to anaerobic ATP production is 4-fold greater than that of phosphocreatine during 30 s of maximal isokinetic cycling exercise (Hultman et al. 1991). The decrease in
muscle glycogen concentration observed after high-intensity exercise could theoretically contribute to impaired exercise performance via a reduction in substrate for phosphorylase and subsequent glycolytic flux (Hargreaves et al. 1998). However, the importance of muscle glycogen for short-term high-intensity exercise is less well established than during endurance exercise, and results of different studies are somewhat controversial.

*Pre-exercise muscle glycogen depletion contributes to the decline in athletic capacity:*

Several investigators have concluded that a substantial reduction of muscle glycogen availability may limit performance during high-intensity exercise (Hepburn and Maughan 1982; Casey et al. 1996). A decrease in maximal isokinetic force generation by the leg extensors was reported 1 hour following exercise designed to deplete intramuscular glycogen (Jacobs et al. 1981, 1982). Moreover, glycogen exhaustion from slow twitch and fast twitch fiber types was associated with impaired maximal muscular strength by leg extensors, produced during a single dynamic contraction, 1 hour following exercise (Jacobs et al. 1981). However this decrease in maximal isokinetic force generation cannot be attributed only to the lack of glycogen, as the previous prolonged exercise (to deplete muscular glycogen) played a large role in the development of fatigue (Sherman et al. 1984; Symons and Jacobs 1989). Other investigations have attempted to dissociate the effect of glycogen depletion from the effects of exercise used to deplete glycogen by evaluating performance after various time intervals and reported a decrease in isometric endurance of the leg extensors in the low glycogen condition.
(Hepburn and Maughan 1982; Symons and Jacobs 1989). From this study, they concluded that glycogen availability might be a limiting factor in the performance of isometric exercise, and that the reduction in lactate formation was a direct result of low glycogen-induced decreased work time. The importance of muscle glycogen reserves to prevent fatigue is also highlighted by studies that manipulated muscle glycogen stores. Performance during supramaximal intermittent exercise was decreased in the low carbohydrate diet group, while a moderate and high consumption of dietary carbohydrate can at least maintain supramaximal intermittent exercise performance (Jenkins et al. 1993).

Because the major pathways for anaerobic ATP resynthesis are the breakdown of creatine phosphate and the degradation of muscle glycogen to lactic acid, reduced glycogen availability may contribute to this decline in anaerobic energy production and exercise performance (Fig. 1.1; Klausen et al. 1973; Hargreaves et al. 1998). The exercise time during two intense exercise bouts separated by 1 h was maintained in a leg with elevated muscle glycogen, whereas it was reduced in the contralateral leg with reduced muscle glycogen (Bangsbo et al. 1992b). Comparing the effects of a glycogen-loading regimen on performance during maximal exercise, a decrease in time to fatigue during anaerobic exercise was noticed in the low carbohydrate diet group, although muscle glycogen concentrations were not reported (Maugan and Poole 1981).

Although muscle glycogen availability limit anaerobic capacity, the lack of an effect of glycogen depletion on aerobic capacity has been well documented. Similar oxygen consumptions during exercise were reported despite varying the initial muscle
glycogen concentration in humans (Hedman 1957; Bergström and Hultman 1967; Hermansen et al. 1967; Bangsbo et al. 1992b).

Pre-exercise muscle glycogen depletion does not interfere with athletic capacity:

Several investigators reported controversial results regarding the role of muscle glycogen availability on high-intensity exercise performance. It has been reported that high-intensity exercise performance was not impaired by low intramuscular glycogen concentration (Symons and Jacobs 1988; Symons and Jacobs 1989; Housh et al. 1990 Hargreaves et al. 1997) and that the decline in exercise performance during high-intensity, intermittent exercise was not related to a reduction in muscle glycogen (Hargreaves et al. 1998). Symons and Jacobs (1989) found no effect of lowering muscle glycogen on electrically evoked muscle force, maximal voluntary isometric force, or repeated maximal isokinetic leg extensions. Moreover, glycogenolytic and glycolytic rates were not influenced by pre-exercise availability of muscle glycogen (Bangsbo et al. 1992b). However in these studies, muscle glycogen stores were not severely depleted and it can be argued that a greater degree of glycogen depletion is required before performance is affected during high-intensity exercise. Conversely, some authors maintain that variation in glycogen should have no effect upon short-term maximal exercise, because intramuscular levels remain high at the point at which fatigue develops (Hermansen 1981; Greenhaff et al. 1987; Symons and Jacobs 1989). However, as previously mentioned, intramuscular glycogen is an important substrate during this specific type of exercise (Saltin and Karlsson 1971; Klausen et al. 1973; Jacobs 1981; Symons and Jacobs 1989). Furthermore, selective depletion of glycogen in individual
muscle fibers may contribute to hasten the onset of fatigue (Rankin 2000). In particular, selective depletion of muscle glycogen associated with SR may impair the contraction process by altering calcium flux (Chin and Allen 1997; Rankin 2000; Lees et al. 2001).

*Effect of muscle glycogen availability on lactate production:*

In humans, blood lactate concentration after high-intensity exercise was lower after a low carbohydrate diet when compared to a high carbohydrate diet (Greenhaff et al. 1987, 1988). One explanation for the decrease in lactate concentration would be a lack of substrate for glycogenolysis and glycolysis during anaerobic metabolism and/or a reduction in work performed (Hepburn and Maughan 1982; Casey et al. 1996). The importance of glycogenolysis during short-term high-intensity exercise was illustrated by Saltin and Karlsson (1971) who observed that the rate of glycogen utilization by the quadriceps during cycling exercise increased in a positive exponential manner as a function of exercise intensity expressed relative to VO_{2\text{max}}. However, the existence of a direct relationship between muscle glycogen content and blood lactate accumulation has been questioned in humans (Jacobs 1981; Greenhaff et al. 1988) with similar post-exercise blood lactate concentrations being observed over a wide range of pre-exercise muscle glycogen levels. Moreover, it has been reported that the rate of muscle glycogen degradation and of lactate production during short, intense contraction is not affected by the initial glycogen concentration (Ren et al. 1990; Vandenberghe et al. 1995).
Why is it a controversial issue?

One explanation for these controversial results could be the diversity of exercise protocols and experimental designs. In some studies, intramuscular glycogen concentrations were assumed to be low but were not measured, raising the speculation as to whether the respective protocols were indeed sufficient to alter glycogen levels (Maughan and Poole 1981; Casey et al. 1996). Moreover, the challenge of such investigations resides in the difficulty of controlling the extraneous factors. For instance, the role of muscle glycogen stores as a limiting factor for high-intensity exercise performance has been questionable because the previous exercise may be a more potent determinant of fatigue than glycogen availability (Grisdale et al. 1990).

A key factor in the interpretation of these studies is the extent to which the pre-exercise muscle glycogen levels influenced performance under each experimental condition (Widrick et al. 1993). Because a muscle glycogen concentration of 150 mmol.kg⁻¹ dw may give sufficient fuel to perform high-intensity exercise during 115 and 405 s (Casey et al. 1996), any study with an experimental design that does not substantially deplete muscle glycogen stores may fail to demonstrate an effect of pre-existing reduction in muscle glycogen concentration on athletic performance. Therefore, it would appear that glycogen availability impairs performance when severely depleted (Casey et al. 1996).
1.1.4.2 Effect of muscle glycogen availability on high-intensity exercise performance in horses

Horses are among the greatest athletes because they have a much greater capacity for energy production via aerobic and anaerobic metabolism than might otherwise be predicted from normal body scaling (Snow et al. 1985; Harris et al. 1987). For instance, racehorses trotting a distance of 1600 to 2600 m rely heavily on anaerobic metabolism, evidenced by high plasma lactate and glucose-6-phosphate (G-6-P) concentrations, and low ATP and phosphocreatine concentrations after racing and maximal treadmill exercise (Valberg et al. 1987; Ronéus and Essén-Gustavsson 1997; Shuback and Essén-Gustavsson 1998). Glycogenolysis and secondary lactate accumulation are greater during maximal exercise in horses than in humans over comparable running distance (Snow and Mackenzie 1977; Nimmo and Snow 1983; Snow et al. 1985; Harris et al. 1987). Therefore, decreased availability of glycogen could contribute to a decline in high-intensity-exercise performance in horses although this issue has received scarce attention. Some authors argue that muscle glycogen is not a limiting factor during exercise because muscle glycogen was only partially depleted (25% of reduction) when fatigue occurred (Miller and Lawrence 1986; Harris et al. 1987). Moreover, Davie and colleague (1996) reported that a 22% decrease of muscle glycogen concentration did not have a measurable effect on high-intensity exercise performance. A concern with these studies is that muscle glycogen concentration might not have been reduced sufficiently to produce detectable effects on athletic performance. In contrast, a 41% decrease in muscle glycogen concentration impaired the capacity for anaerobic work in horses dragging a
sled (Topliff et al. 1985), leading the authors to suggest a reduction in anaerobic capacity. Furthermore, blood lactate concentration at the end of exercise was lower for the glycogen-depleted phase compared to the glycogen repletion phase (Topliff et al. 1983). One explanation for the decreased in lactate concentration would be a lack of substrate for glycogenolysis and glycolysis during anaerobic metabolism and/or by a reduction in work performed. However, a concern with Topliff’s study (1983, 1985) is the inadequate assessment of anaerobic capacity. In these studies, anaerobic capacity was assessed by measuring the work performed by horses dragging a sled, and it may be speculative to apply these results to high-intensity exercise. Overall, there is little evidence to support or refute a significant effect of low muscle glycogen concentration on aerobic and anaerobic capacity of horses. Although the effects of energy availability and dietary manipulation on exercise capacity have been well investigated in human exercise physiology, to our knowledge, no study conclusively demonstrates that manipulation of muscle glycogen stores affects athletic capacity of horses. Such demonstration would provide impetus to examine the methods and mechanisms underlying effects of dietary modifications on muscle glycogen replenishment and athletic capacity of horses.

1.2 MUSCLE GLYCOGEN SYNTHESIS AND NUTRITION

Because glycogen is a limiting fuel for the contracting muscle, the effect of dietary manipulations on post-exercise muscle glycogen resynthesis has been widely studied in humans and rats. Following exercise, carbohydrate supplementation hastens
glycogenesis by increasing glucose availability and insulin release. Raised concentrations of insulin activate protein phosphatase, which converts glycogen synthase from its inactive form (D) to its active form (I) and inhibits glycogenolytic enzymes (phosphorylase a). Under physiological conditions, glycogen synthesis is largely controlled by the glycogen synthase (I form), due to the inhibition of the D form by ATP, ADP and Pi. In humans, muscle glycogen content is closely correlated to glycogen synthase I activity (Bergström et al. 1972). Insulin release also activates the translocation of glucose transporter (type GLUT-4) at the plasma membrane (Kuo et al. 1999). Once the glucose is inside the muscle cell, it is transformed to G-6-P through the activation of hexokinase and becomes trapped in the muscle cell (Fig. 1.2).

1.2.1 Muscle glycogen resynthesis in humans

Because muscle glycogen stores limit performance, it is important for the athlete to ingest an adequate amount of carbohydrate to help to ensure the normalization of muscle glycogen on a daily basis. Early studies found that at least 48 hours were required to replenish muscle glycogen after exercise, but more recent studies have demonstrated that muscle glycogen stores can be normalized within 24 hours after glycogen-depleting exercise and high-intensity intermittent exercise, provided that adequate carbohydrate is consumed in the diet (Bergström et al. 1972; MacDougall et al. 1977; Costill et al. 1981; Sherman and Lamb 1988). In general, it is recommended that athletes eat diets that have
at least 60-70% and up to 65-85% of energy as carbohydrate to ensure normalization of muscle glycogen on a daily basis (Costill et al. 1981).

The rate of glycogen resynthesis varies based on fiber types: the initial muscle glycogen resynthesis occurs in type I fibers, where the rate is about 25% greater than in type II (Casey et al. 1995). The rate declined in type I fibers, while it is maintained in type II fibers. At 24 hours after exercise, no difference in the glycogenesis rate is noticed between fiber types (Casey et al. 1995). This difference has been attributed to the greater number of glucose transporters GLUT-4 at the membrane of type I fibers (Goodyear et al. 1991b). The rate of muscle glycogen resynthesis in man is also affected by the initial muscle glycogen concentration, the time of carbohydrate administration after exercise, and the amount and nature of carbohydrate supplementation (Ivy 1998).

1.2.1.1 Effect of carbohydrate administration on muscle glycogen resynthesis after exercise

The time of administration of carbohydrate after exercise critically affects muscle glycogen restoration (Ivy et al. 1988a, 1998). A mean rate of muscle glycogen resynthesis of 6-7 µmol/g ww/hour has been reported when carbohydrate supplements are provided immediately after exercise (Yvy et al. 1988a, 1998; Reed et al. 1989). This rate declines by approximately 50% 2 hours after carbohydrate supplementation as blood glucose and insulin decline to values similar to those recorded before exercise (Yvy et al. 1988a). However, if a carbohydrate supplement is provided every 2 hours after exercise, the rate of muscle glycogen resynthesis remains constant (Blom et al. 1987). If carbohydrate
supplementation is withheld during the initial 2 hours after exercise, the rate of muscle
glycogen resynthesis declines by about 50% (with a mean rate of 4 mmol/kg/h ww)
despite significant increase in blood glucose and insulin concentrations (Ivy et al. 1988a).
Some authors speculated that when carbohydrate intake is delayed for several hours, the
muscle becomes insulin resistant, reducing the rate of muscle glucose uptake and
glycogen resynthesis (Ivy 1998). Furthermore, little muscle glycogen resynthesis occurs
during the time between the end of exercise and the consumption of carbohydrate. For
instance, muscle glycogen synthesis occurs during recovery under fasting conditions
despite low glucose concentrations at a rate of 1.8 mmol/kg/h wet weight for the first 4
hours after exercise (Maehlum and Hermansen 1978). In conclusion, it appears that
carbohydrate should be ingested immediately after exercise to optimize muscle glycogen
resynthesis.

Amount of carbohydrate administrated:

A critical amount of glucose administered after exercise is necessary for optimal
muscle glycogen resynthesis (Ivy et al. 1988b). Blom and colleagues (1987) first found
that when carbohydrate supplements of 0.35 g/kg of bw were provided at 2-hour
intervals, the mean 6 h post-exercise rate of muscle glycogen resynthesis was 2.1
mmol/kg/h ww. When the glucose load was doubled (0.7 g glucose/kg of bw), the rate of
muscle glycogen resynthesis increased by almost three-fold (5.8 mmol/kg/h ww). The
rate of resynthesis increased in a curvilinear pattern with an increasing amount of
carbohydrate ingested and then plateaued as the carbohydrate supplement approached 1
to 1.5 g/kg of bw (Ivy 1998). Doubling the amount of carbohydrate (3 g/kg) did not have additional effect on muscle glycogen replenishment (Ivy et al. 1988b). The observation of this plateau could be associated with the rate-limiting steps in muscle glycogen resynthesis, which involves either glucose transport or the rate-limiting enzyme in the glycogen synthetic pathway, namely glycogen synthase.

*Nature of diet and of carbohydrate supplementation:*

Because insulin stimulates both muscle glucose transport and glycogen synthase activity, some authors have studied means of enhancing insulin secretion during muscle glycogen resynthesis.

Certain amino acids such as arginine have been found to synergistically increase the blood insulin response to a carbohydrate load when carbohydrate and amino acids are administered in combination (Ivy 1998). Moreover, protein meals and supplements have also been found to enhance the insulin response to a carbohydrate load. Zawadzki et al. (1992) found that administration of carbohydrate plus protein (ratio of 1 gram protein to 2.5 gram of carbohydrate) resulted in a synergistic insulin response in conjunction with lower blood glucose and a 40% faster rate of muscle glycogen storage compared with carbohydrate supplement alone. It was also found that the carbohydrate oxidation rates and blood lactate concentration for the carbohydrate-protein and carbohydrate treatment were similar. The authors concluded that the increased rate of muscle glycogen resynthesis was the result of an increased clearance of glucose by the muscle due to an increased blood insulin response. However, the carbohydrate supplementation used in
this study induced only a moderate glycogen resynthesis. When carbohydrate was ingested in sufficient amounts to produce optimal muscle glycogen resynthesis, ingestion of additional protein did not further increase the rate of muscle glycogen replenishment (Van Hall et al. 2000). Furthermore, when isocaloric diets were given, muscle glycogen restoration was not enhanced with the addition of proteins or amino acids to a eucaloric carbohydrate feeding after exhaustive cycling exercise (Carrithers et al. 2000). Therefore, more recent studies concluded that protein supplementation did not provide additional effects on muscle glycogen resynthesis.

Scientists also investigated the type of carbohydrate consumed after exercise to enhance muscle glycogen resynthesis. For instance, Costill and coworkers (1981) compared the effect of a simple and complex (starch) carbohydrate on muscle glycogen resynthesis. They found that complex or simple carbohydrate diets resulted in similar muscle glycogen concentration 24 hours after strenuous running (Costill et al. 1981). However, ingestion of a complex carbohydrate diet resulted in significantly greater glycogen resynthesis from 24 to 48 hour after exhaustive running, because complex carbohydrates induced a longer lasting elevation of blood glucose and insulin, compared to the simple carbohydrate (Costill et al. 1981). Furthermore, Blom et al. (1987) found that ingestion of glucose and sucrose was twice as effective as fructose for restoration of muscle glycogen after exhaustive bicycle exercise. They suggested that this response is due to the difference in sugar metabolism. Fructose metabolism takes place predominantly in the liver whereas the majority of glucose appears to bypass the liver and is stored or oxidized by the muscle. Similar results were found by Van Den Bergh and
coworkers (1996), using $^{13}$C-nuclear magnetic resonance spectroscopy to measure muscle glycogen stores. When infused, fructose has been found to result in a four times greater liver glycogen storage than glucose (Nilsson and Hultman 1974). On the other hand, higher glycogen storage has been demonstrated in skeletal muscle after glucose than after fructose infusion. Because sucrose is a disaccharide of glucose and fructose, consumption of a glucose polymer promotes a more rapid storage of carbohydrate in the skeletal muscle than an isoenergetic sucrose drink (Bowtell et al. 2000).

Carbohydrate in solid or liquid form can be consumed immediately after exercise with similar results, and it appears that gastric emptying does not limit muscle glycogen resynthesis (Reed et al. 1989). However, liquids are recommended because they are easy to digest, and less filling, and they provide a source of fluid for rapid rehydration (Ivy 1998).

1.2.1.2 Glycogen supercompensation

It has been demonstrated that a combination of glycogen depletion and dietary manipulation in humans has a dramatic effect on muscle glycogen storage and this observation led to the “classical” glycogen supercompensation regimen (Bergström et al. 1967): 6 subjects consumed a mixed diet for 3 days, exercised to exhaustion at $75\%$ $\text{VO}_2\text{max}$, and consumed a low carbohydrate diet for 3 days. Muscle glycogen decreased from 106 mmol/kg to 11 mmol/kg after the glycogen-depleting exercise, and increased only to 40 mmol/kg 3 days later. The same experiment was repeated except that the subjects consumed a high carbohydrate diet for 3 days after the glycogen-depleting exercise. The
authors demonstrated that ingestion of a high carbohydrate diet following glycogen-depleting exercise markedly increased muscle glycogen stores by 1.9 fold (204 mmol/kg instead of 100 mmol/kg). The authors also demonstrated that muscle glycogen resynthesis was more rapid during the first hours following the glycogen-depleting exercise and was attributed to the activation of glycogen synthase, although this speculative mechanism remains controversial (Greiwe et al. 1999). These early findings have since been confirmed by further studies (Alborg et al. 1967) and have led to the common practice of carbohydrate loading among athletes. However, this regimen, which requires exhaustive exercise with non-realistic diets (% of calories from carbohydrate), is not practical for athletes under training or before a competition, and less strenuous exercise with modified diet composition has been shown to produce similar muscle glycogen supercompensation (Sherman et al. 1981). More recently, Fairchild and coworkers (2002) obtained muscle glycogen surpercompensation within 24 hours, following a combination of short-term bouts of high-intensity exercise and high-carbohydrate intake.

Both the “classical regimen” and the modified regimens have been shown to have a beneficial effect on exercise performance by increasing pre-exercise muscle glycogen stores. The pioneering work done by Christensen and Hansen (1939) demonstrated a clear relationship between pre-exercise feeding, muscle glycogen concentration and exercise performance. Furthermore, in the study by Bergström et al. in 1967 (described above), run time to fatigue was decreased by 55% in the subjects fed the low
carbohydrate diet and increased by 50\% for the subjects fed the high carbohydrate diet compared to values observed in the group fed a normal diet.

The mechanisms of glycogen supercompensation after strenuous exercise are still incompletely understood (Bergström and Hultman 1966). Recently, Adamo and Graham (1998) have demonstrated the presence of 2 different pools of glycogen in human skeletal muscle (macroglycogen and proglycogen). These 2 pools are metabolically distinct under physiological conditions (Asp et al. 1999) and an increase in the macroglycogen pool is responsible for the supercompensation observed after exercise and carbohydrate supplementation (Adamo et al. 1998).

1.2.1.3 Training and glycogen resynthesis

Exercise conditioning results in a protective effect against the depletion of carbohydrate stores during exercise, called “glycogen-sparing effect”. For instance James and Kraegen (1984) demonstrated that exercise training was associated with a 60-150\% increase in glycogen synthase and 50-70\% increase in glycogen content in the soleus muscle. Moreover, endurance exercise training results in an increased ability to accumulate muscle glycogen after exercise (Hickner et al. 1997).

1.2.1.4 Biochemical mechanisms for post-exercise muscle glycogen resynthesis

After strenuous exercise, replenishment of muscle glycogen stores is of high priority and glycogen in muscle will be repleted before glycogen synthesis occurs in the liver (Hultman 1978). The rate-limiting enzyme for glycogen resynthesis in skeletal
muscle is glycogen synthase, which is regulated by muscle glycogen concentration (inversely related), glucose-6-phosphate and insulin (Kochan et al. 1979; Bak and Petersen 1990). Glycogen synthase activity plays a key role in determining the rate of glucose uptake into the muscle. Low muscle glycogen content following glycogen-depleting exercise is the major initial stimulus in rats and humans for increasing the rate of glucose transport into the muscle and the rate of glycogen resynthesis (Conlee et al. 1978; Fell et al. 1982). After exercise, during the early insulin-independent phase, it has been demonstrated, in vitro, that glycogen depletion rather than glucose 6-P accumulation controls early glycogen synthesis in humans (Montell et al. 1999). During the insulin-dependent phase, a high carbohydrate diet enhances glycogen synthase activity by dephosphorylation of the enzyme (Kochan et al. 1979; Wojtaszewski et al. 2000).

There is growing evidence that glucose transport is the rate-limiting step for glucose uptake and glycogen resynthesis in skeletal muscle. It has been demonstrated in rat epitrochlearis muscle that glucose transport is the rate limiting step for glycogen synthesis unless the percent of glycogen synthase I is below the fasting range, in which case glycogen synthase activity also becomes a limiting step for glycogen replenishment (Fisher et al. 2002). Basal entry of glucose into the muscle cells occurs by the GLUT-1 transporter, but with insulin and/or exercise stimulation another glucose transporter, GLUT 4, is translocated to the sarcolemma from the intracellular pool (Hayashi et al. 1997). It has been demonstrated using GLUT-4 null mice, that GLUT-4 is the major transporter responsible for exercise-induced glucose transport in the early phase of glycogen resynthesis following exhaustive exercise (Ryder et al. 1999). Furthermore,
ingestion of a carbohydrate supplement with a high-glycemic index after exercise enhances the exercise-induced increase in GLUT-4 protein concentration in muscle (Kuo et al. 1999).

1.2.2 Muscle glycogen resynthesis in horses and dietary considerations

1.2.2.1 Rate of muscle glycogen resynthesis

In contrast to humans, restoration of normal muscle glycogen concentration takes up to 72 hours after single or repeated bouts of exercise on the racetrack in horses fed a conventional diet (Snow and Harris 1991; Hyyppä et al. 1997). This is slower than the rate of muscle glycogen resynthesis in human athletes, which is complete within 24 hours (Costill et al. 1981). Partial repletion of muscle glycogen stores takes place within 24 hours after exercise (Davie et al. 1994, 1995; Snow and Harris 1991) or may be negligible for the first 24 hours (Hyyppä et al. 1997). Complete glycogen repletion occurs first in type IIB fibers, then in type I and IIA fibers 24 and 48 hours, respectively after endurance ride (Hodgson et al. 1984b). Moreover, glycogenolysis may persist for up to 4 hours after exercise (Hyyppä et al. 1997). Feeding after exercise prevents further decreases in muscle glycogen concentration (Brewster et al. 1995). Therefore, developing nutritional strategies to optimize muscle substrate availability for horses competing in several events in the same day, or on successive days may be more crucial than for humans due to the slower rate of muscle glycogen resynthesis in horses.
1.2.2.2 Effect of carbohydrate administration on muscle glycogen stores

Muscle glycogen stores are much higher in horses than in humans: 500 to 650 and 320 to 400 mmol glucosyl units/kg dw, respectively (Snow and Valberg 1994). During brief intense exercise, the catabolism of carbohydrate may account for the majority of energy used, and most of the carbohydrate will be derived from muscle glycogen (Lawrence 1994). Whereas muscle glycogen is the key fuel for exercising skeletal muscle and decreased muscle glycogen concentration is commonly observed after exercise, there is little information regarding factors affecting muscle glycogen storage in horses.

Some authors reported no significant effect of carbohydrate administration on glycogen replenishment in equine skeletal muscle. For instance, 4 Standardbreds received 1.5 g/kg of glucose polymer as 20% solution orally, with a second dose 3 hours later or 3 g/kg of glucose in one treatment or water at an equivalent volume (Davie et al. 1994) 30 min after exercise, without an effect of treatment on muscle glycogen restoration. However, one could argue that the statistical power might not have been sufficient to produce a detectable effect of treatment (n = 4). Pösö and Hyyppä (1999) investigated if muscle glycogen resynthesis could be hastened 22.5 h after glycogen-depleting exercise by providing propionic acid (a gluconeogenic precursor) or by increasing insulin secretion through the administration of leucine. However, in addition to leucine and propionic acid, only 37.8 ± 6.4 g of glucose was administered per horse, which is a small amount of glucose given that humans consume at least 1.5 g/kg. This small amount of glucose administered could partly explain the lack of treatment effect on muscle glycogen resynthesis.
In contrast, several authors have demonstrated an effect of carbohydrate administration on muscle glycogen resynthesis. Evidence that glycogen resynthesis is enhanced by intravenous dextrose infusion (6 g/kg) administered after exercise has been clearly demonstrated (Davie et al. 1995). Snow et al. (1986) studied the effect of diets with different carbohydrate content on muscle glycogen repletion following exercise, and demonstrated that glycogen content was higher for the horses fed the high soluble carbohydrate diet and moderate carbohydrate diet compared to the low soluble carbohydrate diet only at 28 hours post-exercise. However, this study did not provide isoenergetic diets - the low soluble carbohydrate diet provided 20.7 Mcal/day, whereas the high and normal soluble carbohydrate diet provided 34.0 and 32 Mcal/day, respectively. Moreover, Topliff et al. (1983, 1985) demonstrated a significant increase in muscle glycogen concentration when 6 mature horses were fed a high soluble carbohydrate diet during a 3-day rest period following glycogen-depleting exercise when compared to horses fed a normal amount of soluble carbohydrate, although these differences were not present after exercise. In the same study, significant increases in muscle glycogen were also found in horses fed a high soluble carbohydrate diet compared to a high fat diet, although these diets were not isocaloric. Similar results were found by Kline and Albert (1981) in that there was a slight increase in muscle glycogen concentration above normal values in the group of horses fed the high soluble carbohydrate diets compared to the isocaloric moderate and low soluble carbohydrate diets. Therefore, it appears that, in contrast to humans, enhancement of muscle glycogen in horses is possible only to a small extent and that glycogen supercompensation has not
been observed. Most of these studies only considered the effect of pre-exercise feeding on muscle glycogen resynthesis, whereas feeding a high soluble carbohydrate diet immediately after exercise may be beneficial for long-term performance by optimizing muscle glycogen replenishment.

A wide range of rates of muscle glycogen resynthesis has been reported in horses, and it is largely determined by the amount of carbohydrate supplementation, the extent of glycogen depletion, and the time elapsed between exercise and carbohydrate supplementation. In humans, the maximal rate of muscle glycogen resynthesis occurs during the first 4 hours after exercise, providing that carbohydrate supplementation is given immediately after exercise (Ivy 1998). In horses, the mean rate of glycogen resynthesis varies from 7.8 mmol/kg/h dw in the first 4 hours to 2.6 mmol/kg/h dw between 12 to 24 hours (Hodgson 1984). The maximal rate of glycogen resynthesis has been reported the first 6 hours after intravenous dextrose infusion (6 g/kg) following glycogen-depleting exercise (19.8 ± 3.8 mmol/kg/h dw) and this rate was about twice compared to the control group (Davie et al. 1995). The rate rapidly decreased to 7.1 ± 1.6 mmol/kg/h for the first 24 hours (Davie et al. 1995). The slower repletion in muscle glycogen stores observed in horses compared to humans may be related to the extent of substrate depletion and the true difference in the rate of glycogen resynthesis. Post-exercise carbohydrate consumption generally results in a rate of glycogen resynthesis for the first hours of 6-7 mmol/kg ww/h (Ivy 1998), whereas a rate of 3 mmol/kg ww/h has been reported for the first hours following the consumption of high soluble carbohydrate diet in horses (Snow et al. 1987). Similar to humans, feeding a diet with a high-glycemic
index increases the rate of muscle glycogen resynthesis compared to feeding a low glycemic index diet (Kline and Albert 1981; Topliff et al. 1983, 1985; Snow et al. 1987) or moderate glycemic index diet (Essén-Gustavsson et al. 1991), although some studies did not provide isoenergetic diets (Topliff et al. 1983, 1985; Snow et al. 1987). In these former studies, the increase of muscle glycogen resynthesis after a high carbohydrate diet may only reflect the increase in energy provided by this diet compared to the low carbohydrate diet and mixed diet. Overall, there is lack of scientific evidence that dietary manipulation affects post-exercise muscle glycogen resynthesis in horses.

In summary, adequate amounts of a high carbohydrate diet immediately after exercise hastens muscle glycogen restoration in humans. It appears that post-exercise repletion of muscle glycogen is slower in horses compared to humans and rats, and that horses performing endurance events or horses racing several heats could potentially benefit from high soluble carbohydrate diet to hasten muscle glycogen resynthesis between exercise bouts, although this issue needs further investigation.

1.2.2.3 Effect of fat-supplemented diet on muscle glycogen stores

Because ingestion of large amounts of oral carbohydrate may have detrimental effects on the horse’s health, fat supplemented diets have been used as safe and effective ways to increase energy intake in the diet of endurance horses and horses undertaking intense training (Potter 1999).

Fat supplemented diets have been reported to increase muscle glycogen concentration at rest (Meyer et al. 1987; Oldham et al. 1989; Jones et al. 1991; Scott et al.
1991; Harkins et al. 1992; Hughes et al. 1995), while other investigators have found no effect of fat supplemented diet on resting muscle glycogen content (Essén-Gustavsson et al. 1991; Eaton et al. 1995). Furthermore, fat supplemented diets (up to 16%) only marginally increased liver glycogen concentration (Hambleton et al. 1980).

Fat supplementation during training or prior to competition may also attenuate the drop in blood glucose concentration observed during submaximal exercise (Pagan et al. 1995) and delays the decrease in muscle glycogen stores (Potter 1999). However, glycogen utilization can be greater during exercise (Scott et al. 1991; Hughes et al. 1995) or unchanged (Hyyppä et al. 1999) when horses are fed a fat supplemented diet compared to a control diet, challenging the theory of the “glycogen-sparing” effect. The effects of high-fat diets on performance are also equivocal with reports of improvement (Oldham et al. 1989; Eaton et al. 1995) or no change in performance (Topliff et al. 1983). When improved performance was noted, it was thought to be due to either the increased glycogen stores following fat supplementation or the increased fat utilization during exercise with a “glycogen-sparing” effect. These varying results may be explained by the difference in the type and amount of fat given to the horses, the duration of treatment in relation to metabolic adaptations (Orme et al. 1997), and the use of a washout period between treatments.

During the recovery period, free fatty acid concentration is low, which may redirect glucose for energy production (Hyyppä et al. 1997; Pösö and Hyyppä 1999). Therefore, feeding a fat-supplemented diet may spare muscle glycogen stores by increasing lipid availability for energy production (Hyyppä et al. 1997; Pösö and Hyyppä
Few studies have investigated the effect of a fat supplemented diet on the rate of muscle glycogen resynthesis. A fat supplemented diet (5% of DM) slows the rate of muscle glycogen resynthesis when horses were not adapted to fat feeding, whereas, after a 3 week adaptation period, a fat supplemented diet does not alter the rate of post-exercise muscle glycogen resynthesis when compared to the rate with a normal diet (Hyyppä et al. 1999). In summary, the advantage of a fat supplemented diet to enhance muscle glycogen replenishment remains uncertain.

In conclusion, prolonged submaximal fatiguing exercise or repeated bouts of maximal intensity exertion result in significant depletion in muscle glycogen stores both in humans and horses. It has been demonstrated that muscle glycogen pool plays a significant role in limiting human athletic capacity during endurance exercise. High-intensity exercise performance may be affected only by a marked reduction in muscle glycogen availability prior to exercise. However, our understanding of the biochemical and cellular mechanisms that explain why glycogen is essential to the ability of the muscle fiber to maintain high force output remains limited despite almost fifty years of research in this field. The effects of diet composition on muscle glycogen stores and exercise capacity have received a lot of attention in human exercise physiology, and these studies have resulted in significant improvements in nutrition and the performance of human athletes. Similar to humans, muscle glycogen availability may influence exercise performance, although this has received scarce attention in equine exercise physiology. While the rate of muscle glycogen repletion appears slower in horses compared to
1.3 REGULATION OF SKELETAL MUSCLE GLUCOSE UPTAKE IN THE POST-EXERCISE STATE

Following exercise and the consumption of a high carbohydrate diet, the increase in glucose uptake by muscle arises from both an increase in blood glucose delivery secondary to increased blood flow, and an increased glucose extraction secondary to increased number and/or activity of glucose transporters.

1.3.1 Physiological background and local factors

Under most physiological conditions, glucose entrance across plasma membranes into the muscle cell is the rate-limiting step in glucose utilization, at least in rats and humans (Hayashi et al. 1997). Glucose transport occurs primarily by facilitated diffusion that uses a family of structurally related proteins (GLUT-1 to GLUT-8) as glucose carriers (Hayashi et al. 1997). The facilitative glucose transporter family is expressed in a tissue-specific manner (Hayashi et al. 1997). For instance, GLUT-4 is the major isoform in the skeletal muscle (Hayashi et al. 1997). Whereas GLUT-1 and GLUT-5 isoforms are mainly associated with the cell surface and are not insulin-stimulated, the translocation of
the GLUT-4 protein from an intracellular (non active) pool to the plasma membrane (active site) is largely regulated by an insulin- and contraction- dependent processes (Holloszy et al.1986).

Glucose transport in skeletal muscle follows saturation kinetics with an increase in the maximal velocity of transport (Vmax) after exercise without affecting Km, which is the substrate concentration at which glucose transport is half-maximal (King et al. 1989; Ploug et al. 1992; Hayashi et al. 1997). This increase in transport Vmax may occur through an increase in the rate that each carrier protein transports glucose (transporter turnover number or intrinsic activity), or an increase in the number of functional glucose transporter proteins present in the plasma membrane, or both (King et al. 1989; Goodyear et al. 1990). Numerous studies utilizing cytochalasin B binding and Western blot analysis have demonstrated that an increase in Vmax after exercise is due to an increase in the number of glucose transporters in the plasma membrane, as a result of translocation from an intracellular storage (King et al. 1989; Goodyear et al. 1990, 1991a; Ploug et al. 1992).

The major inducers of increased glucose transport activity in muscle are fiber contractions and insulin, although numerous other factors including catecholamines, hypoxia, growth factors, and corticosteroids can alter glucose transport (Hayashi et al. 1997). Exercise-induced stimulation of glucose uptake in skeletal muscle involves the recruitment of glucose transporters to the plasma membrane and is mediated solely by a process associated with contraction (Goodyear et al. 1990, 1991a). For instance, it has been demonstrated that contractile activity can increase muscle glucose uptake and plasma membrane glucose transporters in the absence of insulin (Ritcher et al.1985). The
signaling mechanisms for exercised-stimulated glucose transport include calcium, 5’-AMP-activated protein kinase, protein kinase C and autocrine metabolism (such as nitric oxide, adenosine, kallikrein and bradykinin). For more information on this subject, the reader should refer to an excellent review by Hayashi and coworkers (1997).

**1.3.2 Hormonal regulations**

Similar to the effects of physical exercise, insulin also causes GLUT-4 translocation at the plasma membrane in rat skeletal muscle (Ploug et al. 1992) and more recently in human skeletal muscle (Thorell et al. 1999). Several studies have observed that the effects of insulin and contraction in muscle are additive (Thorell et al. 1999), implying that insulin and exercise stimulate the translocation of glucose transporter via different mechanisms. These authors speculate that insulin and exercise activate different pools of glucose transporters (Ploug et al. 1992, 1993; Hayashi et al. 1997).

After an acute bout of exercise, increase in insulin sensitivity, which is defined as a shift in the dose-response curve to the left, results in an increased GLUT-4 translocation at the plasma membrane in rat skeletal muscle (Hansen et al. 1998). Insulin stimulation results in the rapid phosphorylation of the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) on tyrosine residues and activation of phosphatidylinositol 3-kinase (PI 3-kinase), which is necessary for the stimulation of GLUT-4 translocation (Hayashi et al. 1997). It has been demonstrated that post-exercise enhanced insulin sensitivity activated PI 3-kinase and further downstream signaling elements such as protein kinase
B (or called Akt) and glycogen synthase kinase 3 (Thorell et al. 1999; Wojtaszewski et al. 1999), whereas insulin independent of exercise activated both IR and IRS-1 through a different mechanism (Wojtaszewski et al. 2001).

Most studies in adipose cells suggest that insulin acts primarily through increasing transporter exocytosis of small tubulo-vesicular organelles where the majority of intracellular GLUT-4 is located. The molecular mechanism by which exercise and insulin stimulate the movement of these GLUT-4-containing vesicles to the cell surface in skeletal muscle is not well known (Hayashi et al. 1997). However, some proteins have been identified as components of GLUT-4-containing vesicles in skeletal muscle and it seems that insulin may act specifically on the redistribution of Rab4. Rab proteins are guanosine triphosphate (GTP)-binding proteins that are thought to be involved in all vesicular activity (formation, targeting, fusion) and may act as molecular switches, catalyzing membrane trafficking events by conversion from an inactive guanosine diphosphate-bound form to an active GTP-bound form (Hayashi et al. 1997).

1.3.3 Substrate availability

A temporal relationship has been demonstrated between intramuscular glycogen stores and muscle glucose uptake during exercise (Hargreaves et al. 1995). Studies in the exercising rat suggest that muscle glucose uptake is dependent upon a reduction in muscle glycogen (Fell et al. 1982). In perfused, contracting rat skeletal muscle, increased pre-exercise muscle glycogen concentration reduces glucose uptake, whereas low pre-
exercise muscle glycogen concentration enhances glucose uptake (Fell et al. 1982). Recent evidence has demonstrated that glycogen may also play a regulatory role in muscle glycogen resynthesis by influencing post-exercise insulin sensitivity (Ritcher et al. 2001). Reduction in muscle glycogen stores is associated with an increase in basal glucose transport and insulin sensitivity in muscle (Host et al. 1998). Depletion of muscle glycogen stores may increase the ability of insulin to activate Akt (Derave 2000; Wojtaszewski et al. 2001), which will activate GLUT-4 translocation at the plasma membrane. Recently, Derave and coworkers demonstrated a negative correlation between glycogen content and cell surface GLUT-4 content in rats, and concluded that glycogen stores exert a negative feedback signal to stop the GLUT-4 recruitment at the plasma membrane (Derave et al. 2000). Furthermore, supercompensation of muscle glycogen stores prevents the increase in GLUT-4 translocation at the plasma membrane induced by exercise training, and high muscle glycogen reverses the increase in insulin sensitivity normally observed after exercise and feeding (Host et al. 1998). Because GLUT-4 translocation can be inhibited by high glycogen concentration, it has been speculated that glycogen particles may be directly bound to the GLUT-4 containing vesicles, preventing their translocation at the plasma membrane (Derave et al. 2000). However, the role of glycogen in regulating translocation of GLUT-4 remains controversial and the mechanisms uncertain.
1.3.4 Muscle glucose metabolism in the post-exercise state

After strenuous exercise, glycogen synthesis is of high priority in the exercised muscle. Both glycogen synthase activity and glucose transport increase after exercise. There are two distinct phases associated with glucose transport: an initial short insulin-independent phase and the second phase which is characterized by a large increase in insulin sensitivity (Hansen et al. 1998). The initial increase in muscle glucose uptake is due to an increased translocation of GLUT-4 through a contraction-mediated process (Ryder et al. 1999). The increased insulin sensitivity that may last up to 48 hours in humans contributes to the restoration or supercompensation of muscle glycogen stores by increasing recruitment of GLUT-4 through an insulin-dependent process (Ritcher et al. 2001). Furthermore, ingestion of a carbohydrate supplement with a high-glycemic index after exercise enhances the exercise-induced increase in GLUT-4 protein concentration in muscle, suggesting a complex interaction between exercise, carbohydrate supplementation and GLUT-4 protein content (Kuo et al. 1999).

In horses, no study has been conducted to assess glucose transport following exercise and feeding, especially insulin-sensitive glucose transporter GLUT-4 in skeletal muscle. Only one study investigated the change of GLUT-4 protein content after 6 weeks of training (McCutcheon et al. 2002). This study demonstrated that pre-exercise muscle GLUT-4 protein increased two-fold after training. However, no significant change in GLUT-4 protein content was observed immediately after exercise (McCutcheon et al. 2002).
In summary, GLUT-4 glucose transporters play a crucial role in the post-exercise state to enhance muscle glycogen resynthesis in humans and in rats, although the mechanisms of its regulations remain uncertain. The role of glucose transporters during the post-exercise state remains to be determined in equine skeletal muscle.
Figure 1.1: Biochemical pathways of glycogenolysis and anaerobic ATP production in the skeletal muscle.
Figure 1.2: Biochemical pathways of glycogen synthesis in the skeletal muscle after a high carbohydrate diet.
1.4 REFERENCES


CHAPTER 2

EXERCISE THAT INDUCES SUBSTANTIAL MUSCLE GLYCOGEN DEPLETION IMPAIRS SUBSEQUENT ANAEROBIC CAPACITY
2.1 SUMMARY

The purpose of the first study was to develop a model of muscle glycogen depletion and to study the effect of this model on aerobic and anaerobic capacity of horses. The maximal rate of oxygen consumption (VO$_2$ max), maximal accumulated oxygen deficit, muscle glycogen concentration and blood lactate concentration of 6 fit Standardbred horses were measured on 3 occasions 7 days apart (Trials 1, 2 and 3).

Between trials 2 and 3, strenuous exercise intended to deplete muscle glycogen was performed by exercising horses on the treadmill on 3 consecutive days. Strenuous exercise resulted in reduction of muscle glycogen concentration by at least 55% (from 155.1 ± 5.6 mmol/kg -wet weight- before Trial 2 to 55.4 ± 5.5 mmol/kg before Trial 3; mean ± SE, P < 0.05). VO$_2$ max was similar in Trials 2 and 3 (140.4 ± 5.4 ml O$_2$/kg and 141.8 ml ± 6.2 ml O$_2$/kg, respectively). Run time to fatigue during a single high-speed exercise test (253.9 ± 33.3 s and 153.8 ± 16.4 s, P < 0.05), accumulated oxygen deficit (95 ± 13.2 ml O$_2$/kg and 35 ± 13.9 ml O$_2$/kg, P < 0.05) and blood lactate concentration at the end of the sprint (17 ± 1.2 mmol/l and 10.5 ± 1.1 mmol/l, P < 0.05) were less during Trial 3 than Trial 2.

These data suggested that repeated strenuous exercise that causes muscle glycogen depletion results in impairment of anaerobic but not aerobic metabolism. However, because of the confounding effects of other exercise-induced changes, we could not conclude that pre-exercise muscle glycogen depletion impaired anaerobic capacity of horses.
2.2 INTRODUCTION

Pre-existing muscle glycogen depletion in humans decreases endurance time and increases the time required to cover a given distance during submaximal exercise (Ahlborg et al. 1967; Karlsson and Saltin 1971). However, the importance of muscle glycogen concentration for short-term high-intensity exercise is less well established. It has been reported that high-intensity exercise performance was not impaired by low intramuscular glycogen concentration (Symons and Jacobs 1989; Hargreaves et al. 1997) and that the decline in exercise performance during high-intensity, intermittent exercise was not related to a reduction in muscle glycogen (Hargreaves et al. 1998). However in both of these studies, muscle glycogen concentration was not severely depleted. Conversely, several investigators have concluded that a substantial reduction of muscle glycogen availability may limit performance during high-intensity exercise (Hepburn and Maughan 1982; Casey et al. 1996). A decrease in maximal isokinetic force generation by the leg extensors was reported 1 hour following exercise designed to deplete intramuscular glycogen (Jacobs 1981). However this decrease in maximal isokinetic force generation cannot be attributed only to the lack of glycogen since the previous prolonged activity may have played a large role in the development of fatigue (Sherman et al. 1984; Symons and Jacobs 1989). Other investigations have attempted to dissociate the effect of glycogen depletion from the effects of exercise used to deplete glycogen by evaluating performance after various time intervals and reported a decrease in isometric endurance of the leg extensors in the low glycogen condition (Hepburn and Maughan
A decrease in time to fatigue during anaerobic exercise was noticed in subjects receiving a low carbohydrate diet although muscle glycogen concentrations were not reported (Maughan and Poole 1981).

In horses, there is extensive evidence that running, repeated bouts of maximal intensity exertion, or a combination of prolonged running and repeated sprints, results in significant declines of muscle glycogen concentration up to 50% (Lindholm et al. 1974; Hodgson et al. 1984a,b; Snow et al. 1985; Harris et al. 1987; White and Snow 1987; Essén-Gustavsson et al. 1989; Snow and Harris 1991). It is possible that such a reduction in muscle glycogen stores contribute to a decline in high-intensity-exercise performance in horses although this has received scarce attention.

In horses, the reduction of muscle glycogen concentration by 22% did not have a measurable effect on high-intensity exercise performance (Davie et al. 1996). Overall, there is little evidence to support or refute the effect of low muscle glycogen concentration on aerobic and anaerobic capacity of horses. Although the effects of energy availability and dietary manipulation on exercise capacity have been well investigated in human exercise physiology, to our knowledge, no study demonstrates conclusively that manipulation of muscle glycogen stores affects athletic capacity of horses.

The purpose of this study was to establish a protocol that reduced muscle glycogen concentration by at least 55% and to determine if this exercise model was associated with a decrease in aerobic and/or anaerobic capacity.
2.3 MATERIALS AND METHODS

Design:

The effect of three days of intense exercise that depleted muscle glycogen concentration by at least 55% was examined in a longitudinal study. Each horse served as its own control with pre-depletion measures of aerobic and anaerobic capacity collected twice with a one-week interval (Trials 1 and 2). After 2 days of rest, horses then performed 3 days of strenuous exercise (details below) before aerobic and anaerobic capacities were again assessed (Trial 3). The maximal rate of oxygen consumption (VO$_2$ \textsubscript{max}), measured during an incremental exercise test, and maximal accumulated oxygen deficit (MAOD), measured during a single high-speed exercise test at 120% VO$_2$ \textsubscript{max}, were used to estimate, respectively, the aerobic and anaerobic capacity of the horses. This protocol was approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University.

Animals:

The subjects were 6 Standardbred horses aged, 2 to 7 years. The animals had been accustomed to the treadmill barn (Fig. 2.1), to walking and running on the treadmill, and to wearing the mask of the open circuit indirect calorimeter for one month. A basic level of fitness had been established by running the horses on the treadmill 5 days per week for 5 months. Except during the 3 days of strenuous exercise and Trial 3, the horses were fed grass hay and mixed grain in a sufficient quantity to maintain bodyweight. During the 3
days of strenuous exercise and Trial 3, the horses were fed free choice grass hay. Trace mineralized salt blocks were available at all times.

*Experimental protocol:*

**Aerobic capacity:** The maximal rate of oxygen consumption was measured by indirect calorimetry (Oxymax-XL, Columbus instruments, Columbus, OH; Hinchcliff et al. 1993) during an incremental exercise test that consisted of the horses running on a treadmill inclined at 4 degrees for 90 seconds at 4m/s, then at 7 m/s for 90 s with subsequent increases of 1 m/s every 90 s until the horses were unable to maintain their position on the treadmill (Fig. 2.2; Hinchcliff et al. 1993). The VO$_2$ max was determined as the value of oxygen consumption, which reached a plateau (defined as a change in VO$_2$ < 4 ml/min/kg) (Hinchcliff et al. 1996).

**Anaerobic capacity:** Anaerobic capacity was estimated by the determination of the maximal accumulated oxygen deficit during a single high-speed exercise test (Medbø et al. 1988; Eaton et al. 1995). The single high-speed exercise test consisted of the horses running on a treadmill (4 degrees incline) for 5 min at 3 m/s (warm up), at the speed calculated to require an oxygen consumption of 120% of the maximum oxygen consumption (120% VO$_{2\text{max}}$) until fatigue, and 5 min at 3 m/s (cool down). Oxygen deficit was calculated by subtracting the actual oxygen consumption from the estimated oxygen demand. Oxygen demand was calculated from the speed-VO$_2$ relationship determined by measuring the rate of oxygen consumption at each speed during the incremental exercise test. The regression equation describing the speed-VO$_2$ relationship
for each horse was developed from these values, and used to estimate oxygen demand at higher speeds (Hinchcliff et al. 1996). The speed used to elicit 120% of VO₂ max (11.1 ± 0.1 m/s) was determined for each horse from measurements made during the first incremental exercise test, and was the same for each of the 3 trials.

Muscle glycogen-depleting exercise: Between Trials 2 and 3, the horses performed 3 days of strenuous exercise intended to deplete muscle glycogen stores. This strenuous, repeated exercise consisted of running horses on a treadmill (with a 4° slope) for 5 minutes at 4 m/s, 15 minutes at 70% of VO₂ max, 5 minutes at 90% of VO₂ max, and, after 30 minutes rest, 6 sprints of one minute at 100% VO₂ max with 5 minutes walking between each sprint. Sprint exercise was not performed on the first day of strenuous exercise. The third incremental exercise test and single high-speed exercise test were repeated 14 hours (range 12-16) and 38 hours (range 36-40), respectively after the last bout of repeated exercise.

Muscle glycogen: Muscle samples were collected by needle biopsy in the middle gluteal muscle at a depth of 8 cm at the same location in each horse before each incremental exercise test and single high-speed exercise test. The muscle samples were flash frozen in liquid nitrogen and then frozen at –70°C until analysis. Muscle glycogen concentrations were determined with a fluorometer (Sequoia-Turner model 112) after acid hydrolysis. Water content of each muscle sample was determined by measuring the difference of muscle sample weight after drying the sample in an oven at 90°C until weight stabilization.
Weight: The horses were weighed before each incremental exercise test and each single high-speed exercise test.

Blood samples: Blood samples were collected before exercise and at the end of each speed increment during the incremental exercise test and single high-speed exercise test, through a catheter placed in a jugular vein. Venous samples for measurement of blood lactate concentration were collected in total blood lactate tubes (YSI total blood lactate tube, Yellow Springs Instruments, Yellow Springs, OH). Blood lactate was determined by an electrochemical method (YSI 1500 Sport lactate analyzer, Yellow Springs Instruments). Venous blood was also placed into a glass tube containing EDTA for measurement of hematocrit and plasma total protein concentration, and which were measured by the microhematocrit technique and refractometry, respectively.

Statistical analysis:

Statistical analyses were performed using the Student t-test for paired data, or one way or two repeated measures analysis of variance, as appropriate. The null hypothesis was rejected at $P < 0.05$. All results are expressed as mean ± standard error.

2.4 RESULTS

Muscle glycogen concentration: There was no difference in the glycogen concentration of muscle collected before Trials 1 and 2. However, there was a significant ($P < 0.05$) reduction in muscle glycogen concentration (by 60.6% and 64.5%, respectively)
immediately before the incremental exercise test and the single high-speed exercise test of Trial 3, compared with values for Trials 1 and 2 (Fig. 2.3).

Run time to fatigue, oxygen consumption, and accumulated oxygen deficit:

Run time during the incremental exercise test was significantly (P < 0.05) less after the three days of strenuous exercise (from 722.4 ± 20.6 s to 676 ± 29.3 s, for Trials 2 and 3, respectively). Run time to fatigue during the single high-speed exercise test of Trial 3 was 38.9 ± 4.1% less than that of Trial 2 (253.9 ± 33.3 s and 153.8 ± 16.4 s, P < 0.001). There was a significant (P = 0.02) correlation between the difference of run time to fatigue of the single high-speed exercise test of Trial 2 and 3 and muscle glycogen concentration prior to the third single high-speed exercise test (Fig. 2.4). Despite a decrease in run time to fatigue, there was no significant reduction in rate of maximum oxygen consumption between Trial 2 and Trial 3 (140.4 ± 5.4 ml O₂/kg and 141.8 ml ± 6.2 ml O₂/kg, respectively). There was a 63% reduction in MAOD in Trial 3 compared with Trial 2 (35 ± 13.9 ml O₂ equivalent/kg and 95 ± 13.2 ml O₂ equivalent/kg, respectively, P < 0.05).

Blood lactate concentration:

Maximum blood lactate concentration and blood lactate concentration at the end of each speed increment of the incremental exercise test were lower in Trial 3 than in Trial 2 (Fig. 2.5). The speed at which a blood lactate concentration of 4 mmol/l was reached (VL₄) was 19.7% higher in Trial 3 than in Trial 2 (from 7.98 ± 0.33 m/s during Trial 2 to 9.46 ± 0.3 m/s during Trial 3). Blood lactate concentration at the end of the sprint and at the end of the recovery during the single high-speed exercise test was significantly (P <
0.0001) lower in Trial 3 than in Trial 2 (Fig. 2.6). However, the apparent rate of lactate appearance during the sprint period of the single high-speed exercise test was not statistically different between Trial 2 and Trial 3 (0.07 ± 0.01 mmol/kg/s and 0.07 ± 0.01 mmol/kg/s, respectively).

*Other measurements:*

Water content in muscle samples collected immediately prior to the incremental exercise test was not statistically different between Trials 2 and 3 (77.72 ± 2.2% and 78.6 ± 1.0%, respectively). There was a significant (P < 0.001) reduction in bodyweight by 5.4% in Trial 3, compared to values before Trial 1 (Table 2.1). Hematocrit and total protein concentration at the end of the sprint during the single high-speed exercise test were lower (P < 0.05) in Trial 3 than in Trial 2 (Table 2.1).
Figure 2.1: Picture of a horse (“Fred”) at the Alice L. Finley Memorial Farm (research facility).

Figure 2.2: Picture of a horse running on the treadmill during the incremental exercise test. The horse wears a loose-fitting mask for measurement of the respiratory gas exchange.
Figure 2.3: Muscle glycogen concentration (mmol/kg, wet weight) of 6 horses prior to an incremental exercise test (IET) and a single high-speed exercise test (SHSET) for Trials 1-3. * P < 0.05 versus corresponding value for Trials 1 and 2. Values are means ± SE.
Figure 2.4: Relationship between run time to fatigue during the single high-speed exercise test and muscle glycogen concentration.

$R^2 = 0.769$

$P = 0.022$
Figure 2.5: Blood lactate concentration of 6 horses during each of the 3 incremental exercise tests. * P < 0.05 versus corresponding value for Trials 1 and 2. Values are means ± SE.
Figure 2.6: Blood lactate concentration at rest, at the end of the warm up, at the end of the sprint and at the end of the recovery during each single high-speed exercise test. * P < 0.05 versus corresponding value for Trials 1 and 2. Values are means ± SE for 6 horses.
Table 2.1: Bodyweight immediately before the incremental exercise test and single high-speed exercise test, and hematocrit and total protein concentration during the IET and at the end of the sprint phase of SHSET (mean ± s.e.). IET: incremental exercise test, SHSET: single high-speed exercise test, Bw = Bodyweight, Hct = hematocrit, TP = total protein. * = Significant difference due to treatment (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial End</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IET</td>
<td>SHSET</td>
<td>IET</td>
</tr>
<tr>
<td>Bw (kg)</td>
<td>422.6 ± 8.0</td>
<td>419.3 ± 8.1</td>
<td>416.5 ± 7.9</td>
</tr>
<tr>
<td>Hct (l/l)</td>
<td>53.7 ± 1.2</td>
<td>55.1 ± 1.1</td>
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</tr>
<tr>
<td>TP (g/dl)</td>
<td>7.1 ± 0.1</td>
<td>7.3 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
</tbody>
</table>
2.5 DISCUSSION

These results demonstrated that 3 days of repeated strenuous exercise caused substantial muscle glycogen depletion in horses, similar to that previously reported (Davie et al. 1995). Associated with the reduction in muscle glycogen concentration was a decrease in run time to fatigue during the single high-speed exercise test and a significant reduction in maximal accumulated oxygen deficit. In contrast, 3 days of strenuous exercise did not affect VO₂ max. We conclude that 3 days of exercise that depletes muscle glycogen concentration by at least 55% impairs subsequent anaerobic but not aerobic capacity of horses.

To our knowledge, this is the first study to show an association between glycogen-depleting exercise and subsequent high-intensity exercise capacity in horses. The results of our study are in contrast to the findings of Davie et al. (1996), who reported that a 22% decrease of muscle glycogen concentration did not have a measurable effect on high-intensity exercise performance. A concern with the study of Davie and coworkers is that the muscle glycogen concentration might not have been reduced sufficiently to produce measurable effects on athletic performance. For instance, a decrease in pre-exercise muscle glycogen concentration by 41% impaired the capacity for anaerobic work in horses dragging a sled (Topliff et al. 1985). These inconsistencies relative to the effect of muscle glycogen depletion on athletic performance have been observed both in human and equine studies and might be related to the degree of depletion of pre-exercise glycogen concentration. For instance, it has been speculated
from human studies that muscle glycogen availability may impair high-intensity exercise performance only when it is substantially reduced prior to exercise (Casey et al. 1996).

The lack of an effect of glycogen depletion on aerobic capacity noted in this study is in accordance with previous work reported both in humans and horses. Similar oxygen consumptions during exercise were reported despite varying the initial muscle glycogen concentration in humans (Bergström and Hultman 1967), and a change in \( \text{VO}_2 \text{max} \) was not detected in horses with muscle glycogen depletion (Davie 1995).

Alterations of pre-exercise muscle glycogen reserves by exercise manipulations have in general served to establish a close relationship between muscle glycogen and fatigue resistance during endurance exercise in humans. However, in the present study, impairment of anaerobic capacity evidenced by a decrease in MAOD and run time to fatigue cannot be attributed only to the decrease in muscle glycogen stores since the previous high-intensity exercise may have played another role in the development of fatigue. Other mechanisms that may have reduced performance during high-intensity exercise include decreased creatine phosphate availability, increased muscle \( \text{H}^+ \) concentration and impairment of sarcoplasmic reticulum function (Hargreaves et al. 1998). Additionally, factors such as dehydration and musculoskeletal pain may have decreased performance.

Another limitation of this study is the method used to assess anaerobic capacity in horses. The use of MAOD test as a gold standard for the assessment of anaerobic capacity in horses is questionable because MAOD is an indirect test of anaerobic capacity
and the accuracy of the test could not be assessed because of the absence of other measures of anaerobic capacity (Medbø et al. 1988; Eaton et al. 1995).

Following 3 days of strenuous exercise, a marked reduction in blood lactate concentration was noticed during the incremental exercise and the single high-speed exercise tests, illustrated by the increase in $\text{VL}_{4}$ and decreased maximum blood lactate concentration at the end of the sprint. In humans, several investigators have found that prolonged exercise inducing severe glycogen depletion resulted in diminished capacity to raise blood lactate concentrations but the direct effects of glycogen depletion were not demonstrated (Karlsson and Saltin 1971). In humans, blood lactate concentration after high-intensity exercise was lower after a low carbohydrate diet when compared to a high carbohydrate diet (Greenhaff et al. 1988). Furthermore, blood lactate concentration at the end of exercise was lower for the glycogen-depleted phase compared to the glycogen repletion phase (Topliff et al. 1983). One explanation for the decreased in lactate concentration would be a lack of substrate for glycogenolysis and glycolysis during anaerobic metabolism and/or by a reduction in work performed (Casey et al. 1996).

However, the existence of a direct relationship between pre-exercise muscle glycogen, the rate of glycogenolysis and secondary lactate production remains controversial in humans (Saltin and Karlsson 1971; Jacobs 1981; Greenhaff et al. 1988; Ren et al. 1990). The present study demonstrates that low muscle glycogen concentration decreases blood lactate concentration at identical work intensities, suggesting reduced glycogenolysis in these horses.
Evidence to support an inhibition of anaerobic metabolism includes a reduction in run time, MAOD and blood lactate concentration during the single high-speed exercise test. Although not demonstrated conclusively, we speculate from these data that decreased availability of glycogen stores reduces the rate of anaerobic glycogenolysis, lactate production and therefore anaerobic ATP synthesis. We speculate that a failure to maintain ADP homeostasis at the contractile site due to a relative impairment of ATP resynthesis may result in the decrease of anaerobic power generation (Broberg and Sahlin 1989). We believe that our results demonstrate a potential role for glycogen depletion in limiting high-intensity exercise capacity of horses.

In summary, three days of repeated, intense exercise, that decreased muscle glycogen concentration, impairs anaerobic but not aerobic metabolism during exercise. These results suggest that glycogen availability may contribute to this decline in anaerobic power generation during high-intensity exercise but do not conclusively demonstrate such an effect because of the confounding effects of other exercise-induced changes. Therefore, further research is required to determine the exact role of muscle glycogen stores on the physiological response to high-intensity exercise in horses.
2.6 REFERENCES


CHAPTER 3

MUSCLE GLYCOGEN DEPLETION AND SUBSEQUENT REPLENISHMENT

AFFECT ANAEROBIC CAPACITY OF HORSES.
3.1 SUMMARY

The purpose of this second study was to determine the effect of muscle glycogen depletion and subsequent replenishment on anaerobic capacity of horses. In a blinded crossover study, seven fit horses performed glycogen-depleting exercise on two occasions. Horses were infused after glycogen-depleting exercise with either 6 g/kg body weight of glucose as a 13.5% solution in 0.9% NaCl (GLU) or with 0.9% NaCl (SAL) of equivalent volume. Subsequently, horses performed a high-speed exercise test (120% VO₂max) to estimate maximum accumulated oxygen deficit (MAOD).

Replenishment of muscle glycogen was greater (P < 0.05) in GLU (from mean ± s.e. 24.7 ± 7.2 mmol/kg to 116.5 ± 7 mmol/kg ww, respectively, before and after infusion) than in SAL (from 23.4 ± 7.2 mmol/kg to 47.8 ± 5.7 mmol/kg ww). Run time to fatigue during the high-speed exercise test (97.3 ± 8.2 s and 70.8 ± 8.3 s, P < 0.05), MAOD (105.7 ± 9.3 ml O₂eq/kg and 82.4 ± 10.3 ml O₂eq/kg, P < 0.05) and blood lactate concentration at the end of the high-speed exercise test (11.1 ± 1.4 mmol/l and 9.2 ± 3.7 mmol/l, P < 0.05) were greater for GLU than for SAL, respectively.

We concluded that decreased availability of skeletal muscle glycogen stores diminishes anaerobic power generation and capacity for high-intensity exercise in horses.
Carbohydrate in the form of muscle glycogen is the energy source for anaerobic glycolysis during vigorous exercise in humans and horses. However, repeated bouts of maximal intensity exertion or a combination of prolonged running and repeated sprints results in declines of up to 50% in muscle glycogen concentration of horses (Hodgson et al. 1984; Snow et al. 1985; Harris et al. 1987; Snow and Harris 1991; Davie et al. 1996). Furthermore, many horses undertake several events in a single day and the interval between exercise bouts may be inadequate for complete restoration of the muscle glycogen pool (Snow and Harris 1991). It is possible that a reduction in muscle glycogen stores contributes to a decline in subsequent high-intensity exercise performance in horses, although this issue has received scarce attention. While a reduction in muscle glycogen concentration by 22% did not have a measurable effect on duration of high-intensity exercise (Davie et al. 1996), exercise that depleted muscle glycogen by at least 55% of its initial value was associated with a marked reduction in anaerobic power generation during subsequent high-speed exercise (Chapter 2). These latter results suggest that lower glycogen availability may contribute to a decline in anaerobic power generation during high-intensity exercise. However, this latter study did not conclusively demonstrate such an effect of muscle glycogen depletion because of the confounding residual effects of other exercise-induced changes, such as dehydration and musculoskeletal pain, on athletic capacity. Therefore, the effect of pre-exercise muscle glycogen depletion on athletic capacity of horses remains uncertain.
It is well recognized that provision of supplemental carbohydrate to humans increases the time-to-fatigue during moderate to mild exercise (Coyle 1992). Ingestion of foods with a high glycemic index by humans before exertion increases pre-exercise muscle glycogen concentration and the time-to-fatigue, and delays the decrease in muscle glycogen concentration with a subsequent increase in duration of exercise (Neufer et al. 1987, Jenkins et al. 1993; Sherman et al. 1993). Consumption of a high carbohydrate diet for 3 to 4 days before exercise improves exercise capacity during high-intensity exercise, although this effect is less consistent than that with endurance exercise (Madsen et al. 1990; Maughan et al. 1997). While the observation that provision of supplemental glucose to horses performing endurance exercise on the treadmill increases the duration of exercise provides evidence that energy supplies limit performance in endurance exercise of horses (Farris et al. 1995), most athletic events involving horses are brief or require repeated bouts of exercise in shorter periods of time. Furthermore, as previously noted in Chapter 2, intramuscular glycogen stores may be a limiting factor during this type of activity in horses. Our hypothesis was that an exercise-induced reduction in muscle glycogen concentration of horses would reduce anaerobic capacity, and that subsequent replenishment of muscle glycogen stores by glucose infusion would increase anaerobic capacity over values for horses with continued muscle glycogen depletion.
3.3 MATERIALS AND METHODS

Experimental design:

The effect of muscle glycogen depletion on exercise performance was examined in a longitudinal and blinded study, using a partially counterbalanced randomized crossover design. Seven fit adult Standardbred horses performed two trials separated by a 16-day interval (Trials 1 and 2). Both trials involved the horses completing three consecutive days of strenuous exercise that depleted muscle glycogen by at least 55% of its initial values. After the last bout of glycogen-depleting exercise in each trial, horses were infused intravenously with either 6 g/kg body weight of glucose as a 13.5% solution in 0.9% NaCl or with equivalent volume of isotonic (0.9%) NaCl. The maximal rate of oxygen consumption (VO\textsubscript{2 max}), measured during an incremental exercise test, and maximal accumulated oxygen deficit (MAOD), measured during a single high-speed exercise test at 120% VO\textsubscript{2 max}, were used to estimate, respectively, aerobic and anaerobic capacities of the horses. The MAOD and VO\textsubscript{2 max} were measured 3 and 4 days before the horses undertook the glycogen-depleting exercise, respectively, and 12 hours and 36 hours after the end of glucose or saline administration. The order of trials for each horse was randomized but the overall design was balanced. The horses performed aerobic and anaerobic tests both in the depleted state and after repletion of the muscle glycogen stores (Fig. 3.1). This protocol was approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University.
**Horses:**

The subjects were seven clinically normal horses (6 Standardbreds and 1 Thoroughbred; 2 geldings and 5 females) with ages ranging from 2 to 7 years and had body weight of 436 ± 7 kg (mean ± SE).

The animals had been accustomed to the treadmill barn, to walking and running on the treadmill, and to wearing the mask of the open circuit indirect calorimeter for one month. Horses were conditioned by running on the treadmill (at a 2° incline) 5 days per week for 2 months. The conditioning consisted of the horses trotting at 4 m/s for 5 min, walking at 2 m/s for 2 min, trotting at 4 m/s for 5 minutes, walking at 2 m/s for 2 min, and galloping for 5 minutes at 8 m/s. To maintain a basic level of fitness during the experimental period, the horses continued to run on the treadmill at least 3 days per week. During the conditioning and experimental period, the horses were housed in box stalls (3 x 4 m) and fed mixed hay (approximately 8.5 kg/horse/day) and mixed grain (approximately 2 kg/horse/day) with an estimated digestible energy intake of 19 Mcal/horse/day, which was sufficient to maintain bodyweight. Extending from forty-eight hours prior to the glycogen-depleting exercise until the end of the trial, the horses were fed 8.5 kg of mixed hay per day with an estimated digestible energy of 13 Mcal/horse/day. Feed was withheld for twelve hours prior to the first day of the glycogen-depleting exercise. Trace mineralized salt blocks and water were available at all times.
**Indirect calorimetry:**

The horses were positioned on the treadmill and a loose-fitting mask was applied on their face for measurement of respiratory gas exchange. Oxygen consumption and carbon dioxide production were measured with an open circuit indirect calorimeter (Oxymax-XL, Columbus Instruments, Columbus, OH) during MAOD and VO₂ tests. Flow through the system was approximately 1500 l/min with the horse stationary and 10,000 l/min during running (see below for details). The oxygen and carbon dioxide sensors of the open circuit calorimeter (electrochemical cell and single-bean nondispersive infrared sensor, respectively) were calibrated against gases of known composition within 10 minutes of the start of each exercise test, during which the horse stood quietly on the treadmill. The overall accuracy of the system was verified daily by nitrogen dilution. Discrepancy between stimulated VO₂ produced by nitrogen dilution and the values measured by the system was ± 3% at nitrogen flow rates equivalent to a VO₂ of 54 l/min (approximately 140 ml/min/kg for a 385 kg horse).

**Determination of maximal O₂ consumption:**

The maximal rate of oxygen consumption of each horse was measured by indirect calorimetry during an incremental exercise test 4 days before the glycogen-depleting exercise and 2 days after the glycogen-depleting exercise, following glucose or saline infusion (Fig. 3.1). The incremental exercise test consisted of running horses on a treadmill inclined at 4 degrees for 90 seconds at 4 m/s, with subsequent increases of 1 m/s every 90 s until the horses were unable to maintain their position on the treadmill. VO₂ was measured every 10 seconds. VO₂ max was determined as the value of oxygen...
consumption when it reached a plateau (defined as a change in VO$_2$ < 4 ml/min/kg for an increase in speed). The speed:VO$_2$ relationship for each horse was determined by linear regression of oxygen consumption and speed during the incremental exercise test.

**Determination of maximum accumulated oxygen deficit:**

Anaerobic capacity was estimated by the calculation of the maximal accumulated oxygen deficit during a single high-speed exercise test 3 days prior to and 1 day after the glycogen-depleting exercise. The single high-speed exercise test consisted of running the horses on a treadmill (4° incline) for 5 min at 3 m/s (warm up), at the speed calculated to require an oxygen consumption 120% of the maximum oxygen consumption (120% VO$_2$ max) until fatigue (sprint), and 5 min at 3 m/s (cool down). Fatigue was assessed by the same observer who was blinded to treatment and was based on the horse’s inability to maintain its position on the treadmill despite vigorous, humane (verbal) encouragement. Time-to-fatigue was recorded for each horse. Oxygen deficit was calculated by subtracting the actual oxygen consumption, measured during the single high-speed exercise test, from the estimated oxygen demand (Eaton et al. 1995). Oxygen demand was calculated from the speed-VO$_2$ relationship determined by measuring the rate of oxygen consumption at each speed during the incremental exercise test. The regression equation describing the speed-VO$_2$ relationship for each horse was developed from these values, and used to estimate oxygen demand at higher speeds.

**Glycogen-depleting exercise protocol:**

The horses performed 3 days of strenuous exercise intended to deplete muscle glycogen stores. Exercise for each horse was individualized based on the assessment of
aerobic capacity. This strenuous, repeated exercise consisted of running horses on a treadmill (with a 4° slope) for 5 minutes at 4 m/s, 15 minutes at 70% of VO$_2$ max, 5 minutes at 90% of VO$_2$ max, and after 30 minutes rest, 6 sprints of one minute at 100% VO$_2$ max with 5 minutes walking between each sprint. We have demonstrated that this exercise protocol depletes muscle glycogen by at least 55% of its initial value (Chapter 2). The single high-speed exercise test and the incremental exercise test were repeated 24 and 48 hours, respectively, after the last bout of the glycogen-depleting exercise for each trial.

_Treatment infusions:_

For each trial, thirty minutes after the last bout of the glycogen-depleting exercise, the horses received, randomly, and in a blinded fashion either 6 g/kg bw of glucose as a 13.5% solution in isotonic saline (GLU) or isotonic (0.9%) saline solution (SAL) of equivalent volume delivered at an equivalent rate to that of the glucose infusion. Fluid administration began within 30 minutes of the end of the strenuous exercise. Fluid was administered at a mean rate of 225.5 g of glucose/hour for 11.9 ± 0.1 h (mean ± SE), through a catheter (14-gauge, 5.25 in., Angiocath, Deseret, Sandy, UT) placed in a jugular vein. Similar glucose infusion has previously been shown to replenish at least 80% of muscle glycogen concentration in horses (Davie et al. 1995). The infusion was repeated 30 minutes after the end of the single high-speed exercise test, following the protocol described above, to ensure partial or complete restoration of muscle glycogen concentration before the incremental exercise test. The horses were confined in their stalls during the infusion and until the subsequent exercise test.
Weight: The horses were weighed on entry to the laboratory before each exercise test.

Biochemical analysis:

Muscle samples were collected by needle biopsy of the middle gluteal muscle at a depth of 6 cm, under aseptic conditions after desensitization of the area with 2% mepivacaine (Carbocaine, Abbott, North Chicago, IL). Samples were collected before each incremental exercise and single high-speed exercise test, and within 10 minutes of the end of the last bout of glycogen-depleting exercise. The muscle samples were flash frozen in liquid nitrogen and then stored at -70°C until analysis. Muscle glycogen concentration was determined in duplicate with a fluorometer (Sequoia-Turner model 112, Turner Design, Sunnyvale, CA) after acid hydrolysis (Passoneau and Lauderdale, 1974).

Blood samples were collected through a catheter (14-gauge, 5.25 in., Angiocath, Deseret, Sandy, UT) placed in a jugular vein before exercise and at the end of each speed increment during the incremental exercise test, after desensitization of the overlying skin. Blood samples were collected before each single high-speed exercise test, at the end of the warm up, at the end of the sprint, and 5 and 10 minutes after the end of the test. All the tubes from each collection were centrifuged at 3,000 rpm (1500 x g) at 4°C for 20 min and plasma was stored into plastic cryogenic storage tubes at –20°C until analysis. Venous blood samples were placed into a glass tube containing EDTA (Vacutainer, Becton Dickinson, Parsippany, NJ) for measurement of hematocrit and plasma total protein concentration, that were measured in triplicate by the microhematocrit technique and refractometry (Cambridge Instruments, Buffalo, NY) respectively. Samples for
glucose assays were collected into chilled 5 ml evacuated tubes containing potassium oxalate and sodium fluoride. Plasma glucose concentrations were measured using an automatic analyzer (YSI model 1500, Yellow springs, instrument, Columbus, Ohio, USA). The area under the curve for blood glucose concentration was calculated using the trapezoidal rule. Venous samples for measurement of blood lactate concentration were collected into total blood lactate tubes (YSI total blood lactate tube with 2315 YSI blood lactate preservative kit, Yellow Springs Instruments, Yellow Springs, OH). Blood lactate concentration was determined in duplicate by an electrochemical method (YSI 1500 Sport lactate analyzer, Yellow Springs Instruments, Yellow Springs, OH). The onset of blood lactate accumulation was estimated by measuring the variable VL4, which represented the speed at which a blood lactate concentration of 4 mmol/l was reached during the incremental exercise test. The apparent rate of lactate production (expressed in mmol/l/s) was calculated using the following equation:

\[(\text{[Lac]}_{\text{end}} - \text{[Lac]}_{\text{bef}}) / \text{Time}_{\text{sprint}}\]

where \([\text{Lac}]{\text{end}}\) is the blood lactate concentration at the end of the sprint (expressed in mmol/l), \([\text{Lac}]{\text{bef}}\) is the blood lactate concentration at the end of the warm up (expressed in mmol/l) and \(\text{Time}_{\text{sprint}}\) is the time of sprint measured in seconds.

Statistical analysis: Before the study, we calculated that the power to detect a 10% difference in MAOD and an 8% difference in VO\textsubscript{2 max} using 7 horses was 90%. Statistical analyses were performed using either a two-way repeated measures analysis of variance (repeated measures on time and treatment) or a three-way repeated measures analysis of variance (time, treatment and trial), as appropriate for the dependent variable. The null
hypothesis (no effect of glucose infusion on muscle glycogen concentration and exercise performance) was rejected at P < 0.05. Significant differences between means (P < 0.05) were identified using Student-Newman-Keul’s test. All results are expressed as mean ± standard error.

3.4 RESULTS

Muscle glycogen concentration: Three days of strenuous exercise resulted in substantial reductions (P < 0.001) in muscle glycogen concentration for GLU and SAL (78% and 79% reduction from values before depletion, respectively, Fig. 3.2). Intravenous infusion of glucose resulted in replenishment of muscle glycogen concentration by 78.8% from the values before infusion, after the glycogen-depleting exercise (from 24.7 ± 7.2 mmol/kg wet weight [ww] to 116.5 ± 7.0 mmol/kg ww, before and after infusion respectively, P < 0.05), whereas saline infusion resulted in a significantly smaller increase in muscle glycogen concentration (from 23.4 ± 7.2 mmol/kg ww to 47.8 ± 5.7 mmol/kg ww, P < 0.05) (Fig. 3.2).

Maximal oxygen consumption and speed-VO₂ relationship: VO₂ max of the 7 horses prior to the glycogen-depleting exercise of Trial 1 was 142 ± 15 ml O₂.min⁻¹.kg⁻¹ at a treadmill speed of 9.9 ± 0.4 m/s. The average correlation coefficient of the speed-VO₂ relationship was 0.992 ± 0.002 (P < 0.01), the slope of the regression line was 14.7 ± 0.6 ml O₂.min⁻².kg⁻¹ and the ordinate intercept was 3.7 ± 0.9 ml O₂.min⁻¹.kg⁻¹. There was no significant difference in the rate of total maximum oxygen consumption (l.min⁻¹) or in the rate of
maximum oxygen consumption per unit body weight (ml.kg⁻¹.min⁻¹) between treatments before and after the glycogen-depleting exercise (Table 3.1). Maximum oxygen consumption per unit body weight (ml.kg⁻¹.min⁻¹), but not total VO₂ (l.min⁻¹), was increased in both groups after the glycogen-depleting exercise (Table 3.1).

Maximum accumulated oxygen deficit and run time to fatigue: Replenishment of muscle glycogen concentration by glucose infusion was associated with a 30% greater (P < 0.05) MAOD compared to values after saline infusion. A significant reduction in MAOD was observed for the saline treatment after the glycogen-depleting exercise (from 111.3 ± 9.3 ml O₂eq.kg⁻¹ for values before depletion to 82.4 ± 10.3 ml O₂eq.kg⁻¹ for values after depletion, P < 0.05), but not in the glucose supplemented horses (from 100.5 ± 9.3 ml O₂eq.kg⁻¹ to 105.7 ± 9.3 ml O₂eq.kg⁻¹). A significant (P < 0.05) reduction in run time to fatigue during the single-high speed exercise test was observed for the saline treatment after depletion of the muscle glycogen stores (from 129.9 ± 8.2 s to 70.8 ± 8.6 s, respectively before and after depletion), whereas there was no significant reduction in run time to fatigue for the glucose treatment (from 112.1 ± 8.2 s to 97.3 ± 8.2 s, respectively before and after infusion). Run time during the single high-speed exercise test was 28% longer in horses with glycogen replenishment compared to horses with persistent glycogen depletion.

Biochemical analysis: There was a significant treatment effect (P < 0.05) on blood glucose concentration at each speed, starting at 6 m/s during the incremental exercise test (Fig. 3.3). The area under the curve was significantly greater (P = 0.03) after GLU than SAL treatment. Maximum concentration of venous blood glucose during the incremental
exercise test was significantly higher for horses that received the glucose infusion after the glycogen-depleting exercise (from 4.04 mmol/l before the glycogen-depleting exercise to 5.83 mmol/l after the glycogen-depleting exercise, \( P = 0.02 \)). No significant difference in blood glucose concentration was observed during the single-high speed exercise test after glucose or saline treatment (Table 3.2). Blood lactate during the incremental exercise was significantly lower (\( P < 0.05 \)) for the saline treatment group after depletion compared to values before depletion, starting at 7 m/s (Fig. 3.4). Blood lactate concentration was significantly (\( P < 0.05 \)) lower for the saline group compared to the glucose treatment group after the glycogen-depleting exercise starting at 7 m/s. \( VL_4 \) during the incremental exercise test was significantly higher after saline treatment (7.6 ± 0.3 m/s and 8.5 ± 0.3 m/s, before and after depletion, \( P = 0.025 \)), whereas \( VL_4 \) was not significantly affected by glucose administration (from 7.2 ± 0.3 m/s to 7.3 ± 0.3 m/s). Venous blood lactate concentration at the end of the sprint and during recovery (5 min and 10 min) during the single-high speed exercise test was significantly (\( P < 0.05 \)) lower after saline treatment than after glucose treatment (Fig. 3.5). There was no treatment effect on the apparent rate of lactate production during the single high-speed exercise test after the glycogen-depleting exercise (0.10 ± 0.01 and 0.11 ± 0.01 mmol/l/s, respectively for SAL and GLU, \( P = 0.6 \)). Moreover, the apparent rate of lactate production during the sprint period was not statistically different before and after the glycogen-depleting exercise (0.09 ± 0.01 and 0.10 ± 0.01 mmol/l/s, respectively, \( P = 0.06 \)). The highest hematocrit and plasma total protein concentrations, measured during the incremental exercise test, were similar for both treatments after the glycogen-depleting exercise.
(Table 3.1). Hematocrit and total protein concentration were similar among treatment groups before, during and after each single-high speed exercise test. Hematocrit values were significantly (P < 0.05) lower after the glycogen-depleting exercise for each speed level during the single-high speed exercise test (Table 3.2).

Body weight: Body weight before the incremental exercise test decreased after the glycogen-depleting exercise for both treatments (from 432.3 ± 7.7 kg and 430.5 ± 7.7 kg, to 414.3 ± 7.7 kg and 421.4 ± 7.7 kg, P < 0.001 for SAL and GLU, respectively). In a similar fashion, body weights measured before the single high-speed exercise test decreased after the glycogen-depleting exercise for both treatments (from 430.1 ± 7.1 kg and 427.6 ± 7.1 kg, to 406.1 ± 7.1 and 412.9 ± 7.1, P < 0.001 for the saline and glucose treatments, respectively). However, body weight was not affected by treatment after the glycogen-depleting exercise (414.3 ± 7.7 kg and 421.4 ± 7.7 kg before the incremental exercise test, and 406.1 ± 7.1 kg and 412.9 ± 7.1 kg before single-high speed exercise test, respectively for Sal and Glucose).
Figure 3.1: Time-line of the experimental protocol. MB: muscle biopsy, IET: incremental exercise test, SHET: single high-speed exercise test, GLU: glucose treatment, SAL: saline treatment. Numbers in parenthesis represented numbers of horses receiving treatment.
Table 3.1: VO_{2\text{max}}, hematocrit and total protein concentration during the incremental exercise test. Values are mean ± SE for 7 horses. PCV, hematocrit; TP, total protein. * = P < 0.05 after depletion and GLU or SAL infusion.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VO_{2\text{max}} (ml.kg^{-1}.min^{-1})</th>
<th>VO_{2\text{max}} (l.min^{-1})</th>
<th>Highest PCV (%)</th>
<th>Highest TP (g/dl)</th>
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<td></td>
<td>Saline</td>
<td>Glucose</td>
<td>Saline</td>
<td>Glucose</td>
</tr>
<tr>
<td>Before depletion</td>
<td>143.3 ± 6</td>
<td>139 ± 4</td>
<td>61.7 ± 2.1</td>
<td>59.9 ± 2.1</td>
</tr>
<tr>
<td>After depletion</td>
<td>147.3 ± 6*</td>
<td>147.7 ± 7.7*</td>
<td>60.8 ± 2.1</td>
<td>62.0 ± 2.1</td>
</tr>
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</table>
Table 3.2: Hematocrit, total protein and glucose concentrations during the single-high speed exercise test. Values are mean ± SE for 7 horses. PCV, hematocrit; TP, total protein; SAL, saline group; GLU, glucose group; R, Rest; W, warm up; S, Sprint; R5, 5 min postrecovery; R10, 10 min postrecovery. * = P < 0.05 after depletion and GLU or SAL infusion.

<table>
<thead>
<tr>
<th></th>
<th>Before depletion</th>
<th></th>
<th>After depletion and GLU or SAL infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>W</td>
<td>S</td>
</tr>
<tr>
<td>TP SAL (g/dl)</td>
<td>6.3 ± 0.2</td>
<td>6.8 ± 0.2</td>
<td>7.4 ± 0.2</td>
</tr>
<tr>
<td>PCV SAL (%)</td>
<td>41.1 ± 1.4</td>
<td>49.4 ± 0.8</td>
<td>54.5 ± 1.0</td>
</tr>
<tr>
<td>Glucose SAL (mmol/l)</td>
<td>4.4 ± 0.2</td>
<td>4.3 ± 0.2</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Glucose GLU (mmol/l)</td>
<td>4.7 ± 0.2</td>
<td>4.7 ± 0.3</td>
<td>4.4 ± 0.2</td>
</tr>
</tbody>
</table>
Figure 3.2: Muscle glycogen concentration (mmol glucosyl units/kg, wet weight [ww]) before, at the end of the glycogen-depleting exercise (GDE), and after the intravenous glucose or saline infusion. For each occasion, muscle samples were collected before the high-speed exercise test (MAOD) or the incremental exercise test (VO_2max). Values are mean ± SE for 7 horses. *: P < 0.05 vs. corresponding value before glycogen depletion for treatment and time factors. †: P < 0.05 vs. corresponding value before glycogen depletion for time factor.
Figure 3.3: Plasma glucose (mmol/l) during the incremental exercise test before (Pre), and after (Post) glucose or saline infusion. # Significant difference before and after glycogen depletion, P < 0.05. Values are mean ± SE for 7 horses. * Significant difference between treatments, P < 0.05. GLU: glucose treatment, SAL: saline treatment, PRE: before glycogen-depleting exercise, POST: after glycogen-depleting exercise. Sample of 2 horses was used to calculate mean ± SE at 11 m/s.
Figure 3.4: Blood lactate concentration (mmol/l) during the incremental exercise. Values are mean ± SE for 7 horses. *P < 0.05 vs. corresponding value before glycogen-depleting exercise for SAL group; **P < 0.05 vs. corresponding value between treatment after glycogen-depleting exercise. GLU: glucose treatment, SAL: saline treatment, PRE: before glycogen-depleting exercise, POST: after glycogen-depleting exercise.
Figure 3.5: Blood lactate concentration (mmol/l) at rest, at the end of the warm up, at the end of the sprint and at the end of the recovery during each single-high speed exercise test, and before (a) and after (b) glycogen-depleting exercise. * P < 0.05 vs. corresponding value before glycogen depletion. R-5: 5 min post-recovery; R-10: 10 min post-recovery.
3.5 DISCUSSION

We demonstrated that, in horses, muscle glycogen depletion was associated with significant decreases in run time to fatigue, maximal accumulated oxygen deficit, and blood lactate concentration during a single high-speed exercise test. Furthermore, replenishment of muscle glycogen stores after substantial depletion was associated with restoration of the maximum accumulated oxygen deficit, run time to fatigue and blood lactate concentration to values similar to those recorded before glycogen depletion. In contrast, depletion and subsequent replenishment of muscle glycogen stores were not associated with a change in maximal rate of oxygen consumption. Demonstration that replenishment of muscle glycogen concentration by glucose infusion restores anaerobic capacity confirms a role for muscle glycogen concentration in limiting anaerobic capacity of horses. To our knowledge, this is the first evidence that provision of supplemental energy as glucose to horses, a herbivore that relies on short chain (volatile) fatty acid production for a significant proportion of its resting energy needs, restored muscle glycogen pool and preserved anaerobic capacity during high-intensity exercise.

Muscle glycogen and exercise performance: Prolonged submaximal running, repeated bouts of maximal intensity exertion, or a combination of prolonged running and repeated sprints, results in significant declines in muscle glycogen concentration in horses (Hodgson et al. 1984; Snow et al. 1985; Harris et al. 1987; Snow and Harris 1991; Davie et al. 1996). Moreover, repletion of muscle glycogen stores does not occur within 24 hours in Thoroughbred racehorses after a high-speed training run (Snow and Harris 1991). Therefore, exercise causes muscle glycogen depletion that may persist at the time
of subsequent exercise. While pre-existing muscle glycogen depletion in humans decreases endurance time and increases the time required to cover a given distance during submaximal exercise (Ahlborg et al. 1967; Karlsson and Saltin 1971), the effect of pre-existing muscle glycogen depletion on high-intensity exercise is less well established.

During high-intensity exercise, the major pathways for ATP resynthesis are the breakdown of creatine phosphate (CP) and the degradation of muscle glycogen to lactic acid (Hargreaves et al. 1998). It has been demonstrated that the contribution of glycogen to anaerobic ATP provision is 4-fold greater than that of CP during 30 s of maximal isokinetic cycling exercise (Hultman et al. 1991). The decline in muscle glycogen that occurs during repeated, high-intensity exercise could theoretically contribute to impaired exercise performance via a reduction in substrate for phosphorylase and subsequent glycolytic flux (Hargreaves et al. 1998). Several investigators reported conflicting results regarding the role of low intramuscular glycogen concentration on high-intensity exercise performance in humans. High-intensity exercise performance was not impaired by low intramuscular glycogen concentration (Symons and Jacobs 1989; Hargreaves et al. 1997) and the decline in exercise performance during high-intensity, intermittent exercise was not related to a reduction in muscle glycogen but was more likely induced by reduced CP availability, impairment in SR function, or some other fatigue-inducing agents (Hargreaves et al. 1998). Similarly, Symons and Jacobs (1989) found no effect of lowering muscle glycogen concentration on electrically evoked muscle force, maximal voluntary isometric force, or repeated maximal isokinetic leg extensions. However, in these studies, muscle glycogen pool was not severely depleted and it can be argued that a
greater degree of glycogen depletion is required before changes in performance during high-intensity exercise can be detected. Theoretically, given that in vitro Km of glycogen phosphorylase is reported to be low, a muscle glycogen concentration of 150 mmol.kg\(^{-1}\) dry weight will provide sufficient fuel to perform high-intensity exercise for 115 to 405 s (Casey et al. 1996). In the present study, the decrease in exercise duration in SAL was likely related to the greater reduction in pre-exercise muscle glycogen concentration with that treatment. Conversely, some authors maintain that variations in muscle glycogen concentration should have no effect on duration of short-term maximal exercise because intramuscular concentration remains high at the point at which fatigue develops (Symons and Jacobs 1989). However, at an exertion intensity of 150% VO\(_{2}\)\(_{\text{max}}\), which was maintained for approximately 8 minutes, quadriceps femoris muscle glycogen concentration declined from 62 mmol/kg to 13 mmol/kg (Gollnick et al. 1974). Therefore, short-term high-intensity exercise can induce substantial depletion of muscle glycogen stores.

Because the major pathways for anaerobic ATP resynthesis are the breakdown of creatine phosphate and the degradation of muscle glycogen to lactic acid, reduced glycogen availability could contribute to a decline in anaerobic energy production and exercise performance (Klausen et al. 1989; Hargreaves et al. 1998), leading several investigators to speculate that substantial reduction of muscle glycogen availability may limit performance during high-intensity exercise (Jacobs 1981; Hepburn and Maughan 1982). In support of this theory is the observation that a decrease in maximal isokinetic force generation by the leg extensors was reported 1 hour after exercise designed to
deplete intramuscular glycogen (Jacobs et al. 1981, 1982). Similarly, intense knee extensor performance, during two exercise bouts separated by 1h, is maintained in one leg with elevated muscle glycogen, whereas performance is reduced in the contralateral leg with reduced muscle glycogen (Bangsbo et al. 1992). In the present study, evidence to support an inhibition of anaerobic metabolism by glycogen depletion included a reduction in run time, MAOD and blood lactate concentration in the saline treatment group during the single high-speed exercise test.

The importance of muscle glycogen reserves to prevent fatigue is also highlighted by studies that manipulated muscle glycogen stores with different diets. A decrease in time-to-fatigue during anaerobic exercise occurs in humans fed a low carbohydrate diet (Maughan et al. 1981; Langfort et al. 1997) and performance during supramaximal intermittent exercise was decreased in the low carbohydrate diet group (Jenkins et al. 1993). When compared to a low carbohydrate diet, a moderate and high consumption of dietary carbohydrate can at least maintain supramaximal intermittent exercise performance (Jenkins et al. 1993).

In horses, it is possible that a reduction in pre-exercise muscle glycogen stores contributed to a decline in high-intensity-exercise performance, although this issue has received scarce attention. Davie et al. (1996) reported that a 22% decrease of muscle glycogen concentration did not have a measurable effect on high-intensity exercise performance. A concern with Davie's study is that the muscle glycogen concentration might not have been reduced sufficiently to produce detectable effects on athletic performance, given the small number of animals included in the study. In contrast, a
decrease in pre-exercise muscle glycogen concentration by 41% impaired the capacity for anaerobic work in horses dragging a sled (Topliff et al. 1985), leading the authors to suggest a reduction in anaerobic capacity. We have also previously demonstrated that exercise that induces substantial depletion of muscle glycogen stores in horses is associated with decreased run time to fatigue and decreased anaerobic capacity during a subsequent high-speed exercise test (Chapter 2). Furthermore, low muscle glycogen concentration is associated with a decrease in blood lactate concentration at identical work intensities, suggesting reduced glycogenolysis in these horses. However, in this study, impairment of anaerobic capacity evidenced by a decrease in MAOD and run time to fatigue cannot be attributed only to the decrease in muscle glycogen stores, since the previous high-intensity exercise may have played another role in the development of fatigue.

In summary, the effect of muscle glycogen depletion on short-term high-intensity exercise duration has remained controversial in both human and equine studies. One explanation for this controversy could be the diversity of exercise protocols and experimental designs, and might be related to differences in pre-exercise muscle glycogen concentration. For instance, it appears that glycogen availability impairs performance when muscle glycogen stores are severely depleted (Casey et al. 1996). Moreover, in some studies, intramuscular glycogen concentrations were assumed to be low but were not measured; raising the issue as to what extent the described protocols actually altered muscle glycogen concentration (Maughan et al. 1981; Casey et al. 1993; Jenkins et al. 1993). A further challenge to investigations of the relationship between
muscle glycogen and athletic capacity resides in the difficulty to control extraneous factors that may also affect athletic capacity. For instance, the role of muscle glycogen stores as a factor limiting high-intensity exercise performance has been questionable because the previous exercise may be a more potent determinant of fatigue than glycogen availability (Grisdale et al. 1990). However, in the present study, the restoration of muscle glycogen depletion by glucose infusion accounted for the effect of such extraneous factors and confirmed the role of muscle glycogen as a factor limiting anaerobic capacity during high-intensity exercise in horses. Another limitation when investigating the role of glycogen substrate availability on anaerobic capacity is the method used to assess anaerobic capacity. In the present study, the use of the MAOD test as a gold standard for the assessment of anaerobic capacity in horses is problematic because MAOD is an indirect measured of anaerobic capacity. Despite accurate measurement of submaximal VO₂, the MAOD technique relies on the extrapolation of the VO₂ vs. speed regression to predict the O₂ demand for exercise at supramaximal intensities (Eaton et al. 1995). Therefore, the accuracy of estimation of oxygen demand at supramaximal intensity is unknown, and hence, the accuracy of the MAOD test can not be assessed because of the absence of other measures of anaerobic capacity (Eaton et al. 1995). However, these assumptions have been widely accepted, and MAOD have been used as a measure of anaerobic capacity in horses (Eaton et al. 1995).

The lack of an effect of glycogen depletion on aerobic capacity noted in the present study is consistent with previous reports in both humans and horses. Similar oxygen consumptions during exercise were reported despite varying the initial muscle
glycogen concentration in humans (Bergström and Hultman 1967), and a change in VO$_\text{max}$ was not detected in horses with muscle glycogen depletion (Davie 1995; Chapter 2).

*Glycogen and glycogenolysis.* A marked reduction in blood lactate concentration was apparent in horses with pre-existing muscle glycogen depletion at the end of the sprint and during recovery from the single high-speed exercise test. Furthermore, replenishment of muscle glycogen stores after substantial depletion was associated with restoration of blood lactate concentration to those recorded before glycogen depletion during the single high-speed exercise test to values similar. Blood lactate concentration of humans after high-intensity exercise is lower after a low carbohydrate diet and higher after a high carbohydrate diet (Greenhaff et al. 1988). One explanation for the decreased lactate concentration in glycogen-depleted state would be a lack of substrate for glycogenolysis and glycolysis during anaerobic metabolism and/or by a reduction in work performed (Hepburn and Maughan 1982; Casey et al. 1996). However, the existence of a direct relationship between muscle glycogen content and blood lactate accumulation has been questioned in humans with similar post-exercise blood lactate concentrations being observed over a wide range of pre-exercise muscle glycogen concentrations (Jacobs 1981; Greenhaff et al. 1988). Moreover, it has been reported that the rate of muscle glycogen degradation and of lactate production during short, intense contraction is not affected by the initial glycogen concentration (Ren et al. 1990; Bangsbo et al. 1992).

The decrease in high-intensity exercise performance, observed when glycogen levels are low, may be attributable to the alteration of the blood acid-base status induced
by exercise and dietary intervention. Furthermore, glycogen depletion-induced reductions in lactic acid production reduce cellular acid production, which plays a prominent role in the regulation of sympathetic vasoconstriction, through the activation of the skeletal muscle metaboreflex system. Therefore, glycogen depletion-induced reduction in lactate production may attenuate sympathetic vasoconstriction by reduction of the metaboreceptor stimulation (Synoway et al. 1992) and may contribute to the decrease in high-intensity exercise performance.

_Glycogen and fatigue._ Decreased availability of muscle glycogen stores likely reduces the rate of anaerobic glycogenolysis and lactate production, and therefore limits anaerobic ATP synthesis. We speculate that failure to maintain ADP homeostasis at the contractile site due to a relative impairment of ATP resynthesis secondary to reduced glycogen availability results in decreased anaerobic power generation (Green 1997). Potential intracellular sites where ADP could limit the muscular contraction process are: the actin-myosin interaction, the reuptake of calcium by the sarcoplasmic reticulum (SR), the maintenance of the Na⁺-K⁺ gradient, the membrane potential over the sarcolemma and the signal transduction between T-tubuli and SR (Sahlin 1992; Green 1997). Exercise with low initial glycogen stores results in rapid development of fatigue and in a more pronounced formation of IMP and NH₃ than exercise with normal glycogen levels, which may reflect the large increases in free ADP and AMP at the enzymatic site during the contraction (Ren et al. 1990; Spencer et al. 1992). Therefore, the decrease in substrate availability may impair glycolysis as evidenced by the decreased phosphofructokinase activity and tricarboxylic acid cycle intermediates in humans with depleted glycogen.
stores (Spencer et al. 1992). Moreover, because of the possible functional coupling between ATP supplied by glycolysis and ATP utilized within the SR-T tubule, reduction in muscular force, inhibition of contractile proteins and failure of calcium release observed during fatigue have been linked to reduced muscle glycogen stores (Chin and Allen 1997). Therefore, in the present study, all these mechanisms of interaction between muscular fatigue and substrate glycogen availability may partly explain the accelerated onset of fatigue observed in horses in a glycogen depleted state.

In summary, these data demonstrate that repletion of muscle glycogen stores restored anaerobic capacity of horses with pre-existing exercise induced depletion of muscle glycogen. Therefore, we concluded that decreased availability of skeletal muscle glycogen stores causes a decline in anaerobic power generation in horses.
3.6 REFERENCES


CHAPTER 4

INSULIN-SENSITIVE GLUCOSE-TRANSPORTER (GLUT-4) PROTEIN CONTENT IN EQUINE SKELETAL MUSCLE: CHARACTERIZATION; EFFECTS OF EXERCISE AND CARBOHYDRATE ADMINISTRATION.
4.1 SUMMARY

The objectives of this study were to characterize insulin-sensitive glucose-transporter GLUT-4 protein content in equine tissues, and to determine the effect of exercise and carbohydrate administration on GLUT-4 protein content in equine skeletal muscle.

Western blot analyses were performed on crude membrane preparations of equine and rat tissues to characterize GLUT-4. In a crossover, randomized and blinded study, horses received intravenously either 13.5% glucose (Glu) or an isotonic NaCl (Con) at similar infusion rates for 12.1 h after exercise. Muscle samples were collected from the middle gluteal muscle for measurements of glycogen concentration and total GLUT-4 protein content. Venous blood was collected for measurement of glucose concentration.

Western blot analysis of crude membrane preparation revealed immunoreactive bands for GLUT-4 in equine insulin-sensitive tissues, with a molecular weight (~ 53 kDa) similar to that present in rats. GLUT-4 protein content increased by 27.3% 22.2 h after exercise for Con group (P < 0.05). Intravenous infusion of glucose resulted in higher rate of glycogenesis over 22.2 h after exercise compared to the Con group (P < 0.001). Despite enhanced glycogenesis, there was no further increase in GLUT-4 protein content 10.1 h after Glu infusion.

We concluded that 1) horses express GLUT-4 protein in skeletal and cardiac muscles; 2) exercise increases total GLUT-4 protein content of skeletal muscle; and 3) replenishment of muscle glycogen stores after glucose infusion attenuates the exercise-induced increase in GLUT-4 protein content of equine skeletal muscle.
4.2 INTRODUCTION

During the recovery period from exercise, skeletal muscle is the major tissue responsible for glucose uptake and utilization, both of which are largely regulated by insulin. Glucose entrance across plasma membranes into the muscle cell is the rate-limiting step in glucose utilization, at least in rats and humans (Hayashi et al. 1997). Glucose transport across muscle sarcolemma occurs primarily by facilitated diffusion via a family of structurally related proteins (GLUT-1 to GLUT-8) (Hayashi et al. 1997). GLUT-4 is the major isoform in rodent and human muscle cells (Hayashi et al. 1997). Whereas GLUT-1 and GLUT-5 isoforms are mainly associated with the cell surface and are not insulin-stimulated, the translocation of the GLUT-4 protein from an intracellular (non active) pool to the plasma membrane (active site) is largely regulated in non-exercising animals and humans by an insulin-dependent process (Holloszy et al. 1986). The major inducers of increased glucose transport activity in muscle are fiber contractions and insulin, although numerous other factors including catecholamines, hypoxia, growth factors, and corticosteroids can also alter glucose transport (Hayashi et al. 1997). Recent studies suggest that glycogen concentration also plays a regulatory role in glucose transport (Derave et al. 1999).

In contrast to the extensive investigations carried out in humans and rats, little information is available in regard to glucose transport in other mammals. The GLUT-4-like protein has been characterized by immunoblot analysis in insulin-responsive tissues from calves, goats, and sheep (Aso et al. 1995; Hocquette et al. 1995, 1996a). However,
attempts to characterize GLUT-4 in ruminants have led to equivocal results because of
the lack of controlled experiments in some studies (Hocquette et al. 1995; Aso et al.
1995). Furthermore, glucose transport and GLUT-4 protein content appear to be different
in ruminants compared to single-stomached animals possibly because of the peculiarities
of glucose metabolism in ruminants. Because horses are also herbivores, it is likely that
there are differences in the physiological role of glucose transporters in horses compared
to non-herbivorous animals. GLUT-4 protein content and glucose transport of horses
increases with short-term exercise conditioning (McCutcheon et al. 2002). However,
there is little other information regarding factors influencing GLUT-4 content in equine
skeletal muscle.

After exercise, replenishment of muscle glycogen stores is of high priority for the
skeletal muscle. While it has been demonstrated that glucose transport is the rate-limiting
step for glucose uptake and glycogen resynthesis in rodent and humans skeletal muscles
(Fisher et al. 2002), the effect of post-exercise carbohydrate supplementation on the
glucose transporter has yet to be investigated in horses. Our first objective was to
characterize GLUT-4 transporters in non-exercising animals by demonstrating the
specificity of the immunoreactive bands detected by immunoblotting. Our second
objective was to evaluate the effects of exercise and carbohydrate administration on total
GLUT-4 content in equine skeletal muscle. Our hypothesis was that exercise and
carbohydrate administration would increase GLUT-4 protein content in equine skeletal
muscle.
4.3 MATERIAL AND METHODS

*Animals and experimental design:*

*Characterization of GLUT-4:* GLUT-4 protein was isolated from 3 muscle samples, 2 liver samples and 2 heart samples of horses. Muscle samples were collected by needle biopsy from the middle gluteal muscle of an adult horse over a 3 week-period (Lindholm and Piehl 1974). The horse was a healthy Standardbred gelding of 443 kg, which was housed in a box stall and fed mixed hay and mixed grain, enough to maintain body weight. Equine liver and heart samples were collected within 20 minutes after euthanasia (pentobarbital IV) of 2 horses, which were donated to Ohio State University for non-related medical conditions. The muscle and liver samples were flash frozen in liquid nitrogen and then stored at –80°C until analysis. Several experiments using immunoblot analysis were performed to specifically isolate GLUT-4 protein in these equine tissues.

*Effect of exercise and glucose infusion:* Six fit horses participated in a balanced, blinded, randomized crossover study. The horses were 5 Standardbreds and 1 Thoroughbred, with ages ranging from 2 to 7 years, with body weight of 433 ± 7 kg (mean ± SE) and with a maximum aerobic capacity of 133.2 ± 6.6 ml/kg/min. Each horse performed two trials separated by a 10-day interval. Both trials involved the horses completing three consecutive days of exercise that depleted muscle glycogen by at least 60% of its initial values. The exercise protocol was individualized based on the assessment of each horse’s maximum aerobic capacity. After the last bout of exercise in each trial, horses were
infused intravenously with either 6 g/kg body weight of glucose as a 13.5% solution in 0.9% NaCl (Glu) or with an equivalent volume of isotonic (0.9%) NaCl (Con). Within 30 minutes of the end of the strenuous exercise, glucose and isotonic NaCl infusions were administered for 12.1 ± 0.4 h (mean ± SE) at a mean rate of 1.68 ± 0.06 liters/hour through a catheter placed in a jugular vein. Feed was withheld during the period of glucose or saline infusion. Treatment and control experiments were performed at the same time of the day for each horse. Muscle samples were collected by biopsy of the middle gluteal muscle before exercise, immediately after exercise and the morning after the saline/glucose infusion (10.1 ± 0.4 h after the infusion, mean ± SE) for measurement of muscle glycogen concentration and total GLUT-4 protein content. Venous blood samples were collected before exercise, before beginning infusion, every 4 hours during the infusion, and 10.1 h after the infusion for determination of glucose concentration. For further details on this study, please refer to Chapter 3. This experimental design allowed us to investigate the effect of exercise on GLUT-4 transporters (Con group) and; 2) the combined effect of exercise and glucose infusion on GLUT-4 transporters (Glu group). All procedures were approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University.

_Biochemistry analysis:_ Muscle samples were collected by a needle biopsy from the middle gluteal muscle, under aseptic conditions after desensitization of the area with 2% mepivacaine (Carbocaine, Abbott, North Chicago, IL). The muscle samples were flash frozen in liquid nitrogen and then stored at –80°C until analysis. Glycogen content was
determined fluorometrically as previously described (Passoneau and Lauderdale 1974). Blood samples were collected through a catheter (14-gauge, 5.25 in., Angiocath, Deseret, Sandy, UT) placed in a jugular vein, after desensitization of the overlying skin. Venous blood samples for glucose assays were collected into chilled 5 ml evacuated tubes containing potassium oxalate and sodium fluoride. Plasma glucose concentrations were measured in duplicate using an automatic analyzer (YSI model 1500, Yellow Springs Instruments, Yellow Springs, Ohio, USA).

**Western blot analysis:** Total crude muscle membranes were obtained by the method of Ploug et al. (1993). Frozen tissues samples (30 to 60 mg) were homogenized in 4 ml of homogenizing buffer (210 mM sucrose, 40 mM NaCl, 2 mM ethylene glycol-bis (β-aminoethylether) N,N’,N’-tetraacetic acid, 30 mM HEPES (pH 7.4), 0.35 mg/ml PMSF at pH 7.4) using polytron fitted with a generator (Kinematica, Switzerland). The homogenate was mixed with 3 ml of 58.3 mM of sodium metaperiodate and 1.17 mM KCl and left on ice for 15 min. Total muscle membranes were then recovered by centrifugation at 100 000 g at 4 °C for 90 min (70 Ti rotor, Ultracentrifuge, Beckman Instruments, Palo Alto, CA). The pellets were resuspended in 400 µl of 10 mM Tris – HCl and 1 mM EDTA (pH = 7.4), and then 100 µl of 20% SDS was added. Samples were then centrifuged at 1100 g for 25 min. Protein concentrations of the supernatant was determined spectrophotometrically (Microplate reader, Versamax, Molecular Devices Corporation, Sunnyvale, CA) using a detergent compatible method with BSA as a standard (Pierce, Rockford, IL). Total crude muscle membranes were analyzed for
GLUT-4 transporters by electrophoresis and subsequent western blotting (Johannsson et al. 1996). Samples were diluted in 1:2 Laemmli sample buffer (2% SDS, 25% glycerol, 0.01% bromophenol blue, 350 mM DTT, 65.2 mM Tris-HCl, pH = 6.8) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% resolving gel. The proteins were transferred electrophoretically to a PVDF membrane (Millipore, Bedford, MA) for 90 min, using a semi-dry blotting. The membrane was then blocked overnight in a 10% non-fat dry milk solution at 4°C. After blocking, the membrane was incubated for 1 hour with a polyclonal antibody directed against the last 12 COOH-terminal amino acids of the GLUT-4 protein (Biogenesis Inc, Sandown, NH). After three washes in 0.1% Tris-buffered saline Tween (150 mM NaCl, 25 mM Tris, 0.1% Tween-20, pH = 7.4), the membrane was incubated for 1 hour with an anti-rabbit horseradish peroxidase (HRP) (Calbiochem, San Diego, CA). Quantitative determination of GLUT-4 protein was performed by autoradiography after revealing the antibody-bound transporter protein by enhanced chemiluminescence reaction (Kirkegaard & Perry Laboratories, Maryland, USA), according to manufacture recommendations. The density of the bands on scanned autoradiograph was quantified using a computerized densitometry program. Molecular weight marker (Biorad, Hercules, CA) was used for each gel to determine the relative molecular weight of the labeled bands. Cardiac crude membrane preparation was used as a standard and density was expressed as relative units (%).
Statistical analyses were performed using either a one-way or a two-way repeated measures analysis of variance (repeated measures on time and treatment). The null hypothesis (no effect of exercise and/or carbohydrate administration on measured parameters) was rejected at P < 0.05. Significant differences between means (P < 0.05) were identified using Student-Newman-Keul’s test. All results are expressed as mean ± standard error.

4.4 RESULTS

Characterization of GLUT-4: Polyclonal antibodies directed against rabbit GLUT-4 recognized a protein of similar size in crude membranes prepared from rat heart (positive control), and equine skeletal and cardiac muscles. According to the standards, the calculation of the molecular mass of the band representing GLUT-4 gave a theoretical value of 53.3 ± 0.5 kDa [50-55 kDa]. The bands at 50 kDa were present in cardiac and skeletal muscles of horses, whereas no signal was obtained in the negative control (equine liver) (Fig. 4.1). Furthermore, a positive linear correlation was obtained between the amounts of protein loaded on the gel and the density of the bands at 50 kDa (r² = 0.99, P < 0.001, Figs 4.2 and 4.3). However, at least one additional labeled band was observed around 150 kDa in rat and equine muscles. In some experiments, a band around 80 kDa was also detected. These bands could correspond to non-specific bindings of the primary or secondary antibody to the most common proteins present in muscle tissues (myosin
heavy chain or actin) (Hocquette et al. 1995). Therefore, ruling out the presence of non-specific bands was critical for the validation of this technique. These bands remained on the immunoblot despite the use of different blocking solutions (Kirkegaard & Perry Laboratories, Maryland, USA) or after additional washes with Tween TTBS. The bands at 50 kDa were absent when a non-immune rabbit serum (Pierce, Rockford, IL) was used instead of the primary antibody, whereas weak signals were observed at 150 kDa and 80 kDa, suggesting the presence of non-specific bands (results not shown). No immunoreactive band was observed on autoradiograph when the membrane was only incubated with HRP antibody prior to the enhanced chemiluminescence, suggesting the absence of non-specific binding with HRP (results not shown). The 150 kDa, but not the 50 kDa, band was present on the immunoblot when the primary antibody was preliminary incubated for 1 h with GLUT-4 peptide to which the antibody was raised (Biogenesis Inc, Sandown, NH) before incubating the membrane (Fig. 4.4). From these results, we concluded that we had detected the GLUT-4 protein in skeletal muscle of horses, and that the band at 50 kDa, but not at 150 kDa, is specific for GLUT-4 protein in skeletal and cardiac muscles in horses.

Effect of exercise and glucose administration:

Muscle glycogen concentrations: Muscle glycogen concentrations significantly decreased after exercise (P < 0.001; Table 4.1). Muscle glycogen concentrations were not significantly different between treatments before and after exercise. Post-exercise intravenous infusion of glucose resulted in a significant increase in muscle glycogen.
concentrations (91% from pre-exercise value, P < 0.001), whereas a smaller increase in muscle glycogen stores was noted 10.1 ± 0.4 h after the saline infusion (39% from pre-exercise values). Intravenous infusion of glucose resulted in a higher rate of glycogenesis over 22.2 ± 0.3 h after exercise compared to the Con group: 3.98 ± 0.61 and 1.47 ± 0.20 mmol/kg/h wet weight (ww), respectively (P < 0.001). Muscle glycogen concentrations similar to baseline values were obtained 22.2 ± 0.3 h after exercise in the Glu treatment group (P = 0.34), but not in the control treatment group (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Pre Ex</th>
<th>Post Ex</th>
<th>Post Inf</th>
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<tbody>
<tr>
<td>Con</td>
<td>120.2 ± 7.0</td>
<td>16.7 ± 2.7 *</td>
<td>46.6 ± 6.2* &amp;</td>
</tr>
<tr>
<td>Glu</td>
<td>125.3 ± 7.9</td>
<td>26.4 ± 4.8 *</td>
<td>114.3 ± 7.3</td>
</tr>
</tbody>
</table>

Table 4.1: Muscle glycogen concentration (mmol/kg ww) before (Pre Ex) and after exercise (Post Ex), and after glucose or saline infusion (Post Inf). *: P < 0.05 via values before depletion. & P < 0.05 significant difference between treatment group.

**Blood glucose concentrations:** There was a significant time and treatment interaction (P < 0.001) for venous glucose concentrations (Fig. 4.5). Glucose concentration was similar between treatments before and after exercise. Intravenous infusion of glucose resulted in a peak of plasma glucose concentration (7.6 ± 0.9 mM, P < 0.001) at 4 hours after the beginning of the infusion, whereas glucose concentration was unchanged throughout the saline infusion. Blood glucose concentration was significantly lower after exercise.
compared to 10.1 h after infusion for the Con group (P < 0.01). Blood glucose concentrations were similar between treatments 10.1 h after infusion.

*Total GLUT-4 protein content:* There was a significant time effect (P < 0.001) on GLUT-4 protein content (Fig. 4.6). Total GLUT-4 protein content increased by 8.3% immediately after exercise (P < 0.01). Furthermore, total GLUT-4 protein content was significantly (P < 0.001) higher after infusion, compared to values before exercise. GLUT-4 protein content increased by 27.3% and 12.3% 22.2 h after exercise for Con and Glu group, respectively (P < 0.05). Whereas GLUT-4 protein content significantly increased after saline infusion (increase by 12.5% compared to values before infusion, P < 0.05), the increase after Glu infusion was smaller (8.3%, P = 0.054). There was a tendency (P < 0.1) for the GLUT-4 protein content in the Con group to be higher than the values in the Glu group after the infusion. Two values in the Con group were considered outliers, and were removed from the data set. Statistical analysis performed with these outliers also showed a significant time effect on GLUT-4 protein content (P = 0.02).
Figure 4.1: Western blot identification of GLUT-4 protein from crude membrane preparation in horses. A: Molecular weight marker. B: Heart; C: Skeletal muscle; D: Liver.
Figure 4.2: Western blot analysis of GLUT-4 protein in crude membrane of equine skeletal muscle with different amount of protein (µg). A: Molecular weight marker.

Figure 4.3: Relationship between integrated density value and amount of total GLUT-4 protein content.
Figure 4.4: Western blot analysis of GLUT-4 protein in crude membranes of equine gluteal muscle. Western blots were performed in the presence (+) or in the absence (-) of the peptide to which the antibody was raised.
Figure 4.5: Plasma glucose concentration before exercise (Pre Ex), immediately after exercise, and after glucose or saline infusion (Post inf). Con: Control; Glu: Glucose; * P < 0.05 vs. corresponding value after infusion. & P < 0.05 for treatment effect. Values are mean ± SE for 6 horses.
Figure 4.6: Total GLUT-4 protein content before exercise (Pre Ex), immediately after exercise (Post Ex), and after glucose or saline infusion (Post inf). Con: Control; Glu: Glucose; * P < 0.05 vs. corresponding value before exercise. # P < 0.05 vs. corresponding value before infusion.

Figure 4.7: Effect of exercise and carbohydrate administration on GLUT-4 protein content of skeletal muscle. Representation of an immunoblot showing labeled GLUT-4 protein from crude membranes of equine gluteal muscle. 1 (Con), 5 (Glu): before exercise; 2(Con), 3(Glu): after exercise; 4: after Con infusion; 6: after Glu infusion.
4.5 DISCUSSION

These results provided evidence for the presence of GLUT-4 protein in equine skeletal and cardiac muscles. We characterized the GLUT-4 transporters by Western blot in sedentary animals by demonstrating the specificity of the immunoreactive bands detected by immunoblotting, and we determined the molecular weight of the GLUT-4 protein at approximately 50 kDa. We also demonstrated that exercise leads to an increase in GLUT-4 protein content of muscle and that replenishment of muscle glycogen stores by glucose infusion after exercise attenuated the increase in GLUT-4 protein content of muscle.

Performing immunoblotting to specifically identify equine GLUT-4 is somewhat difficult because of the lack of species-specific antibody. Furthermore, ruling out the presence of non-specific bands is critical when using immunoblotting, because non-specific bands can appear in crude homogenates, especially when using non-radioactive systems of detection (Hocquette et al. 1996a). Furthermore, dimmers of GLUT-4 result in the presence of bands of differing molecular weights (Weistein et al. 1991). Our results demonstrated the specific immunoreactivity of the bands corresponding to GLUT-4, which were specifically blocked by preincubation of the primary antibody with the immunizing peptide, whereas the nonspecific bands were not sensitive to the peptide block. Furthermore, this protein was only detected in tissues in which glucose uptake is sensitive to insulin, namely cardiac and skeletal muscles, and not in non-insulin sensitive tissue, namely liver, suggesting the presence of an insulin-sensitive protein. Our results
also confirmed that commercial antibody against rabbit GLUT-4 can be used to detect
GLUT-4 transporters in horses because the polyclonal rabbit antibody is produced against
the last 12 COOH-terminal amino acids of the GLUT-4 antigen, an area well conserved
across several species (McCutcheon et al. 2002). These results also demonstrated that the
use of crude membrane preparation is suitable for sensitive detection of GLUT-4 protein.

Our results also demonstrated that GLUT-4 proteins in rat and equine muscles
have the same molecular weight, which is approximately 50 kDa. Our results are in
agreement with studies conducted in rats and other herbivores. For instance, a molecular
mass of 53 kDa was reported for GLUT-4 protein in rat heart and bovine masseter and
rectus abdominis muscles (Hocquette et al. 1996b). A molecular mass of 42-45 kDa has
also been reported in rats, bovine and avian skeletal muscle (James et al. 1989; Hocquette
et al. 1995; Thomas-Delloye et al. 1999). However, this value is probably
underestimated, since GLUT-4 protein had a slightly reduced mobility during the
electrophoresis compared to actin, which has a molecular weight of 43 kDa (Hocquette et
al. 1996b). Furthermore, these differences in the theoretical calculation of the molecular
mass of GLUT-4 can be explained by different conditions of electrophoresis, or
eventually by the presence of non-specific bindings of the primary antibody to other
proteins in muscle when the specificity of the bands was not preliminary checked
(Hocquette et al. 1996b). In conclusion, these results demonstrated that we detected, by
immunoblot analysis, the insulin-sensitive glucose transporter in equine skeletal and
cardiac muscles.
There are two distinct phases associated with muscle glucose uptake after exercise: an initial short insulin-independent phase, by which GLUT-4 translocation occurs through a contraction-mediated process, and the second longer phase (up to 48 h), which is characterized by an increased muscle insulin sensitivity (Ivy and Kuo 1998). Our study suggests that as in other species, the existence of exercise-induced contraction mediated translocation of the glucose transporters at the plasma membrane for glucose uptake, evidenced by the increase in GLUT-4 protein content after exercise, especially in the Con group. Similar to our findings, others have demonstrated that GLUT-4 protein content increased only slightly immediately after exercise in horses, despite increased glucose uptake (McCutcheon et al. 2002). Furthermore, the amplitude of the increased GLUT-4 content 22.1 h after exercise was lower than that reported in rodents. In our study, GLUT-4 protein content increased up to 28% after exercise in the Con group, whereas a 43% increase in GLUT-4 protein concentration was reported after exercise in fasted rats (Kuo et al. 1999a). Furthermore, the persistent increase in GLUT-4 protein content 22.1 h after exercise suggested that insulin-mediated glucose translocation also occurs in horses (Hansen et al. 1998). Similar to humans and rats, muscle glycogen synthesis occurs during recovery under fasting conditions (Maehlum and Hermansen 1978), suggesting that the increased GLUT-4 protein content observed after exercise contributed to the resynthesis of muscle glycogen stores. In conclusion, the results from the Con group suggested that, similar to other species, exercise induced an increase in GLUT-4 protein content in a biphasic manner, and that this increase was associated with post-exercise glycogenesis.
Our results also demonstrated that in horses, similar to humans, intravenous infusion of glucose hastened post-exercise muscle glycogen resynthesis (Blom et al. 1987; Ivy et al. 1988). However, despite significant increased glucose availability and hastened muscle glycogenesis, GLUT-4 protein content was not significantly different before and after the Glu infusion, whereas there was a significant increase after saline infusion. These results in horses are in contrast with results reported in humans and rats: ingestion of a carbohydrate supplementation after exercise enhances the exercise-induced increase in GLUT-4 protein concentrations after exercise (Kuo et al. 1999a,b). Because GLUT-4 protein content is positively correlated with muscle glycogen stores in other species (r = 0.81; Kuo et al. 1999a), we should expect an increased GLUT-4 protein content after Glu infusion. However, because muscle glycogen concentration of resting horses is higher than other species (Snow and Valberg 1994), high muscle glycogen (observed after Glu infusion) may reverse the increase in insulin sensitivity normally observed after exercise and carbohydrate supplementation (Host et al. 1998), and prevent further increased GLUT-4 translocation at the plasma membrane by a negative feedback (Derave et al. 1999). This phenomenon has been well described in humans and rodents with muscle glycogen supercompensation after glycogen-depleting exercise and carbohydrate supplementation (Host et al. 1998). Therefore, these results provide some evidence that muscle glycogen concentration plays a regulatory role in GLUT-4 recruitment at the plasma membrane (Derave et al. 1999). Furthermore, these results provide some evidence that GLUT-4 glucose transporters are the rate-limiting step for glucose uptake and secondary glycogenesis (Kuo et al. 1999a; Fisher et al. 2002). Indeed,
we speculate that as soon as normal muscle glycogen concentration is restored after carbohydrate administration, muscle glycogen inhibited further GLUT-4 translocation at the plasma membrane (Derave et al. 1999), which will then limit further muscle glycogen replenishment. To support this theory is the observation that muscle glycogen supercompensation is not observed after glycogen-depleting exercise and carbohydrate administration in horses (Topliff et al. 1983; see Chapter 5).

The lower GLUT-4 protein content after Glu infusion may also be related to the peculiarities of glucose metabolism observed in herbivores. In roughage fed animals, a limited amount of glucose is absorbed in the small intestine because the majority of glucose supply is from volatile fatty acids produced in the large colon (Argenzio 1990). Therefore, similar to ruminants, peripheral tissue of horses might be less sensitive to insulin with secondary decreased glucose uptake and GLUT-4 translocation at the plasma membrane (Hocquette et al. 1995). However, it should be mentioned that one limitation in this study is that GLUT-4 protein content was isolated from crude membrane preparation and not from plasma membrane isolation, allowing quantification of total GLUT-4 protein content and not quantification of glucose transporters available at the plasma membrane for glucose uptake. However, studies in rodent and humans have shown that exercise and feeding increased GLUT-4 protein content using total crude membrane preparation (Kuo et al. 1999a, Bruce et al. 2001). Therefore, the differences in glucose transporters observed in this study may reflect differences in glucose metabolism in horses, rather than inadequate muscle preparation.
In conclusion, to our knowledge, this is the first study that provided some biochemical and functional evidences for the presence of insulin-sensitive glucose-transporter (GLUT-4) protein in equine skeletal muscles. The results of this study also suggested that muscle glycogen stores play a regulatory role in recruitment of GLUT-4 transporters at the plasma membrane of equine skeletal muscle. However, further studies are required to confirm the physiological role of glucose transporters in horses using plasma membrane isolation.
4.6 REFERENCES


CHAPTER 5

POST-EXERCISE FEEDING OF MEALS OF VARYING GLYCEMIC INDICES AFFECTS MUSCLE GLYCOGEN RESYNTHESIS IN HORSES.
5.1 SUMMARY

Our objectives were to study the effect of isocaloric diets of varying soluble carbohydrate content on the rates of muscle glycogen synthesis after exercise, using seven fit Standardbred gelding horses.

In a three-way crossover study, horses received each of the 3 isoenergetic diets: a high soluble carbohydrate diet (HCO) or a low soluble carbohydrate diet (LCO) or a mixed diet (M) every 8 hours for 72 hours after undertaking glycogen-depleting exercise. Muscle samples were collected for measurements of glycogen concentration, GLUT-4 protein content and glycogen synthase activity. Venous blood was collected for measurement of glucose and insulin concentrations, and other plasma variables.

Feeding HCO diet resulted in higher glycemix index (P = 0.005) for 72 h, and greater muscle glycogen concentration at 48 h and 72 h after exercise, compared to M and LCO meals (133.6 ± 3.6, 106.8 ± 3.6 and 102.6 ± 3.6 mmol/kg, respectively, 72 h after exercise; P < 0.001). Muscle glycogen concentrations similar to baseline were obtained 72 h after exercise in horses fed the HCO diet. The rate of glycogen resynthesis was higher for horses fed HCO diet, compared to horses fed M and LCO diets (1.51 ± 0.15 vs. 1.12 ± 0.11 vs. 0.97 ± 0.10 mmol/kg/h, respectively; P < 0.001). Glycogen synthase activity was inversely related to glycogen content (P < 0.001). GLUT-4 protein content was lower at 72 h after exercise (P < 0.001).

We concluded that muscle glycogen resynthesis is slow in horses. Feeding of high-glycemic meals after exercise hastened muscle glycogen replenishment compared to LCO and M diets by increasing blood glucose availability to the skeletal muscle.
5.2 INTRODUCTION

Exercise depletes stores of muscle glycogen, which is the principal energy source for skeletal muscle during vigorous exercise. Horses frequently perform endurance exercise or multiple bouts of moderate to intense fatiguing exercise during show jumping, training and racing. Single or repeated bouts of galloping by Thoroughbred horses or trotting by Standardbred racehorses deplete muscle glycogen stores by approximately 30-40% (Lindholm and Piehl 1974; Snow et al. 1985; Harris et al. 1987; Snow and Harris 1991). Participation in an 80- or 160-km endurance ride depletes muscle glycogen of horses by 50 to 75% (Snow et al. 1981, 1982). Moreover, complete repletion of muscle glycogen stores requires up to 48-72 hours after exercise (Snow and Harris 1991; Hyypä et al. 1997). Therefore, because many horses undertake several events in a single day, the interval between exercise bouts appears to be inadequate for complete restoration of the muscle glycogen pool (Snow and Harris 1991). Similar to humans, depletion of intramuscular glycogen stores has been associated with hastened fatigue during endurance exercise (Snow et al. 1981) and during high-intensity exercise in horses (Chapters 2 and 3). Therefore, enhanced replenishment of energy substrate stores after exercise might confer significant advantages in performance to equine athletes.

The effects of diet composition on muscle glycogen availability and exercise capacity have received much attention in human exercise physiology, and these studies have resulted in significant improvements in nutrition and the performance of human athletes. The variation of glucose responses after the ingestion of carbohydrate diets has
led to the establishment of the glycemic index, which classifies dietary carbohydrates on the basis of the rise in blood glucose relative to the same amount of carbohydrate taken as glucose. Ingestion of foods with a high-glycemic index by humans after exhaustive exercise increased by two-fold the rate of muscle glycogen resynthesis 6 hours after exercise compared to a low glycemic index meal (Kiens et al. 1990). Furthermore, feeding a high-glycemic index meal increased by two fold muscle glycogen concentration 24 h after glycogen-depleting exercise compared to a low glycemic index meal (Burke et al. 1993), although its effect remains controversial (Burke et al. 1995).

While the effects of dietary manipulation on muscle glycogen availability have been well investigated in human exercise physiology, there is little information regarding dietary factors affecting post-exercise muscle glycogen resynthesis in horses and the conclusions drawn from human studies could not be applied to horses because of the physiological differences, especially in regard to carbohydrate metabolism. For instance, horses have approximately two-fold greater muscle glycogen concentrations (Snow and Valberg 1994) and they have a greater capacity for energy production via aerobic and anaerobic metabolism compared to humans (Wagner 1995; Shuback and Essén-Gustavsson 1998). Furthermore, these herbivorous animals primarily rely on volatile fatty acids, which result from the breakdown of carbohydrate by microbial enzymes in the large intestine, for hepatic gluconeogenesis (Argenzio 1990). Perhaps of most importance, muscle glycogen resynthesis appear to be slow in horses, although no mechanistic explanation has yet been provided. Because of the slow muscle glycogenesis, dietary manipulations after exercise appear to be crucial in horses to optimize muscle
glycogen replenishment. Whereas intravenous dextrose and glucose infusion (6 g/kg) hastens muscle glycogen replenishment compared to saline infusion (Davie et al. 1995; Chapter 4), no study has demonstrated conclusively an effect of diets of varying glycemic indices on rates of post-exercise muscle glycogen resynthesis in horses. Therefore, our objectives were to investigate the rate of glycogen resynthesis after exercise and to compare the effects of post-exercise feedings of 3 isoenergetic diets of varying soluble carbohydrate content on muscle glycogen replenishment. Our hypotheses were that muscle glycogen resynthesis will be low after strenuous exercise, and that feeding a high soluble carbohydrate diet would hasten replenishment of muscle glycogen stores after exercise by increasing blood glucose availability to the muscle compared to conventional or low soluble carbohydrate diets.

5.3 MATERIALS AND METHODS

Experimental design: The effect of dietary carbohydrate administration on muscle glycogen resynthesis was examined in a partially balanced, three-way crossover study. Seven fit Standardbred horses performed each of the three trials separated by 10-day intervals (Trials 1, 2 and 3). All trials involved the horses completing three consecutive days of strenuous exercise that depleted muscle glycogen by at least 60% of its initial values (days 1-3). Immediately after the last bout of exercise in each trial, the horses received either a high soluble carbohydrate diet (grain, HCO) or an isoenergetic low
soluble carbohydrate diet (hay, LCO) or an isoenergetic mixed diet (grain and hay, M, control group with conventional diet) every 8 hours for 72 hours (days 3-5). Muscle samples were collected by biopsy of the middle gluteal muscle for measurement of muscle glycogen concentration, glycogen synthase activity and total GLUT-4 protein content. Samples were collected before exercise (on day 1) and 0 h, 3h, 6 h, 12 h, 24 h, 48 h and 72 h after the last day of exercise (day 3). Blood samples for measurement of plasma glucose, serum insulin, plasma lactate, serum free fatty acid, plasma triglyceride, plasma glycerol and total protein concentrations, and hematocrit were collected before exercise on day 1 and immediately after exercise on day 3, then every 60 min for 12 hours after exercise, and at the time of each muscle biopsy. The order of the treatments for each horse during the 3 trials was randomized and the overall design was partially balanced (Fig. 5.1). This protocol was approved by the Institutional Laboratory Animal Care and Use Committee of the Ohio State University.

Animals: The subjects were 7 Standardbred gelding horses aged between 2 and 10 years, with a body weight of 473 ± 9 kg (mean ± SE) and body condition scores of 4.6 ± 0.3. Horses were chosen for the absence of lameness, cardiovascular and respiratory diseases, and for suitable temperament. Horses were placed for 3 weeks in an isolation facility. The animals were then accustomed, during a 2-week period, to the treadmill barn, to walk and run on the treadmill, and to wearing the mask of the open circuit indirect calorimeter. Horses were conditioned by running on the treadmill (at a 2° incline) 5 days per week for 5 weeks. To maintain a basic level of fitness between each experimental trial, the horses continued to run on the treadmill at least 3 days per week. During the conditioning and
between each trial, the horses were housed in box stalls (3 x 4 m) and fed timothy grass and alfalfa hay (approximately 8.5 kg/horse/day) and mixed grain (approximately 2 kg/horse/day) at an estimated digestible energy intake of 19 Mcal/horse/day, which was sufficient to maintain bodyweight. Trace mineralized salt blocks and fresh water were available at all times.

**Preliminary testing:** Prior to exercise studies, basal aerobic capacity was assessed for each horse by measuring the maximal rate of oxygen consumption (VO$_2$ max) during an incremental exercise test on a high-speed treadmill (Sato, Uppsala, Sweden), as previously described (Chapter 3). The speed:VO$_2$ relationship for each horse was determined by linear regression of oxygen consumption and speed during the incremental exercise test.

**Glycogen-depleting exercise:** The horses performed 3 days of strenuous exercise intended to deplete muscle glycogen stores, as previously described (Chapters 2 and 3). The exercise protocol was individualized based on the assessment of each horse’s aerobic capacity. From these studies, we have demonstrated that this exercise protocol depleted muscle glycogen by at least 60% of its initial value.

**Diets:** Immediately at the end of exercise on the 3rd day, the horses received one of the 3 isocaloric diets (HCO, LCO and M) for 72 hours on each of 3 different occasions in a randomized fashion (Table 5.1). The horses fed the mixed diet, which represents a typical diet for horses, were considered the control group. The diets were given every 8 hours for 72 hours. Digestible energy (DE) of the diets was calculated based on the daily energy requirement of horses in light work (NRC 1998). Samples of the diets were retained and
submitted for nutrient analysis to a commercial laboratory (Holmes laboratory Inc., Millesburg, OH). The amount of feed actually consumed by each horse was recorded. Water consumption was recorded for each horse during the 72 hours of dietary interventions. During the experimental period, horses were kept in their box stalls. At the end of the 3 trials, the 7 horses had received each of the 3 diets.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>100% DM basis</th>
<th>HCO</th>
<th>M</th>
<th>LCO</th>
</tr>
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<tbody>
<tr>
<td>Mixed hay</td>
<td>21</td>
<td>66</td>
<td>99</td>
<td></td>
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<tr>
<td>Cracked corn</td>
<td>37</td>
<td>33</td>
<td>_</td>
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<tr>
<td>Oats</td>
<td>20</td>
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<tr>
<td>Cracked barley</td>
<td>20</td>
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<tr>
<td>Calcium carbonate</td>
<td>0.5</td>
<td>_</td>
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<tr>
<td>Vitamin/mineral Premix</td>
<td>1.5</td>
<td>1</td>
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</table>

| Intake* (kg/day/horse)        | 6.5 ± 0.2     | 7.8 ± 0.2 | 9.7 ± 0.3 |
| DE (Mcal/kg/horse)            | 0.041         | 0.041     | 0.041     |
| Estimated starch (%)          | 50.87         | 26.38     | 4.31      |
| NDF (%)                      | 22.20         | 34.68     | 47.08     |

Table 5.1: Composition of experimental diets. DM: Dry matter, DE: digestible energy, NDF: neutral detergent fiber. * Values are reported as mean ± SE.

Weight: The horses were weighed before the first day and the last day of the exercise and every day during the treatment periods. Body condition scores were recorded before and after each trial, according to NRC scoring system (Henneke et al. 1983).
**Muscle glycogen content and glycogen synthase activity:** Muscle samples were collected by a needle biopsy from the middle gluteal muscle, under aseptic conditions after desensitization of the area with 2% mepivacaine (Carbocaine, Abbott, North Chicago, IL). The muscle samples were flash frozen in liquid nitrogen and then stored at –80°C until analysis. Muscle glycogen concentrations were determined in duplicate with a fluorometer (Sequoia-Turner model 112) after acid hydrolysis (Passoneau and Lauderdale 1974), and glycogen content was expressed as millimoles of glucosyl units liberated per kg of wet muscle weight. For determination of glycogen synthase activity, sample from gluteal muscle was homogenized (1:50) in 50% glycerol, 20 mM phosphate buffer (pH = 7.4), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, and 0.02% bovine serum albumin. Glycogen synthase activity was then measured fluorometrically in the absence (active form) or presence (total activity) of 10 mM glucose-6-P (Henriksson et al. 1986). Glycogen synthase activity was expressed as mmol/kg/h and reported as activity ratio (active/total).

**Total GLUT-4 protein content:** Total crude muscle membranes were analyzed for GLUT-4 glucose transporter by electrophoresis and subsequent immunoblotting (Ploug et al. 1993; Johannsson et al. 1996). This technique has been recently validated in horses (Chapter 4).

**Blood chemistry:** Blood samples were collected through a catheter (14-gauge, 5.25 in., Angiocath, Deseret, Sandy, UT) placed in a jugular vein, after desensitization of the overlying skin. Venous blood samples were placed into an evacuated glass tube containing EDTA (Vacutainer, Becton Dickinson, Parsippany, NJ) for measurement of
hematocrit and plasma total protein concentration, which were measured by the microhematocrit technique and refractometry (Cambridge Instruments, Buffalo, NY), respectively. Venous blood samples for glucose and lactate assays were collected into chilled 5 ml evacuated tubes containing potassium oxalate and sodium fluoride. Venous blood samples for measurement of nonesterified fatty acid (NEFA) and insulin concentrations were collected into tubes containing no additive. Venous samples for measurement of glycerol and total triglyceride concentrations were collected into tubes containing heparin. All the tubes from each collection were centrifuged for 15 min at 1,600 x g at 4°C and were stored at –80°C until analysis. Plasma glucose and lactate concentrations were measured spectrophotometrically (Microplate reader, Versamax, Molecular Devices Corporation, Sunnyvale, CA) using commercial kits (Glucose hexokinase 20, Sigma Diagnostic, St Louis, MO; Lactate, Sigma Diagnostic, St Louis, MO, respectively). Plasma triglyceride, glycerol and serum NEFA concentrations were also measured spectrophotometrically using commercial kits (Triglycerides, GPO trender, Sigma Diagnostic, St Louis, MO; NEFA test kit, Wako Chemicals, Dallas TX, respectively). Serum insulin concentration was measured by radioimmunoassay, using a commercial kit validated for the use in horses (125I RIA kit, ICN Pharmaceuticals, Costa Mesa, CA) (Reimers et al. 1982). All the samples were analyzed in duplicate, except samples for determination of glucose concentrations, which were measured in triplicate. Statistical analyses were performed using either a one-way or a two-way repeated measures analysis of variance (repeated measures on time and treatments), as appropriate for the dependent variables. The null hypothesis (no effect of diet on muscle glycogen
replenishment and other variables) was rejected at P < 0.05. Significant differences between means were identified using Student-Newman-Keul’s test. Statistical analyses were performed using the Sigmastat 2.0 software package (Jandel Scientific, San Rafael, CA). All results are expressed as mean ± SE.

5.4 RESULTS

Maximal Oxygen Consumption and Speed-VO₂ relationship: VO₂ max of the horses prior to exercise of Trial 1 was 109.7 ± 6.3 ml O₂.min⁻¹.kg⁻¹ at a treadmill speed of 10.7 ± 0.3 m/s. The average correlation coefficient of the speed-VO₂ relationship was 0.990 ± 0.003 (P < 0.001), the slope of the regression line was 8.5 ± 0.4 ml O₂.min⁻².kg⁻¹ and the ordinate intercept was 3.5 ± 1.4 ml O₂.min⁻¹.kg⁻¹.

Glucose and insulin concentrations (Figs 5.2 and 5.3): Feeding different amounts of soluble carbohydrate produced different glycemic indices. At rest and after exercise, glucose and insulin concentrations were similar for the 3 groups. After exercise, feeding the HCO diet resulted in greater plasma glucose concentrations (up to 4 hours after feeding) compared to LCO and M diets (P = 0.005), whereas plasma glucose concentrations were similar for the horses fed M and LCO diets (4.7 ± 0.1 and 4.8 ± 0.1 mM, respectively). Furthermore, glucose concentrations above pre-exercise values were obtained only in the horses fed the HCO diet from 9 h to 72 h after exercise. The serum immunoreactive insulin concentration curve followed in general the glucose
concentration curve. Feeding horses the HCO diet induced a hyperinsulinemic response in which insulin increased by 70% (from 85.7 ± 11.1 to 144.1 ± 16.8 pM, before and 2 h after feeding, respectively, P < 0.001), although this response was less pronounced after the 2nd meal. Insulin concentration remained elevated at 48 h and 72 h after exercise in horses fed the HCO diet (P < 0.005).

Muscle glycogen concentrations: Feeding diets with different glycemic indices affected muscle glycogen resynthesis. Strenuous exercise resulted in substantial depletion of muscle glycogen stores (from 129.0 ± 3.6 to 28.0 ± 3.6 mmol/kg wet weight –ww- before and after exercise, P < 0.001; Fig. 5.4). Muscle glycogen concentrations were 64.4%, 54.2% and 52.9% from their initial values for horses fed the HCO, LCL and M diets, respectively, at 24 h after exercise. The rate of muscle glycogen resynthesis was higher 3 h compared to 12 h after the strenuous exercise (3.33 ± 0.29 and 2.10 ± 0.29 mmol/kg ww /h, respectively, P < 0.05; Fig. 5.5). The rate of glycogen resynthesis decreased by 50% and 73% at 24 h and 72 h, respectively, after exercise (P < 0.001). Feeding HCO meals resulted in greater muscle glycogen concentration at 48 h and 72 h after exercise compared to M and LCO meals (133.6 ± 3.6, 106.8 ± 3.6 and 102.6 ± 3.6 mmol/kg, respectively, 72 h after exercise; P < 0.001; Fig. 5.4). Muscle glycogen concentrations similar to baseline were obtained 72 h after exercise only in horses fed the HCO diet (123.3 ± 3.6 and 133.6 ± 3.6 mmol/kg before and 72 h after exercise; P = 0.12). Overall, the rate of glycogen resynthesis during 72 h was higher for horses fed the HCO diet, compared to horses fed the M and LCO diets (1.51 ± 0.15 vs. 1.12 ± 0.11 vs. 0.97 ± 0.10 mmol/kg/h, respectively; P < 0.001).
Glycogen synthase I activity increased immediately after exercise and feeding with a peak of activity between 3 and 12h (P < 0.001, Figs 5.6a,b). Glycogen synthase I activity declined in parallel with the rate of glycogen resynthesis. Glycogen synthase I activity similar to baseline values was obtained at 48 h after exercise. Glycogen synthase I activity was higher at 3 h after feeding the HCO diet compared to feeding the other diets (Fig. 5.6a). No statistical significant difference was observed among groups at 3 h after feeding when the results of glycogen synthase activity were expressed as a ratio (Fig. 5.6b). As shown in Fig. 5.7, there was a strong negative correlation between glycogen content and glycogen synthase activity ratio (r² = 0.71, P < 0.001). We also found a linear positive relationship between glycogen synthase activity ratio and the rate of glycogen resynthesis (r² = 0.53, P < 0.05, Fig. 5.8).

Total GLUT-4 protein content: There was a significant time effect on GLUT-4 protein content (P = 0.001). There was a mild increase of GLUT-4 protein content after exercise and feeding (by 8.2% 12h after exercise and feeding for the HCO group, P = 0.24). Values similar or lower than the baseline values were obtained 72 hours after exercise. The decrease in GLUT-4 protein content observed at 72 hours after exercise was statistically significant for the horses fed the HCO diet (P < 0.05, Fig. 5.9).

Other plasma and serum variables: Plasma lactate, glycerol, triglyceride, serum NEFA and total protein concentrations, and hematocrit were significantly higher (P < 0.001) after the end of exercise compared to before exercise (Figs 5.10 – 5.14). There was a tendency for total protein concentrations to be higher after each meal for horses fed the LCO diet compared to horses fed the M and HCO diets, although there was not a
statistical difference. The NEFA concentrations were significantly lower (P < 0.05) 2 h after each meal compared to values before feeding. During the first 8 hours after feeding, there was a tendency for NEFA concentrations to be lower for the horses fed the LCO diet compared to horses fed M and HCO diets, this difference was statistically significant 6 h after exercise (P < 0.05). There was also a tendency for triglyceride concentrations to be higher for the horses fed the HCO diet compared to horses fed the LCO diet up to 8 hours after exercise and feeding, this difference was statistically significant 1 h after the first meal (P < 0.05). Glycerol concentration was significantly higher 1 h after the first meal for the horses fed the HCO diet compared to horses fed the MCO and LCO diets (P < 0.05). There was no significant treatment effect on plasma lactate concentrations.

**Other measurements:** The 7 horses consumed all the meals offered. Water consumption was significant different between treatments over 72 hours: 43.4 ± 3.2, 67.7 ± 7.0, 88.5 ± 7.5 liters for horses fed the HCO, M and LCO diets, respectively (P < 0.001). Body weights were significantly lower after exercise for all the groups (P < 0.001, Fig. 5.15). Body weights were not significantly different throughout the treatment period, except for horses fed the M diet in which a significant increase in body weight was noticed (from 451.6 ± 8.8 to 460 ± 11.1 kgs; 0 and 72 h after exercise; P < 0.05). Body weights were significantly lower for the horses fed the HCO compared to horses fed the LCO or M diets at 48 h and 72 h after exercise (P < 0.05). Body scores were not significantly different between each trial (4.6 ± 0.3 before trial 1, 4.5 ± 0.2 before trial 2, 4.9 ± 0.3 before trial 3, 4.8 ± 0.3 after trial 3).
Figure 5.1: Time-line of the experimental protocol. MB: muscle biopsy. HCO: high soluble carbohydrate diet; M: mixed diet; LCO: low soluble carbohydrate diet.
Figure 5.2: Plasma glucose concentration before and after exercise, and during the 72 hours of dietary interventions. Values are mean ± SE for 7 horses. The arrows indicated the time of feeding. a: $P < 0.05$ HCO vs. corresponding value for M diet; b: $P < 0.05$ HCO vs. corresponding value for LCO diet. &: $P < 0.05$ vs. corresponding value before exercise.
Figure 5.3: Serum insulin concentration before and after exercise, and during the 72 hours of dietary interventions. The arrows indicated the time of feeding. Values are mean ± SE for 7 horses. a: $P < 0.05$ HCO vs. corresponding value for M diet; b: $P < 0.05$ HCO vs. corresponding value for LCO diet; c: $P < 0.05$ M vs. corresponding value for LCO diet.
Figure 5.4: Muscle glycogen concentration before and after exercise, and during the 72 hours of dietary interventions. Values are mean ± SE for 7 horses. a: P < 0.05 HCO vs. corresponding value for M diet; b: P < 0.05 HCO vs. corresponding value for LCO diet. GDE: Glycogen-depleting exercise.
Figure 5.5: Rate of muscle glycogen replenishment from 3 h to 72 h after exercise (all groups included). Values are mean ± SE for 7 horses. a: P < 0.05 vs. corresponding value for [0-3] h; b: P < 0.05 vs. corresponding value for [3-6] h; c: P < 0.05 vs. corresponding value for [6-12] h.
Figure 5.6: Muscle glycogen synthase I activity, expressed as mmol/kg/h ww (A), and muscle glycogen synthase activity ratio (B) before and after exercise, and during the 72 hours of dietary interventions. b: P < 0.05 HCO vs. corresponding value for LCO diet. *: P < 0.05 vs corresponding value before exercise.
Figure 5.7: Relationship between muscle glycogen concentration and muscle glycogen synthase activity ratio after exercise, and during the 72 hours of dietary interventions. Values are mean for 7 horses.

Figure 5.8: Relationship between the rate of muscle glycogen resynthesis and muscle glycogen synthase activity ratio from 3 h to 72 h after exercise. Values are mean for 7 horses.
Figure 5.9: Total GLUT-4 protein content before and after exercise, and during the 72 hours of dietary interventions (B). Representative autoradiogram with labeled GLUT-4 protein appears above each appropriate time point (A). * P < 0.05 corresponding value after exercise.
Figure 5.10: Plasma lactate concentration before and after exercise, and during the 72 hours of dietary interventions. Values are mean ± SE for 7 horses. * P < 0.05 vs. corresponding value before exercise.
Figure 5.11: Plasma glycerol concentration before and after exercise, and during the 72 hours of dietary interventions. The arrows indicated the time of feeding. a: $P < 0.05$ HCO vs. corresponding value for M diet; b: $P < 0.05$ HCO vs. corresponding value for LCO diet. * $P < 0.05$ vs. corresponding value before exercise.
Figure 5.12: Plasma triglyceride concentration before and after exercise, and during the 72 hours of dietary interventions. The arrows indicated the time of feeding a: $P < 0.05$ HCO vs. corresponding value for M diet; b: $P < 0.05$ HCO vs. corresponding value for LCO diet. * $P < 0.05$ vs. corresponding value before exercise.
Figure 5.13: Serum NEFA concentration before and after exercise, and during the 72 hours of dietary interventions. The arrows indicated the time of feeding * P < 0.05 vs. corresponding value after feeding.
Figures 5.14: Total protein concentration (A) and hematocrit (B) before and after exercise, and during the 72 hours of dietary interventions. Values are mean ± SE for 7 horses. The arrows indicated the time of feeding.
Figure 5.15: Weight of the 7 horses before and after exercise, and during the 72 hours of dietary interventions. a: P < 0.05 HCO vs. corresponding value for M diet; b: P < 0.05 HCO vs. corresponding value for LCO diet. Values are mean ± SE.
5.5 DISCUSSION

We demonstrated that at least 72 hours is required after strenuous exercise for restoration of muscle glycogen stores. Furthermore, feeding a high-glycemic index (HCO) diet increases the rate of muscle glycogen resynthesis compared to feeding conventional (M) and LCO diets by increasing glucose availability and insulin release. To our knowledge, this is the first evidence that post-exercise feeding of isocaloric meals of varying glycemic indices affects muscle glycogen resynthesis in horses. Additionally, we did not find evidence of supercompensation of muscle glycogen stores in horses.

Rate of glycogen resynthesis: Our findings are in accordance with other studies, which demonstrated that muscle glycogen replenishment takes up to 72 hours after single or repeated bouts of exercise on the racetrack (Snow and Harris 1991; Hyyppä et al. 1997) in horses fed a conventional diet. While restoration of muscle glycogen stores in human athletes is complete within 24 hours (Costill et al. 1981), muscle glycogen concentration in our study was only 52 - 64% of resting values 24 hours after exercise. In agreement with our findings, partial repletion of muscle glycogen stores takes place within 24 hours after exercise (Davie et al. 1994, 1995; Snow and Harris 1991) or may be negligible for the first 24 hours (Hyyppä et al. 1997). This slower repletion of muscle glycogen stores observed in horses compared to humans is related to the extent of substrate depletion (Snow and Valberg 1994), but also to a difference in the rate of glycogen resynthesis. Similar to humans, the maximal rate of muscle glycogen resynthesis in horses occurs in the first 3 hours providing that carbohydrate
supplementation is given immediately after exercise (Ivy 1998). However, post-exercise carbohydrate consumption generally results in a rate of glycogen resynthesis of 6-7 mmol/kg ww/h for the first 3 hours in humans (Ivy 1998) whereas a rate of 3.2 mmol/kg ww/h occurred during the first 3 hours after consumption of HCO diet in horses in this study. We could speculate that this slower rate of muscle glycogen resynthesis is due to differences in muscle glycogen metabolism compared of humans and horses.

Muscle glycogenesis in horses: Several factors appear to control muscle glycogen resynthesis. In humans and rats, the rate-limiting enzyme for glycogen resynthesis in skeletal muscle is glycogen synthase, which is regulated by muscle glycogen concentration, glucose-6-phosphate and insulin (Kochan et al. 1979; Bak and Peterson 1990). Similar to other species, we demonstrated an inverse linear relationship between glycogen content and glycogen synthase activity. For instance, our results demonstrated that the rate of glycogen resynthesis declined in parallel with progressive replenishment of muscle glycogen stores. Furthermore, glycogen synthase activity increased after exercise when glycogen stores are low, suggesting that glycogen availability plays a regulatory role in muscle glycogen resynthesis by influencing glycogen synthase activity (Nielsen et al. 2001). However, whereas a five- to tenfold increase of glycogen synthase activity ratio has been reported in humans after strenuous exercise (Bergström et al. 1972; Piehl et al. 1974; Maehlum et al. 1977; Kochan et al. 1979), glycogen synthase activity ratio was only increased by 2 fold after exercise in this study. Therefore, the slower rate of glycogen resynthesis observed in horses could be partially explained by the lower glycogen synthase activity compared to values reported in humans and rats. Another
interesting finding in this study was the continuous replenishment of glycogen stores in
the face of low glycogen synthase activity at 48 and 72 hours after exercise, suggesting
that other mechanisms promote glycogen synthesis, such as hexokinase, G-6-P and
GLUT-4 glucose transporters (Piehl 1974). Surprisingly, exercise and feeding resulted
only in a slight increase in total GLUT-4 protein content. Similar to our findings, no
significant change in GLUT-4 protein content was observed immediately after exercise in
horses despite an increased glucose uptake in muscle membrane vesicles (McCutcheon et
al. 2002). One possible explanation could be a reduction of GLUT-4 translocation at the
plasma membranes compared to other species. Similar to ruminants, peripheral tissue
might also be less sensitive to insulin with secondary decreased glucose uptake
(Hocquette et al. 1995). It should be mentioned that the limit from the study of
McCutcheon and coworkers, and our study is that GLUT-4 protein was measured from
total crude muscle homogenates and not specifically from preparation of muscle
membranes. However, evidence showed that exercise and feeding increased total GLUT-
4 protein content in humans and rats (Kuo et al. 1999; Bruce et al. 2001). Therefore, we
could speculate that the slow replenishment of muscle glycogen stores observed in horses
may be related to the low glycogen synthase activity but also to the lower GLUT-4
translocation at the plasma membrane following exercise and feeding. However,
additional studies are required to confirm the physiological role of muscle glucose
transporters in horses, using membrane preparations.

Perhaps of more significance was our finding of little or no supercompensation of
muscle glycogen concentration as observed in humans and rats under similar conditions.
Ingestion of a high carbohydrate diet for 3 days following glycogen-depleting exercise markedly increased muscle glycogen stores by 1.9 fold in humans (204 mmol/kg instead of 100 mmol/kg wet weight) (Bergström et al. 1967). Previous studies failed to replicate the carbohydrate loading program in horses (Kline and Albert 1981; Topliff et al. 1985). Because low muscle glycogen content following exercise is the major initial stimulus for increasing the rate of glycogen resynthesis (Fell et al. 1982), some experimental designs did not optimize muscle glycogen replenishment because of the lack of glycogen-depleting exercise prior to “carbohydrate loading” (Kline and Albert 1981). Despite the fact that horses undertook glycogen-depleting exercise before eating a high soluble carbohydrate diet, muscle glycogen supercompensation was also not observed in this study. Furthermore, GLUT-4 protein content was the lowest when muscle glycogen concentration was the greatest (at 72 h after exercise). Because resting muscle glycogen concentration in horses is almost twice greater than in humans (500-650 and 320-400 mmol glucosyl units/kg dry weight, respectively; Snow and Valberg 1994), we could speculate that this high glycogen level in horses has a direct negative feedback on muscle glycogen resynthesis at later stage, preventing further muscle glycogen supercompensation. In humans, supercompensation of muscle glycogen stores prevents the increase in GLUT-4 translocation at the plasma membrane induced by exercise training and high muscle glycogen reverses the increase in insulin sensitivity normally observed after exercise and feeding (Host et al. 1998). It has been concluded that high glycogen concentration exerts a negative feedback signal to stop GLUT-4 recruitment at the plasma membrane (Derave et al. 1999). Because GLUT-4 translocation can be
inhibited by high glycogen level, it has been speculated that glycogen particles may be directly bound to the GLUT-4 containing vesicles, preventing their translocation at the plasma membrane (Derave et al. 1999). Therefore, we could speculate that the lack of supercompensation may be explained by the negative feedback of the high glycogen level on GLUT-4 recruitment. However, its exact mechanism remains to be elucidated in horses.

Glycemic indices: To our knowledge, this is the first study that investigated the effect of post-exercise feeding of isocaloric meals with different soluble carbohydrate content on glycemic indices. Few studies have investigated the effect of post-exercise feeding on the glycemic responses (De la Corte et al. 1999; St. Lawrence et al. 2002). In agreement with these studies, post-exercise feeding induced post-prandial glucose and insulin peaks within 2 hours after feeding, and blood glucose and insulin concentrations returned to pre-feeding value within 5 hours. However, the increased glucose concentrations were lower compared to horses at rest, for which a concentration peak of 6.5 to 7.5 mmol/l was reported within 2 hours after feeding common equine diets (Stull and Rodiek 1988). Furthermore, in our study, glucose concentrations after the first meal were not statistically difference then the resting values for the 3 groups, suggesting that exercise attenuates glucose response. This decreased glycemic index in the first hours after exercise is well recognized in humans (Ploug et al. 1984; Ritcher et al. 1984; Cartee et al. 1989) and more recently in horses (Brewster-Barnes et al. 1995; De la Corte et al. 1999), and is due to an increased glucose transport in the skeletal muscle. There are two distinct phases associated with glucose transport: an initial short insulin-independent
phase, by which muscle glucose uptake is initially caused by an increased translocation of GLUT-4 through a contraction-mediated process, and the second long phase which is characterized by a large increase in muscle insulin sensitivity (Ivy and Kuo 1998).

Although, little is yet known in horses, it has been speculated that exercise-attenuated glucose response results from the independent and synergistic effects of exercise and insulin (De la Corte et al. 1999).

Our study demonstrated that post-exercise feeding of isocaloric diets with different soluble carbohydrate content produced different glycemic indices. As we hypothesized, post-exercise feeding of a grain-based diet (HCO), which contained the highest % of starch, resulted in higher post-prandial glucose and insulin peaks within 2 hours after feeding compared to the LCO and M diet. Feeding roughage (LCO) resulted in low to moderate glycemic indices because the bulk of the glucose arise from gluconeogenesis of the volatile fatty acids, secondary to fermentation in the large colon (Van der Walt and Linington 1989). Surprisingly, feeding roughage and conventional diets produced similar glycemic responses. Few studies have investigated the glycemic responses following feeding common equine diets. Post-prandial peak glucose levels were higher in horses fed isocaloric diets of corn or a mixture of corn and alfalfa (50-50%) compared to the horses fed only alfalfa before exercise (Stull and Rodiek 1988). In contrast to this study, Pagan and Harris (1999) demonstrated that feeding hay during the grain meal significantly reduced glycemic responses compared to feeding grain meal. In our study, feeding only hay (LCO) resulted in an increased plasma total protein concentration within 1 hour after feeding compared to the other diets, although the
difference was not statistically significant. The increased plasma protein concentrations in the hay fed group were probably due to the increased water consumption, and increased gut fill and movement of water from the plasma into the gut (Pagan and Harris 1999). For instance, in agreement with other studies (Ellis et al. 2002), water consumption for the horses fed the LCO diet was 104% and 30% higher compared to values reported in the M and HCO groups. Moreover, body weights were statistically higher in horses fed the LCO or M diets at 48 hours and 72 hours after exercise, probably related to the higher hay intake and water consumption, and secondary greater saliva and digestive juice productions compared to the horses fed the HCO diet (Pagan and Harris 1999). When feeding a mixed diet, the greater volumes of fluid associated with the hay consumption increased the rate of passage of the grain through the small intestine and therefore, reduces starch digestibility in the small intestine and post-prandial glucose response (Pagan and Harris 1999). Therefore, our results demonstrated that feeding hay along with grains (M) increased water consumption and body weight, and reduced glycemic indices compared to horses fed grain-based diets (HCO) after exercise.

Metabolic responses to dietary interventions: Similar to other studies, the marked increase in NEFA and glycerol concentrations immediately after exercise reflected the increased rate of lipolysis to sustain increased lipid oxidation during exercise (Hyyppä et al. 1997). The lower triglyceride and NEFA concentrations in horses fed the LCO diets, compared to the ones fed M or HCO diets, may reflect an increased lipid uptake at the plasma membrane and suggested that a greater proportion of lipids rather than carbohydrate are oxidized for energy production after exercise in that group (Hyyppä et
al. 1997). Glycerol concentration was lower for horses fed the LCO or M diets 1 h after exercise and feeding, suggesting that glycerol served as a substrate for glycogenesis when soluble carbohydrate are somewhat limited. However, its quantitative importance is unknown.

**Dietary manipulations and glycogen resynthesis:** We demonstrated that post-exercise feeding of HCO diet produced a greater increase in blood glucose and insulin concentrations, and hastened muscle glycogen resynthesis by increasing blood glucose availability to the skeletal muscle and insulin release compared to feeding isocaloric LCO or conventional diets. Our results suggested that raised concentrations of insulin converted glycogen synthase from its inactive form (D) to its active form (I), which then hastened muscle glycogen resynthesis. Our results also suggested that this mechanism was enhanced in horses fed high-glycemic index meal compared to horses fed moderate glycemic index meal, although feeding HCO diet only increased glycogen synthase activity at 3 hours after exercise compared to M and LCO diets. These results are in contradiction with one study, which reported no significant effect of oral glucose supplementation (3 g/kg) on glycogen replenishment in equine skeletal muscle (Davie et al. 1994), although the statistical power may have been low because of the small number of horses (n = 4). Furthermore, muscle glycogen resynthesis could not be hastened after glycogen-depleting exercise by providing propionic acid or by increasing insulin secretion through the administration of leucine (Pösö and Hyyppä 1999). However, in this study the small amount of glucose administered (38 ± 6 g/horse) may explain the lack of treatment effect on muscle glycogen resynthesis. In contrast, several authors have
demonstrated the effect of feeding diets with different soluble carbohydrate content on muscle glycogen repletion. Topliff et al. (1985) demonstrated a significant increase in muscle glycogen when 6 horses were fed a high soluble carbohydrate diet during a 3-day rest period following glycogen-depleting exercise when compared to values before exercise (when horses were under training and fed conventional diets). Muscle glycogen stores were also found higher in horses fed high soluble carbohydrate diet compared to horses fed high fat diet before exercise (Essén-Gustavsson et al. 1991). While these studies considered the effect of pre-exercise feeding on muscle glycogen stores, post-exercise feeding might be more beneficial by optimizing muscle glycogen replenishment. Only one study investigated the effect of post-exercise feeding on muscle glycogen stores. They demonstrated that glycogen content was higher in horses fed a high soluble carbohydrate diet and moderate soluble carbohydrate diet compared to a low soluble carbohydrate diet (Snow et al. 1987) only at 28 hours post-exercise. However, these diets were not isocaloric and the difference in muscle glycogen replenishment found in this study could be solely attributed to the difference in digestible energy intake. To our knowledge, this is the first study that clearly demonstrated that post-exercise feeding of high-glycemic index diet (HCO) resulted in faster rate of glycogen resynthesis and greater muscle glycogen concentrations at 48 and 72h after exercise compared to feeding M or LCO diets.

In summary, the rate of post-exercise replenishment of muscle glycogen is slower in horses compared to humans and rats. We demonstrated a temporal relationship between intramuscular glycogen level and glycogen resynthesis, suggesting that
intramuscular glycogen level plays a regulatory role in post-exercise muscle glycogen
resynthesis. We believe that this study also highlighted some peculiarities of muscle
glycogen metabolism in horses, which may be related to the high resting level of muscle
glycogen, and provide some mechanistic explanations in regard to the slow rate of
glycogen resynthesis. Because of the slow glycogenesis, developing nutritional strategies
to optimize muscle substrate availability for horses competing in several events in the
same day or on successive days may be more crucial than for humans. We demonstrated
that feeding high-glycemic index (HCO) meals after exercise hastened muscle glycogen
replenishment compared to isocaloric conventional diets or LCO diets by increasing
blood glucose availability to the skeletal muscle. Direct practical implications and
applications could be drawn from this study: we suggested that horses performing
endurance events or horses undertaking several events on successive days could benefit
from high soluble Carbohydrate diets after exercise to hasten muscle glycogen
resynthesis. However, further studies are needed to develop optimal nutritional strategies
to optimize post-exercise muscle glycogen replenishment in horses.
5.6 REFERENCES


CONCLUSIONS

Because the major pathways for anaerobic ATP production are the breakdown of creatine phosphate and the degradation of muscle glycogen to lactic acid, reduced glycogen availability could contribute to a decline in anaerobic energy production and exercise performance (Klausen et al. 1973; Hargreaves et al. 1998). While the effects of muscle glycogen availability on exercise performance have been well investigated in humans, this issue has received scarce attention in equine exercise physiology. To our knowledge, this doctoral research provides the first evidence that manipulation of glycogen stores affects anaerobic capacity during high-intensity exercise in horses. In the first study (Chapter 2), we developed an exercise model that depleted muscle glycogen stores. We demonstrated that exercise-induced glycogen depletion was associated with a decreased run time to fatigue, maximum accumulated oxygen deficit and blood lactate concentration, suggesting an impairment of anaerobic capacity during subsequent high-intensity exercise. Furthermore, exercise-induced glycogen depletion was not associated with a change in aerobic capacity. However, the limitation of this study was the presence of confounding residual factors. The decreased anaerobic capacity cannot solely be attributed to the decline in muscle glycogen stores, because the previous strenuous exercise may have contributed to the development of fatigue. The second study
(Chapter 3) was designed to remove these confounding variables through the manipulations of muscle glycogen stores by glucose or saline infusion. The results from this study confirmed our first findings. Exercised-induced muscle glycogen depletion was associated with a decreased anaerobic capacity, without affecting aerobic capacity. Furthermore, replenishment of muscle glycogen stores by IV glucose infusion was associated with an increased run time to fatigue, restoration of MAOD and blood lactate concentrations, compared to values before glycogen depletion. Demonstration that replenishment of muscle glycogen concentration by glucose infusion restores anaerobic capacity confirms a role for muscle glycogen concentration in limiting anaerobic capacity of horses. The limitation when investigating the role of glycogen availability on anaerobic capacity is the method used to assess anaerobic capacity. Because determination of MAOD is an indirect calculation from aerobic capacity, MAOD calculation provides only an estimation of anaerobic capacity. However, in both studies, the calculation of MAOD was well correlated with other variables related to anaerobic capacity (e.g., run time to fatigue and blood lactate concentrations). Furthermore, the assumptions made when calculating MAOD have been well validated in horses (Eaton et al. 1985). In conclusion, both studies demonstrated that substantial muscle glycogen depletion impaired athletic capacity during high-intensity exercise. These results highlight the critical role of muscle glycogen stores in limiting exercise performance in horses. These novel findings may provide some new insights in human exercise physiology where the effect of muscle glycogen depletion on athletic capacity remains controversial during high-intensity exercise. For instance, some authors suggested that
muscle glycogen pool is not a limiting factor during exercise because its intramuscular concentrations remain high at the point at which fatigue develops (Symons and Jacobs 1989). However, one particularity in horses is that muscle glycogen replenishment takes up to 72 hours (Snow and Harris 1991; Hyyppä et al. 1999). Therefore, the time allowed between interval bouts is not sufficient for complete replenishment of muscle glycogen stores for horses undertaking several events in the same days or on consecutive days. Therefore, reduced muscle glycogen stores could persist at the time of subsequent exercise and would then limit athletic performance.

Because muscle glycogen stores are a limiting factor during exercise and muscle glycogen resynthesis is slow in horses, our next critical question was: does dietary manipulation affect muscle glycogen resynthesis in horses? Administration of amino acids and glucose to increase insulin release failed to enhance muscle glycogen resynthesis in horses (Pösö and Hyyppä 1999). Whether feeding fat-supplemented diets increases lipids availability for energy production and therefore spares glycogen stores remains uncertain in horses (Hyyppä et al. 1997; Pösö and Hyyppä 1999). Therefore, the only other means to increase muscle glycogen resynthesis would by increasing substrate availability for glycogenesis by providing glucose supplementation or feeding high soluble carbohydrate diets. In humans, the effect of carbohydrate supplementation on glycogen availability has been well demonstrated in humans, and these studies have resulted in significant improvements in nutrition and performance of human athletes. Whereas intravenous glucose infusion hastened muscle glycogen replenishment compared to saline infusion (as demonstrated in Chapter 4), no study has demonstrated
conclusively an effect of dietary carbohydrate on muscle glycogenesis (Davie et al. 1994; Hyyppä et al. 1999; Pösö and Hyyppä 1999). Therefore, in the last study (Chapter 5), we investigated the effect of feeding high soluble carbohydrate diet on the replenishment of muscle glycogen stores after exercise. To our knowledge, this is the first study that clearly demonstrated that feeding isocaloric diets of different soluble carbohydrate content affects muscle glycogen resynthesis in horses. This study demonstrated that: 1) muscle glycogen resynthesis was slower in horses than in humans; 2) supercompensation of muscle glycogen stores was not obtained; 3) feeding isocaloric high-glycemic index meal hastened muscle glycogen resynthesis and increased muscle glycogen stores at 48 and 72 hours after exercise. This study highlighted the importance of post-exercise feeding to enhance muscle glycogen replenishment and the importance of developing nutritional strategies, related to carbohydrate feeding to optimize muscle glycogen resynthesis. This study also highlighted some critical differences in muscle glycogen metabolism compared to other species and provided for the first time some mechanistic explanations in regard to the limited rate of glycogenesis. Further studies are required 1) to confirm the molecular mechanisms underlying muscle glycogen resynthesis; 2) to develop nutritional strategies to optimize muscle glycogen replenishment.

Because glucose transporters are the rate-limiting step for glucose utilization by muscle, extensive investigations have been done on glucose transporters, especially in rats and in humans. Whereas this aspect of glucose metabolism has recently been investigated in horses (McCutcheon et al. 2002), the technique to isolate GLUT-4 protein in equine tissue has not been validated. With this doctoral research, we characterized
GLUT-4 transporters in sedentary horses by demonstrating the specificity of the immunoreactive bands detected by Western blot, and we determined the molecular weight of the GLUT-4 protein. We also demonstrated that exercise, and to a lesser extent feeding, increased GLUT-4 protein content in skeletal muscle. Our results suggested that glycogen stores play a regulatory role on GLUT-4 glucose transporters in equine skeletal muscle by exerting a negative feedback on GLUT-4 recruitment, once muscle glycogen stores have been restored. Finally, further studies are required to confirm the physiological importance of glucose transporters using plasma membrane isolation and primary antibody species-specific.

In conclusion, we developed a reliable model to deplete muscle glycogen stores and we characterized GLUT-4 glucose transporters in horses. These tools allowed us to further investigate glucose and glycogen metabolism in equine skeletal muscle. This doctoral research demonstrated that: 1) decreased muscle glycogen availability is a contributing factor to fatigue during high-intensity exercise in horses; 2) carbohydrate administration, either intravenous glucose infusion or a high soluble carbohydrate diet, enhances muscle glycogen resynthesis after exercise; 3) exercise increases GLUT-4 protein content, and replenishment of muscle glycogen stores after carbohydrate administration, either by glucose infusion or diet, attenuated the increase in GLUT-4 protein content of skeletal muscle. Our results also suggested that muscle glycogen stores play a regulatory role on muscle glycogen synthesis, in particular on GLUT-4 recruitment in equine skeletal muscle.
Finally, we expect that this research dissertation will advance our understanding of the mechanisms of fatigue during high-intensity exercise and our understanding of the metabolic and physiological events that occur during the recovery period in horses. Furthermore, we hope that we have reached our overall goal, which was to contribute to the improvement of the welfare of the horse.
LIST OF REFERENCES


