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Post-exercise Muscle Glycogen Repletion in Horses

Doctoral Dissertation

Seppo Hyyppä

Animal Production

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To Horses who so faithfully serve us

even beyond their own well-being

MTT Agrifood Research Finland

Animal Production, Equine Research Ypäjä

and

Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine

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Post-exercise Muscle Glycogen Repletion in Horses

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Abstract

Because muscle glycogen has a crucial role as a substrate for energy metabolism, and because subsequent exercise performance may be dependent upon the extent of muscle glycogen resynthesis during recovery, post-exercise muscle glycogen repletion is very important for both human and equine athletes. Muscle glycogen repletion has been extensively studied in man, but due to the physiological differences between the two species, the results of human studies cannot be directly applied to horses.

The major finding of this study was the slow rate of resynthesis of glycogen, indicating that horses may be progressively depleted in muscle glycogen stores during an intensive training period.

The results emphasize the need for an adequate rest:work ratio over the training period to maintain a positive anabolic hormonal balance and to provide sufficient time for repletion of muscle glycogen stores.

When horses consume a normal basal diet, neither extra carbohydrate nor extra fat will enhance the repletion of muscle glycogen stores, but may, especially in unadapted horses, produce undesirable effects.

Maintaining horses in a good state of hydration seems to have a moderate positive effect on repletion of muscle glycogen stores. Providing horses with an isotonic glucose-electrolyte rehydration solution soon after exercise helps to overcome dehydration significantly better than providing them plain water.

In practical situations, changes in body weight serve as a useful indicator of recovery.

Key words: horse, exercise, glycogen, recovery

List of original articles

This thesis is based on the following original papers, referred to in the text by their Roman numerals (I-V).

I. Seppo Hyyppä, Leena A. Räsänen and A. Reeta Pösö (1997) Resynthesis of glycogen in skeletal muscle from Standardbred trotters after repeated bouts of exercise. Am. J. Vet. Res. 58, 162 - 166.

II. A. Reeta Pösö and S. Hyyppä (1999) Metabolic and hormonal changes after exercise in relation to muscle glycogen concentrations. Equine vet. J., Suppl. 30, 332 - 336.

III. S. Hyyppä, M. Saastamoinen and A. R. Pösö (1996) Restoration of water and electrolyte balance in horses after repeated exercise in hot and humid conditions. Equine vet. J., Suppl. 22, 108 - 112.

IV. S. Hyyppä, M. Saastamoinen and A. Reeta Pösö (1999) Effect of a post exercise fat-supplemented diet on muscle glycogen repletion. Equine vet. J., Suppl. 30, 493 – 498.

V. S. Hyyppä (2001) Effects of nandrolone treatment on recovery in horses after strenuous physical exercise. J. Vet. Medicine, Series A. 48, 343 – 352.

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Abbreviations

AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AS	Anabolic steroid
CAMK	Ca^{2+}/calmoduli-dependent protein kinase
CET	Competition exercise test
CS	Citrate synthase
CV	Red cell volume
ET	Exercise test
GE	Isotonic glucose-electrolyte solution
GEL	Isotonic glucose-electrolyte solution supplemented with 50 g of leucine
GEP	Isotonic glucose-electrolyte solution supplemented with 200 ml of propionic acid
GLUT	Sodium-independent glucose transporter protein
HAD	3-hydroxyacyl-CoA dehydrogenase
IGF	Insulin-like growth factor
IR	Insulin receptor
IRS-1	Insulin receptor substrate
MCT	Monocarboxylate transporter
NEFA	Non-esterified fatty acid
NO	Nitric oxide
PI3K	Phosphatidylinosito 3-kinase
P_{La4}	Exercise heart rate causing a blood lactate level of 4 mmol/l
PKC	Ca^{2+}-dependent conventional protein kinase C
PV	Plasma volume
SET	Submaximal exercise test
TG	Triglyceride
$V_{La\ 1.5\ -4}$	Exercise speed causing a blood lactate level of 1.5-4 mmol/l
VO_{2max}	Maximal oxygen consumption

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1 Introduction

Horses engage in a variety of physical activities. Some efforts are sustainable for several hours, whereas others lead to fatigue in one minute or less. Exercise intensity and duration have a major impact on energy expenditure and the physiological changes that occur in the horse.

Thoroughbred racehorses and Quarter horses are able to achieve speeds of 70 km/h over short distances 400-1000 m. Trotters demonstrate both speed and endurance in trotting races, where racing distances vary from 1600 m to 3100 m. In these athletes, a huge increase occurs in the rate of energy expenditure in an attempt to maintain the constant concentration of adenosine triphosphate required for excitation-contraction coupling. The readily available phosphocreatine stores are rapidly depleted. Oxidative metabolism, with carbohydrates being the main fuel source, is pushed to the limit and additional energy must be derived from anaerobic glycolysis. Nimmo and Snow (1983) have reported rates of glycogen utilization up to 149 mmol/kg muscle dry weight per minute, and muscle glycogen concentrations have been shown to decrease by 20-40% during high-intensity, short-distance exercise (Hodgson et al. 1984; Snow et al. 1987; Snow and Harris 1991).

Endurance horses show extreme endurance in long-distance rides of up to 160 km. In the longest rides, riding times exceed 9 h even for the winners. During low-intensity exercise, fat has been shown to be the major energy source for oxidative metabolism, with carbohydrates playing only a minor role (Essén-Gustavsson et al. 1984; Rose et al. 1991). Although the rate of glycogen utilization during low-intensity exercise may be less than 1 mmol/kg muscle dry weight per minute (Essén-Gustavsson et al. 1991), exercise over extended periods of time, as in endurance rides of over 100 km, generally results in depletion of glycogen in most fibres, and total muscle glycogen concentrations have been shown to decrease by 67% (Hodgson et al. 1983; Essén-Gustavsson et al. 1984).

Low muscle glycogen content before exercise could adversely affect subsequent racing performance (Snow and Harris 1991; Lacombe et al. 1999; 2001). Moreover, when glycogen content in muscle is low prior to exercise, an increased rate of protein degradation may occur during exercise (Lemon 1987). This might contribute to the onset of signs of stress and overtraining.

Because muscle glycogen has a crucial role as a substrate for energy metabolism, and because subsequent exercise performance may be dependent upon the extent of muscle glycogen resynthesis during recovery, post-exercise muscle glycogen repletion has been intensively studied in man. The rate of post-exercise muscle glycogen repletion is also important in horses, but only a limited number of equine studies have been conducted. Although both man

and horse are monogastric, they have physiological differences in digestion. Horses have evolved to utilize high-fibre diets. The production of short-chain fatty acids by bacterial fermentation in the caecum and colon, and gluconeogenesis from propionate, have important roles in equine energy metabolism (Hintz 1983). In man, high carbohydrate intake shortly after exercise is known to enhance the repletion of muscle glycogen stores (Bergström and Hultman 1966). The horse has digestive and metabolic limitations with regard to high-grain, highly hydrolysable carbohydrate diets; these diets may cause gastrointestinal disorders or laminitis (Rowe et al. 1994). Large amounts of soluble carbohydrates cannot therefore be recommended for horses.

Due to the physiological differences between the two species, the results of human studies cannot be directly applied to horses.

2 Review of the literature

2.1 Digestion of carbohydrates

Horses have evolved over millions of years as grazers, and therefore, fibrous feeds (e.g., roughages and pastures) provide the primary nutrition for most horses (Meyer 1987). Fibrous feeds are also essential for proper function of the digestive tract and to satisfy the horse's craving to chew. To satisfy the energy requirements of athletic horses, a portion of the fibrous feeds is usually substituted with starchy feeds, mainly cereal grains. All equine diets are high-carbohydrate diets with low protein and low fat content.

Carbohydrates present in plants may be divided into three groups: simple sugars, storage molecules (e.g., starch, fructans) and structural polysaccharides (e.g., hemicelluloses, cellulose) (Meyer 1987). From the standpoint of equine digestive physiology, carbohydrates may be divided into two major groups: (1) carbohydrates with alpha-1,4-linked molecules subject to enzymatic hydrolysis to simple sugars in the small intestine by digestive amylase and other enzymes and (2) carbohydrates with beta-1,4-linked molecules fermented by microbial enzymes to short-chain fatty acids, also known as volatile fatty acids, in the sacculated large intestine, which is made up of the caecum and the colon (Meyer 1987).

2.1.1 Enzymatic digestion in the small intestine

Hydrolysable carbohydrates include disaccharides, some oligosaccharides (e.g., maltotriose) and starch (Meyer 1987). Digestion is initiated primarily in the small intestine by pancreatic alpha-amylase, which cleaves alpha-1,4 linkages in starch molecules, but does not cleave alpha-1,6 linkages or terminal alpha-1,4 linkages (McDonald et al. 1995). Because, unlike in humans, relatively little alpha-amylase is present in equine saliva, very limited starch hydrolysis occurs prior to arrival in the stomach (Meyer 1987). To a limited extent carbohydrates are hydrolysed by gastric acid in the stomach, independently of any enzymes (Meyer 1987). Even the small intestinal capacity of horses for starch digestion is comparatively low owing to fairly low activity of pancreatic alpha-amylase (Foreman 1998). The end products at this stage are disaccharides, oligosaccharides and limit dextrins (Meyer 1987).

To yield free sugars, further carbohydrate breakdown occurs in the brush border of the small intestine. Disaccharases produced by the villi are sucrase, which converts sucrose to glucose and fructose; maltase, which breaks down maltose to two molecules of glucose; in foals lactase, which hydrolyses lactose to one molecule of glucose and one molecule of galactose; and oligo-

1,6-glucosidase, which attacks the alpha-(1-6) bonds in limit dextrins (McDonald et al. 1995). The activity of sucrase is high in the horse (Kienzle and Radicke 1993), but lactase activity decreases as individuals grow older, and in adult horses lactose is usually not broken down in the small intestine (Bracher et al. 1996). Maltase activity is extremely high compared with in man and sheep, and oligo-1,6-glucosidase activity is similar to those reported in omnivorous and carnivorous species (Roberts 1975; Dyer et al. 2002).

2.1.1.1 Absorption of monosaccharides

Presumably glucose, fructose and galactose are absorbed in the equine small intestine, as in other monogastric species.

Glucose and galactose are absorbed rapidly by two methods: transcellular and paracellular. The transcellular route through high-affinity, low-capacity, sodium-dependent glucose-galactose cotransporter type 1 (SGLT1) permits crossing of the cell membranes of small intestinal enterocytes against its concentration gradient. Absorbed carbohydrate travels through the cytoplasm to the basolateral membrane, through which it is carried via sodium-independent glucose transporter (GLUT-2) to interstitial fluid and the portal vein (Zierler 1999; Dyer et al. 2002). At high concentrations of carbohydrates in the intestinal lumen, passive absorption occurs via the paracellular route across the tight junctions between enterocytes (Gato-Pecina and Ponz 1990).

Fructose is mainly absorbed across the brush border membrane by sodium-independent glucose transporter (GLUT-5), and then across the basolateral membrane by GLUT-2 (Cui et al. 2003). On entering the enterocyte, much of the fructose becomes phosphorylated, then converted to glucose, and finally transported in the form of glucose the rest of the way into the interstitial fluid (Thomson and Wild 1997). In humans, absorption of fructose occurs at about half the rate of glucose (Riby et al. 1993), and the limited capacity for fructose absorption frequently leads to abdominal discomfort and diarrhoea when ~1 g/kg body weight fructose is ingested (Erickson et al. 1987; Murray et al. 1989). In horses, at an oral dose of ~0.7 g/kg body weight, fructose was well-absorbed and rapidly converted to glucose, resulting in a similar increase in plasma glucose values as with the same oral dose of glucose (Bullimore et al. 2000). So practically speaking, glucose and fructose sources, such as molasses and honey, are utilized well by horses.

2.1.1.2 Limitations of enzymatic digestion

The breakdown of starch, not the cleavage of liberated disaccharides and oligosaccharides by disaccharidases, is the rate limiting step in starch digestion in the horse (Kienzle and Radicke 1993).

2.1.2 Microbial fermentation in the large intestine

To utilize the large amounts of non-hydrolysable carbohydrates synthesized by plants, horses depend on the cellulolytic action of the microbial flora in their digestive tracts. Fermentable carbohydrates include hemicellulose, cellulose and lignocellulose, soluble fibres, some oligosaccharides (e.g., fructans, galactans) and those starches that have escaped enzymatic hydrolysis (Hoffman et al. 2001). Fermentation occurs predominantly in the large intestine; the caecum and colon, but limited fermentation may occur in any portion of the digestive tract (Frape 1998).

Roughage contains little starch, but relatively large amounts of sugars and structural carbohydrates, including cellulose (McDonald et al. 1995). Of all the digestible carbohydrates in roughage, roughly 5% is hydrolysable in the small intestine, 14% is rapidly fermentable and 81% is slowly fermentable (Hoffman et al. 2001). In horses on an all-roughage ration, the molar proportion of bacterial fermentation end products, i.e.short-chain fatty acids, in caecal or colonic fluid is about 70% acetate, 17% propionate, 8% butyrate and 5% others (e.g. isobutyrate, valerate, isovalerate) (Hintz et al. 1971). The proportion of acetate decreases, while that of propionate increases, as grain replaces hay in the ration (Hintz et al. 1971). The short-chain fatty acids are relatively weak acids with pKa values of ~4.8 (Topping and Clifton 2001). Furthermore, normally rapid absorption of short-chain fatty acids together with bicarbonate and phosphate buffers of the caecum and colon maintain the caecal and colonic pH between 6,6 and 7,5, which, combined with a constant fibre source entering the large intestine, is necessary for normal microbial function (Meyer 1996).

2.1.2.1 Absorption of short-chain fatty acids

Absorption of short-chain fatty acids at their site of production is rapid. Studies in ruminants suggest that absorption through the apical membrane of epithelial cells of the dissociated short-chain fatty acids (the predominant form at physiological pH) is made possible either by competing with Cl^- for binding sites at a common anion-exchange junction or by being absorbed by an short-chain fatty acid anion/HCO_3^- exchanger. On the basolateral membrane of the epithelium, the efflux of short-chain fatty acids is carried out by H^+/monocarboxylate transporters, particularly MCT1 (Bergman 1990; Kramer et al. 1996; Gabel et al. 2002; Enerson and Drewes 2003; Koho et al. 2005). This proton-dependent transporter from the family of monocarboxylate transporters has been detected in hamster caecal cells and in both human and porcine colonic epithelial cells (Garcia et al. 1994; Ritzhaupt et al. 1998; Sepponen et al. 2006). To some extent, simple diffusion of the undissociated form may also be involved (Frape 1998; Topping and Clifton 2001).

Some acetate appears to be oxidized in the enterocytes, with the remainder absorbed unchanged and used for energy or fat synthesis by peripheral tissues (Bergman 1990). Pethick and co-workers (1993) estimated that in horses at rest up to 30% of the energy may be provided by acetate.

Propionate passes across the intestinal wall, where little is converted to lactate and the majority is carried via the portal system to the liver, where it is used for the synthesis of glucose (Hintz 1983, Ford and Simmons 1985, Simmons and Ford 1991). Simmons and Ford (1991) estimated that colonic propionate production on a hay diet was about 150 mg/h per kg body weight, and on a hay and bran diet, about 200 mg/h per kg body weight. The mean total glucose production was around 100 mg/h per kg body weight for the two diets indicating that 50-60% of the glucose was synthesized from propionate produced in the colon.

A major portion of the butyrate is metabolized in the large intestinal enterocytes, where butyrate oxidation supplies some 60-70% of their energy needs (Topping and Clifton 2001). Apparently, in horses, the rest of the butyrate passes readily to blood, whereas in ruminants most of the butyrate is converted by the epithelial cells of the rumen to ketone bodies, especially to beta-hydroxybutyrate (Bergman 1990; Frape 1998). In horses, butyrate is transported by blood to provide energy or carbon for fatty acid synthesis in liver and adipose tissues (Frape 1998).

2.1.2.2 Limitations of microbial fermentation

In monogastric species other than the horse, starches are completely digested by the time the ingesta reach the distal ileum (Foreman 1998). Especially, if starch intake exceeds 3.5-4 g/kg body weight per feeding, the excess escapes small intestinal hydrolysis, allowing non-degraded starch to reach the caecum and colon, where it is readily used by Streptococci and Lactobacilli spp.(Hoffman et al. 2001; Medina et al. 2002). Rapid fermentation of high levels of sugars and starches yields lactate and propionate (Rowe et al. 1994). Apparently lactate is not very well absorbed from intestinal lumen, although MCT1 facilitates, in addition to volatile fatty acids, lactate diffusion across the basolateral membrane of epithelium cells (Frape 1998; Enerson and Drewes 2003). The accumulation of lactic acid, a relatively strong acid with a pK_a value of 3.86, may overpower the buffering mechanisms of the caecum and colon, resulting in severe acidosis (pH below 6). This leads to lysis of the desired bacterial population, depressed efficiency of fibre utilization and a reduced concentration of volatile fatty acids (Margolis et al. 1985; Rowe et al. 1994). This may further increase the risk of endotoxemia, laminitis, and gastrointestinal disorders, notably colic (Sprouse et al. 1987; Clarke et al. 1990; Rowe et al. 1994).

Also fructans, fructose-containing storage oligosaccharides present in grass, have been associated with laminitis. Because of the beta-(1-2)-glucosidic bonds between fructose molecules, fructans are resistant to hydrolysis by small intestinal enzymes, therefore passing through the small intestine undigested into the caecum and colon (Bailey et al. 2002). There, they are rapidly and extensively fermented by caecal and colonic bacteria, producing butyrate and lactate, resulting in acidic pH (Gibson and Wang 1994; Bailey et al. 2002). Although limited quantities of fructans may be beneficial to the balance of the hind gut flora, large amounts arriving to the large intestine are speculated to be responsible for initiation of pasture-induced laminitis (Longland et al. 1999).

2.2 Role of the liver in carbohydrate metabolism

Glucose is an energy substrate for all cells and the principal fuel for the brain, and therefore, an elegant system of homeostasis exists to regulate glucose availability. Endocrinal mechanisms are particularly important and glucose homeostasis is regulated by hormones of the pancreas, anterior pituitary, adrenal cortex and medulla, including insulin, somatostatin, glucagon, adrenocorticotropic hormone, cortisol and catecholamines

The liver is crucial to glucose homeostasis because it functions as an important buffer system to maintain a steady blood glucose concentration. Concomitantly with the post-feeding rise in blood glucose, the rate of glucose uptake and its almost immediate storage into the liver increase (Thorens 1996). The major glucose transporter in liver cells is GLUT-2. This membrane-bound glucose transport protein has high transport capacity and enables the rate of glucose uptake to change in parallel with plasma glucose levels, independently of insulin (Fluckey et al. 2001).

The direction of the glucose flux is dictated by both the nutritional and the hormonal environment. Hyperglycaemia, hyperinsulinaemia and hypoglucagonaemia favour hepatic glucose uptake by increasing glucokinase activity so that glucose is rapidly phosphorylated; entry of glucose into the liver is therefore facilitated by the maintenance of an intracellular glucose concentration lower than that in plasma. Hypoglycaemia, hypoinsulinaemia, hyperglucagonaemia and hypercatecholaminaemia, in turn, favour glucose release by decreasing glucokinase activity and glucose phosphorylation, and the exit of glucose is facilitated by an intracellular glucose concentration higher than that in plasma (Rencurel and Girard 1998). In addition, thyroid hormones upregulate GLUT-2 expression in liver membranes (Weinstein et al. 1994).

Insulin

When blood glucose concentration rises, insulin is released from pancreatic beta-cells. Some of the many effects of insulin on liver are to promote glycogen synthesis, decrease the rate of glycogen breakdown, inhibit gluconeogenesis and increase glycolysis (Newsholme and Dimitriadis 2001). When the quantity of glucose entering liver cells is more than can be stored as glycogen or be used for hepatocyte metabolism, insulin promotes the conversion of this excess glucose into fatty acids, which are either stored in liver as triglycerides or discharged back into the circulation and transported by blood to adipose or other tissues primarily as triglycerides in the form of very low-density lipoproteins (Lawrence 1990; Newsholme and Dimitriadis 2001). The uptake of circulating triglycerides by muscle and adipose tissue is regulated by the activity of endothelial lipoprotein lipase (Lawrence 1990).

Glucagon and catecholamines

A decrease in blood glucose stimulates pancreatic glucagon secretion from alpha-cells, counterbalancing the effects of insulin to increase the glucose concentration to normal levels. Glucagon mainly acts on the liver by decreasing glycogen synthesis, increasing glycogenolysis and increasing gluconeogenesis (Stabenfeldt 1992). During exercise catecholamines, together with glucagon, account for the increased hepatic glucose production and glucose output. By means of beta-adrenoreceptors, adrenaline and noradrenaline promote glycogen breakdown in the liver, increase lipolysis in adipose tissue and inhibit insulin release (Zierler 1999). If hepatic glucose output fails to match the demands of working muscles during, for example, prolonged exercise, the decline in plasma glucose supply contributes to the onset of fatigue (McCutcheon et al. 2002).

2.2.1 Alternative carbon sources for glycogenesis

Gluconeogenesis

The predominant precursor for muscle glycogen is glucose. However, even in the absence of carbohydrate intake, a wide range of animal species has the capacity to replenish some of their muscle glycogen stores, indicating that alternative carbon sources can be used (Fournier et al. 2002). Gluconeogenesis, the synthesis of new glucose from intermediates of glycolysis and the Krebs cycle, plays an essential role, especially in the absence of carbohydrate intake, in the maintenance of normoglycaemia. For example, in humans, gluconeogenesis accounts for almost all glucose production after 42 h of fasting (Landau et al. 1996). Glucagon and the beta-adrenergic agonists adrenaline and noradrenaline stimulate gluconeogenesis, whereas insulin inhibits it (Zierler 1999).

Synthesis of glucose occurs in the liver and to some extent in the kidney. Since skeletal muscle lacks the glucose-6-phosphatase enzyme, it cannot deliver free glucose to blood and therefore seems to undergo gluconeogenesis exclusively as a mechanism to produce glucose to storage as glycogen (McDermott and Bonen 1992).

2.2.1.1 Propionate

In fasting humans and rats, restoration of glycogen stores by glyconeogenesis is slow (Fell et al. 1980; Favier et al. 1987). One distinct difference between humans and horses is the considerable propionate production in the latter due to cellulolytic action of microbial flora in the caecum and colon of the horse. Up to 60% of glucose in equine blood may be synthesized in the liver from propionate via gluconeogenesis (Hintz 1983, Ford and Simmons 1985, Simmons and Ford 1991). Since mean retention times for different forages in the equine hind gut range from 26 to 42 h (Guay et al. 2002), much longer fasting times would be needed in horses than, for example, in humans to study the ability to replenish muscle glycogen stores post-exercise from endogenous carbon sources only.

2.2.1.2 Lactate

After high-intensity exercise, lactate is a significant source of carbon atoms for gluconeogenesis. When post-exercise blood lactate concentration is high, lactate is taken up by the liver and extrahepatic tissues for glycogen resynthesis or for oxidation (Johnson and Bagby 1988). The major fate of lactate taken up by inactive muscles is oxidation (Putman et al. 1999). However, during the early stage of recovery from a short period of high-intensity exercise, the rate of glycogen resynthesis in fasted humans was 3-5 times faster than the rate of synthesis after prolonged moderate-intensity exercise when lactate was unavailable (Krssk et al. 2000). Estimations of lactate's relative contribution, either directly or indirectly via its conversion to plasma glucose mainly by the liver, to glycogen synthesis vary widely from 11% to 50% in human muscles and from 10% to 66% in rat muscles (McDermott and Bonen 1992). The actual contribution of direct lactate glyconeogenesis and indirect Cori cycling to the resynthesis of muscle glycogen in rats and humans is still under dispute (Fournier et al. 2002).

2.2.1.3 Amino acids

When the body's stores of carbohydrates are depleted, moderate quantities of glucose can be formed from amino acids (Fournier et al. 2002).

Protein degradation and amino acid metabolism in the working muscle are enhanced and estimated to constitute 5-15% of energy sources during exer-

cise (Lemon and Nagle 1981). Ammonia is formed at an increased rate in muscle mainly by deamination of adenine nucleotides and to a minor extent by deamination of branched-chain amino acids (Galassetti et al. 1998). To avoid exceedingly high ammonia concentrations, which may interfere with proper muscle function, some ammonia is released in free form, but more is released as alanine and glutamine after reaction with pyruvate and glutamate, respectively (Galassetti et al. 1998). In horses, the greatest increases in alanine concentration during recovery after high-intensity exercise were measured in those horses that were the best performers, suggesting that during exercise the synthesis of alanine from pyryvate is physiologically important for aerobic energy metabolism (Pösö et al. 1987; 1991). Fractional uptake of alanine by the liver, where its carbon chain is used for gluconeogenesis and the amino group for urea synthesis, is high. Already in 1974, Ahlborg and co-workers reported that the glucose-alanine cycle accounted for >40% of endogenous glucose production during prolonged exercise in man. Also, in horses, alanine may be an important gluconeogenic precursor, especially at the end of an endurance ride (Essén-Gustavsson and Jensen-Waern 2002). Thus alanine is the major amino acid for glucose synthesis, but about 60% of the other amino acids can be converted fairly easily into glucose by means of transamination plus several simple interconversions, whereas the branched-chain amino acids (leucine, isoleucine and valine) are not significantly taken up or metabolized by the liver (Pösö et al. 1982; Guyton and Hall 1996).

During starvation, amino acids arising primarily from muscle proteolysis are the principal substrates for hepatic gluconeogenesis (Brosnan 2003). However, gluconeogenesis from amino acids is not limited to fasting periods. The body does not have a store of amino acids, and therefore, dietary amino acids in excess of those required for protein synthesis are rapidly catabolized. Jungas and co-workers (1992) estimated that in humans amino acids serve as a primary fuel for the liver and the primary carbon source for hepatic gluconeogenesis in a normal prandial state. All equine diets have low protein content, suggesting that protein is probably less important as an energy source in horses than in humans in a normal prandial state. However, protein is also used in greater quantities as an energy source also in horses when excessive protein is provided, or when a horse is not consuming sufficient energy through carbohydrates and fats (Harris 1997).

2.2.1.4 Glycerol

Glycerol is only produced metabolically by lipolysis (Wolfe et al. 1990). Glycerol cannot be oxidized directly by skeletal muscle, but it is a substrate for hepatic gluconeogenesis (Miller et al. 1983). Phillips and co-workers (2002) found that in isolated hepatocytes from fasted rats over 90% of the glycerol reaching the cells was converted into glucose.

During prolonged exercise, the rate of gluconeogenesis from glycerol was sufficient to make a significant contribution to energy metabolism in rats but not in humans (Miller et al. 1983). In dogs, less than 9% of the glucose turnover during exercise was derived from glycerol (Shaw et al. 1976).

Post-exercise low plasma insulin and elevated concentrations of glucagon and cortisol allow continued mobilization of fatty acids and glycerol. Glycerol is therefore an important gluconeogenic substrate for hepatic glucose or glycogen synthesis, especially in the absence of food intake (Guyton and Hall 1996). Baba and co-workers (1995) estimated that in humans conversion of glycerol to glucose accounted in the post-absorptive state for 4.5% and after 62-86 h of starvation for 22% of total glucose production.

2.3 Muscle glycogen

2.3.1 Glucose transport across membranes

A crucial and often rate-limiting step in glucose metabolism in skeletal muscle is the ability to transport glucose across the plasma membrane into the cell (Ziel et al. 1988; Richter et al. 2001). Although most of the studies of glucose transport into skeletal muscle have involved laboratory animals, much of the data obtained are probably applicable across the species.

Glucose uptake occurs by regulated facilitated diffusion, made possible by glucose transporter proteins (GLUTs). From the family of GLUTs at least five transport primarily D-glucose; GLUT-1, GLUT-2, GLUT-3, GLUT-4 and GLUT-7 (Zierler 1999). GLUT-5 is thought to function as a fructose transporter and is expressed mainly in the small intestine but also in the kidney, brain, muscle, adipose tissue, testes and sperm (Hundal et al. 1992; Zierler 1999). GLUT-1, -4 and -5 are present in mammalian skeletal muscle (Constable et al. 1988; Douen et al. 1990).

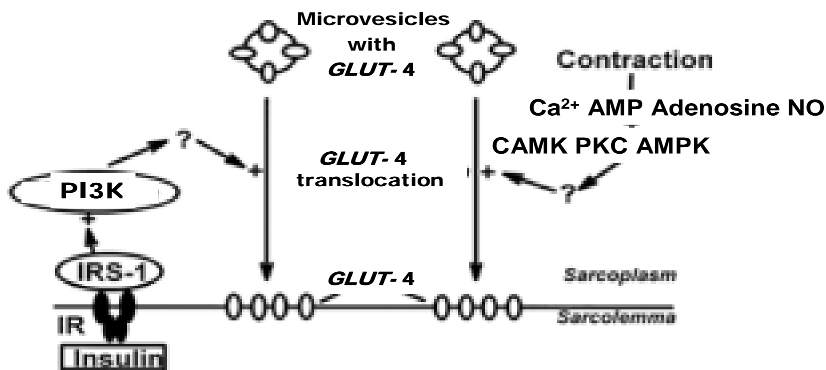
GLUT-1

GLUT-1 is resident in the sarcolemma independently of stimulation with insulin and/or muscle contractions, and its main function is thought to be to provide basal glucose transport (Mueckler 1994). It is expressed at very low levels in muscle, and therefore, the normal resting muscle membrane is only slightly permeable to glucose (Ren et al. 2000). Consequently, during much of the day, muscle tissue depends not on glucose for its energy, but on free fatty acids, including the volatile fatty acids in horses (Zierler 1999).

GLUT-4

GLUT-4 is the most abundant and most important glucose transporter in skeletal muscle. GLUT-4 translocates from an intracellular storage site to the

sarcolemma and T-tubules upon stimulation with insulin, contractions and/or hypoxia (Constable et al. 1988; Douen et al. 1990; Zierler 1999). Insulin and contractions have additive effects on glucose transport, suggesting that they act via at least partially independent pathways (Ploug et al. 1987). This is further supported by the finding that each of the two stimuli recruits distinct pools of intracellular GLUT-4 vesicles to the surface membrane of muscle fibres (Ploug et al. 1998).



AMP	=	adenosinemonophosphate
AMPK	=	AMP-activated protein kinase
CAMK	=	Ca^{2+}/calmoduli-dependent protein kinase
GLUT-4	=	Glucose transporter protein 4
IR	=	insulin receptor
IRS-1	=	insulin receptor substrate
NO	=	nitric oxide
PI3K	=	phosphatidylinositol 3-kinase
PKC	=	Ca^{2+}-dependent conventional protein kinase C

Figure 1. Two mechanisms for translocation of GLUT4 to the sarcolemma of skeletal muscle (modified from Winder and Hardie 1999).

Between meals, the amount of insulin that is secreted is too small to translocate GLUT-4 and promote significant amounts of glucose to enter the muscle cells. During the few hours after a meal, the blood glucose concentration is high and the pancreas secretes large quantities of insulin. The extra insulin causes translocation of GLUT-4 from an intracellular storage site to the sarcolemma, thereby enhancing the glucose transport capacity (Thorell et al. 1999). Subsequently, GLUT-4 returns from the cell surface to the cell interior

via endocytosis (Zierler 1999). Studies in humans have demonstrated that the larger the plasma insulin concentration the greater the increase in plasma membrane GLUT-4 (Thorell et al. 1999). Insulin also seems to be able to increase somewhat the intrinsic activity of GLUT-4, presumably by inducing a change from one configuration to another, thereby enhancing the ability to transport glucose (Zierler 1999). In response to insulin, glucose transport into human and rat muscle cells may increase up to 15-fold (Zierler 1999). During this period, muscle cells use glucose preferentially over fatty acids (Newsholme and Dimitriadis 2001). Despite this, in humans 70% or more of glucose uptake was accounted for by an increase in muscle glycogen (Zierler 1999). Muscle glycogen seems to have a regulatory role in insulin-stimulated glucose transport. For example, in fast-twitch muscle fibres of rats, high glycogen content was associated with a state of insulin resistance to translocate GLUT-4- containing vesicles (Derave et al. 2000). Presumably, high glycogen content affects insulin signalling rather than translocation of GLUT-4-containing vesicles directly (Derave et al. 2000).

Both during and immediately after exercise, the concentration of insulin is slightly decreased mainly because catecholamines inhibit its secretion from the pancreas (Zierler 1999). During this period, glucose uptake is facilitated by the exercise-induced translocation of GLUT-4 (Thorell et al. 1999). The signalling pathways initiating translocation of GLUT-4-containing vesicles from exercise-sensitive intracellular storage sites to the cell surface are still incompletely understood (Richter et al. 2001). Youn and co-workers (1991) showed that in isolated rat muscles the release of calcium from the sarcoplasmic reticulum during the contraction process increases glucose transport in the muscle. In addition to the rise in intracellular calcium concentration, with activation of conventional protein kinase C and calmodulin-dependent protein kinase, other signalling agents, indicators of metabolic stress, such as adenosine, nitric oxide and AMP-activated protein kinase, may be involved (Richter et al. 2001; Fryer et al. 2002; Ojuka et al. 2002; Hardie et al. 2006; Long and Zierath 2006).

Muscle glycogen exerts a regulatory effect on exercise induced glucose uptake. In humans, a 30- to 60-min post-exercise period of rapid synthesis of muscle glycogen occurs which does not require insulin (Jentjens and Jeukendrup 2003). This initial rapid resynthesis phase has been demonstrated only when muscle glycogen is heavily depleted of normal resting levels, whereas moderate depletion results in a more linear glycogen resynthesis pattern (Price et al. 2000). In addition, during exercise glucose uptake is regulated by muscle glycogen, as indicated by the negative correlation between contraction-stimulated surface membrane GLUT-4 content and initial muscle glycogen level in rats (Derave et al. 1999). However, in this experiment, the effect was restricted to the fast-twitch fibres and was not demonstrable in the slow-twitch soleus muscle. At present, the mechanism by which glycogen concentration influences the activity of contraction-stimulated glucose transporters

is unclear. Possible explanations for the inhibitory effect could be that high glycogen levels affect contraction-induced GLUT-4 translocation to surface membrane directly, or high glycogen levels promote contraction-induced glycogen breakdown, giving rise to high concentrations of glucose-6-phosphate, which in turn inhibit hexokinase (Derave et al. 1999).

Studies in exercise-trained rats have demonstrated an exercise-induced increase in the number of glucose transporters in muscle (Ebeling et al. 1993). Hourmard and colleaques (1991) found that in humans GLUT-4 content was nearly doubled after a training programme, and Ebeling and co-workers (1993) reported a positive correlation between GLUT-4 content and VO_{2max} in humans. In horses, Lacombe and co-workers (2003) described an exercise-induced increase in the content of GLUT-4 protein in membrane preparations of equine muscle, but an other study found neither a single bout of exercise nor the combination of exercise followed by hyperglycaemia to increase muscle GLUT-4 protein or mRNA content (Nout et al. 2003). However, McCutcheon and co-workers (2002) reported a 2- to 3-fold increase in muscle GLUT-4 protein content following 6 weeks of moderate-intensity exercise training. Evidence of a post-training reduction in glucose utilization during submaximal exercise indicates that more information is needed to understand the link between the content of GLUT-4 in muscle and its intrinsic activity (Bergman et al. 1999).

2.3.2 Glycogenesis in muscles

The biochemical pathway that allows the incorporation of new glucose units into a growing glycogen molecule, the chief storage form of carbohydrate in animals, is fairly well known and involves the successive action of several enzymes.

In the initial reaction, glucose is phosphorylated by hexokinase enzyme and glucose-6-phosphate is formed. Muscle expresses two isoforms of hexokinase, I and II (Ferrer et al. 2003). Hexokinase I, which accounts for a large propotion of total hexokinase activity in skeletal muscle, has a high affinity for glucose, which is readily converted to glucose-6-phosphate upon entering the muscle cell (Ferrer et al. 2003).

In the second step, glucose-6-phosphate undergoes mutation in which phosphate is transferred to the C-1 position of the glucose molecule. This reaction is catalysed by the enzyme phosphoglucomutase (Beitz 1993). This enzyme does not have tissue-specific isoforms (Ferrer et al. 2003).

In the third step, uridine diphosphoglucose is synthesized by the transfer of glucose from glucose-1-phosphate to uridine triphosphate. This reaction is catalysed by the enzyme uridine diphosphate-glucose pyrophosphorylase

(Beitz 1993). Muscle and liver express the same isoform of this enzyme (Ferrer et al. 2003).

In the fourth step, the glucose moiety of uridine diphosphoglucose is linked, in the presence of a oligosaccharide primer and the enzyme glycogen synthase, to the polysaccharide chain by an alpha-(1-4) bond. This primer, which is a prerequisite to glycogen synthesis, is produced by an autoglycosylating protein, glycogenin (Lomako et al. 1990). Glycogen synthase is regarded as the rate-limiting enzyme in glycogen synthesis (Jentjens and Jeukendrup 2003). Two isoforms of mammalian glycogen synthase have been described. Most organs express the muscle form, whereas the liver isoenzyme appears to be tissue-specific (Ferrer et al. 2003). When the chain length of the polysaccharide reaches a critical level between 11 and 16 glucose units, the brancher enzyme, alpha-glucan glycosyl 4:6 transferase, transfers the terminal 7-residue portion from an alpha-(1-4) bond to an alpha-(1-6) bond (Beitz 1993). The newly established alpha-(1-6) bond thus becomes a branch point in the glycogen molecule. The remaining stub can again be lengthened by the action of glycogen synthase. The result is a complex ramification of chains of glucosyl units (Beitz 1993). A rare inherited glycogen-branching enzyme deficiency leading to death or euthanasia by age 6 weeks has been reported in Quarter horses (Sponseller et al. 2003)

Two forms of glycogen, which can be distinguished on the basis of their solubility in acid, have been described in rats, humans and horses (Lomako et al. 1991, Adamo and Graham 1998**,** Bröjer et al. 2002a). These glycogen pools also differ in size, with the acid-soluble macroglycogen being the larger, at 10000 kDa, and the acid-insoluble proglycogen the smaller, at 400 kDa; both forms contain only a single glycogenin, but different numbers of glucosyl units (Adamo et al. 1998). In human muscle with normal glycogen content, the smaller proglycogen is the predominant form, 20-25% being macroglycogen (Adamo et al. 1998). In horses, proglycogen accounted for about 43% of the total glycogen content in the gluteus medius muscle (Bröjer et al. 2002b). However, the ratio between proglycogen and macroglycogen is not constant, but rather dependent on the total glycogen concentration; when the total glycogen concentration increases the relative percentage of macroglycogen increases, whereas the relative percentage of proglycogen decreases (Adamo and Garaham 1998; Adamo et al. 1998; Bröjer et al. 2002a; 2002b).

Danforth (1965) originally described glycogen synthase as existing in two interconvertible forms, the least phosphorylated and more active I form and the D form.

Activation of glycogen synthase occurs through the dephosphorylation of three specific serine residues (Shulman 1995; Ferrer et al. 2003). For example, insulin enhances the activity of glycogen synthase by promoting the net dephosphorylation through the inactivation of protein kinases, such as glyco-

gen synthase kinase-3, and the activation of protein phosphatases, such as protein phosphatase 1 (Brady and Saltiel 2001; Wojtaszewski et al. 2002). Moreover, allosteric effectors, mainly glucose-6-phosphate, regulate glycogen synthase activity (Gomis et al. 2000). Therefore, the control of glycogen synthesis in muscle is shared by glycogen synthase and glucose transport because glucose is readily converted into glucose-6-phosphate upon entering the muscle cell (Ferrer et al. 2003).

Glycogen concentration also influences glycogen synthase activity. In fact, glycogen concentration may be a more potent regulator of glycogen synthase activity than insulin (Nielsen et al. 2001). Several human studies have shown that glycogen synthase activity was higher in a glycogen-depleted state than in a glycogen-loaded state (Wojtaszewski et al. 2002). This strong inhibitory effect of glycogen on glycogen synthase activity could be attributed to inhibition of the stimulatory effect of insulin on glycogen synthase activity through inactivation of the protein phosphatase enzyme (Mellgren and Coulson 1993; Laurent et al. 2000). Muscle glycogen content may regulate glycogen synthase activity also by regulating glycogen synthase localization. Nielsen and co-workers (2001) have demonstrated that glycogen synthase translocates from the glycogen-enriched membrane fraction to the cytoskeleton as glycogen content is lowered in the muscle cell, and this could have functional consequences.

Experiments in humans have shown that, presumably because of increased glycogen synthase activity, the rate of glycogen synthesis after glycogen depletion is actually higher during low-intensity exercise than at rest (Price et al. 1994b).

Although regulation of glycogen synthesis and glycogen synthase activity has been intensively studied in humans and rats, in horses the mechanisms are poorly defined (McCutcheon et al. 2006).

According to Snow and Guy (1979), in equine muscle the more active I form constitutes only 10% of the total glycogen synthase activity. Neither training, consisting of both submaximal and maximal work for 10 weeks followed by a 5-week period of sprinting, nor detraining had significant effects on the activity of glycogen synthase.

Lacombe and co-workers (2004) found that in equine muscle glycogen synthase activity was inversely related to muscle glycogen content.

McCutcheon and co-workers (2006) demonstrated stimulation of glycogen synthase activity in response to a single one-hour bout of exercise. However, in horses fed normal diets, glycogen contents were restored equally within 2-3 days after modest (19-25%) muscle glycogen depletion regardless of the

intensity of recovery exercise on subsequent days. Horses were either walked for 10-20 min or cantered slowly for 1000 m (Snow and Harris 1991).

2.3.3 Glycogen repletion and fibre type composition

In the majority of studies, the time course of post-exercise glycogen resynthesis has been investigated in mixed fibre muscle biopsy samples, not considering the heterogeneous nature of skeletal muscle. However, some evidence suggest that glycogen repletion may be affected by fibre type composition due to a fibre-dependent pattern of regulation of the phoshorylation state of glycogen synthase and fibre type differences in glucose transporter numbers (Fournier et al. 2002). The muscle glycogen accumulation process may be delayed after different types of muscle-damaging exercise, e.g. delayed accumulation in type I fibres after a competitive marathon in humans (Asp et al. 1999).

Casey and co-workers (1995) found that in humans in the initial 3 h of recovery a 25% higher rate of resynthesis occurred in type I than in type II fibres. Between 3 and 10 h of recovery, resynthesis in type I fibres declined to 50% of the rate in type II fibres, in which the rate was maintained. They speculated that the differences may have arisen because of fibre type differences in glucose transporter numbers and insulin sensitivity and/or regulation of glycogen synthase.

Hodgson and co-workers (1983) found that glycogen repletion after endurance rides occurred in the reverse pattern to depletion, with preferential repletion of type IIB fibres relative to type I fibres. They suggested this could be related either to higher total glycogen synthase levels in type IIB fibres or to an adaptational response to produce the more active I form of glycogen synthase in these fibres.

In rodents, it is well established that glucose uptake capacity is greater in muscles rich in type I fibres than in muscles rich in type II fibres, and one underlying mechanism appears to be the higher level of GLUT-4 expression (Henriksen et al. 1990; Goodyear et al. 1991; Marette et al. 1992). There is some evidence, although not as conclusive, that this applies also to humans (Gaster et al. 2000a). In addition, in humans the GLUT-4 in type I muscle fibres has been suggested to be more insulin-sensitive than in type II fibres, and in type II fibres GLUT-4 may be more responsive to contraction than in type I fibres (Gaster et al. 2000b).

Adamo and co-workers (1998b) have shown that in man proglycogen was resynthesized far faster than macroglycogen and was also much more sensitive to carbohydrate ingestion, suggesting that the two glycogen pools might have metabolic differences in terms of their regulation. However, in horses,

glycogen resynthesis seems to start in the macroglycogen pool (Bröjer et al. 2006).

2.3.4 Muscle glycogen concentrations

Glycogen is found in most mammalian cells, but the liver and skeletal muscles are the tissues that can accumulate glycogen to the largest extent. The liver contains the highest concentration of glycogen, but owing to its greater mass, skeletal muscle contains the largest amount of glycogen (Gomis et al. 2000). However, relatively low amounts of energy are present in the glycogen stores of the body as compared with fats; in a 500-kg horse, these figures are 75300 kJ and 640000 kJ, respectively (Eaton 1994). This is explained in part by glycogen yielding far less energy on a per gram basis than fat. Moreover, since glycogen is stored in a hydrated form (3-5 g of water per gram of glycogen), the energy yield per gram of wet glycogen is decreased further (Bridge and Bridges 1932; Olsson and Saltin 1969; Olsson and Saltin 1970; Fournier et al. 2002). Consequently, muscle glycogen concentration can be expressed either as mmol/kg dry weight or as mmol/kg wet weight; 1 mmol/kg wet weight equals approximately 4 mmol/kg dry weight (Snow and Harris 1991).

In equine muscle, glycogen is found in higher quantities than in human muscle: 550-600 mmol/kg dry weight in equine muscle vs. 300-400 mmol/kg dry weight in human muscle (Essén and Henriksson 1974; Essén-Gustavsson et al. 1984; Snow et al. 1985; Snow and Harris 1991; Hultman and Greenhaff 1993).

A frequent problem encountered in studies of muscle glycogen is the variance between different sampling sites. Specific variance due to site can probably be attributed to fibre variation; type I fibres have a lower glycogen content than type II fibres, and types IIa and IIb have similar levels in horses (White and Snow 1987). On the other hand, if frequent samples are taken close to the previous sample to get an equal fibre distribution, another problem may arise. Because the muscle biopsy is traumatic, the biopsy itself may induce inflammation similar to that resulting from exercise, and if the muscle biopsies are not obtained from a site some distance away from the previous biopsy, there may be an effect of the biopsy on muscle glycogen (Costill et al. 1988a).

Muscle glycogen concentration may also be affected by muscle damage caused by intensive exercise. Usually, the exertional muscle damage is a focal micro-injury, including myofibrillar disruption and mitochondrial changes to a small portion of the fibres in skeletal muscle. Signs may be subclinical, such as a decrease in muscle respiratory capacity and force output. In a more serious case of damaged muscle fibre segments, soreness and stiffness may

be detectable immediately after exercise or 1-3 days after exercise (McEwen and Hulland 1986; Lindholm 1987).

Signs of regeneration usually occur on the 4th day after onset of myopathy. In cases of damaged muscle fibre segments, regeneration occurs rapidly and efficiently from myogenic precursor cells. The predominant source of these is the satellite cells that lie between the sarcolemma and the basement membrane of each fibre. When activated, these cells proliferate rapidly to produce myoblasts that fuse with pre-existing fibres and with one another to replace part or all of the damaged fibres. The normal glycolytic activity of damaged cells returns within 8 days and normal mitochondrial activity within 14 days (Armstrong et al. 1991; Duguez et al. 2002; Partridge 2002).

2.3.5 Muscle glycogen concentration and training

Humans, who exercise regularly, generally have higher muscle glycogen levels than their sedentary counterparts (Greiwe et al. 1999). Lindholm and Piehl (1974) also found significantly higher muscle glycogen concentrations in adult, trained Standardbred horses than in young, untrained Standardbreds, with average figures of 126 and 95 mmol glucose units/kg wet muscle, respectively. Guy and Snow (1977) reported an average increase of 33% in glycogen concentration of six limb muscles after a 10-week training programme involving both aerobic and anaerobic work. In horses undergoing daily intensive training for 5 weeks, glycogen levels decreased slightly after 1-2 weeks of training, remained low during the training period and increased to pretraining levels after one week's cessation of training (Essén-Gustavsson et al. 1989). Geor and co-workers (1999) found no effect of 10-day moderate-intensity exercise in resting muscle glycogen content, but reported a 15% increase in untrained horses after a 6 week moderate-intensity training protocol in another study (Geor et al. 2002). Gansen and co-workers (1999) found 47% and 48% increases, in muscle glycogen concentration after conditioning untrained 2-year-old Haflingers for 6 weeks with exercise of 45-min duration at their individual $V_{La1.5}$ and $V_{La2.5}$, respectively, but exercising horses at their V_{La4} for 25 min did not reveal a measurable effect on muscle glycogen concentration. Based on these data, an increase in muscle glycogen concentration can be concluded to require longer lasting exercise sessions at relatively low intensity over a fairly long period.

2.3.6 Muscle glycogen concentration and exercise performance

Studies of prolonged exercise on successive days in humans have shown a day-by-day reduction in muscle glycogen (Costill et al. 1971). Especially when the exercise is sufficiently severe to cause muscle damage, the time needed for glycogen repletion may be prolonged. For example, low muscle

glycogen concentrations were observed during the 7 days after a marathon, when subjects rested or engaged in short, low-intensity exercise (Sherman et al. 1983, Hikida et al. 1983).

In one study conducted on swimmers, the group who had the lowest muscle glycogen values experienced the greatest difficulty in training (Costill et al. 1988b). Thus suboptimal muscle glycogen content at the onset of endurance exercise is likely to lead to early fatigue. In fact, studies in man have shown that endurance capacity at work intensities of 60-85% VO_{2max} is directly related to the glycogen content of working muscles (Bergström et al. 1967: Hermansen et al. 1967), and exercise has to be stopped or the intensity must be significantly reduced when muscle glycogen reaches low levels, <50 mmol/kg wet weight (Saltin and Karlsson 1971).

In addition, exercise above the anaerobic threshold is potentially limited by the availability of carbohydrate reserves. In humans, whether pre-exercise muscle glycogen concentration was normal (364 mmol/kg dry muscle) or high (568 mmol/kg dry muscle), the amount of glycogen degraded over a 105-s supramaximal exercise bout was identical (Vandenberghe et al. 1995). However, with a glycogen content of <40 mmol/kg dry muscle, the muscles' capacity to produce lactate was compromised and exercise performance was reduced (Jacobs 1981). Thus, provided that pre-exercise glycogen levels are not extremely low during short-term intense exercise, exhaustion often precedes glycogen depletion (Jacobs 1981; Vandenberghe et al. 1995), with ionic disturbances over the cell membrane, decreased function of the sarcoplasmatic reticulum and intracellular accumulation of metabolic end products of glycogen, including lactate, instead accounting for fatigue (Fitts 1994).

Also for horses performing strenuous exercise on consecutive days, a progressive depletion of glycogen stores may become a problem, because even after a modest muscle glycogen depletion (19-25% reduction in muscle glycogen content) in Thoroughbred horses on a normal diet of hay and cubes, glycogen was restored fully only after 2-3 days (Snow and Harris 1991).

Effects of low glycogen levels in horses resemble those in humans. Moderate depletion of glycogen stores does not seem to have a significant impact on high-intensity exercise capacity, especially because glycogen is found in large quantities in well-trained equine muscle. In one study, muscle glycogen concentration in the horse was reduced by 22% without having a significant effect on physical work capacity during high-intensity exercise (Davie et al. 1996). In another study, Davie and co-workers (1999) concluded that 20-30% depletion of glycogen concentration in the middle gluteal muscle resulted in a shift towards fat metabolism, but did not significantly affect heart rate, oxygen uptake or concentrations of plasma glucose and lactate during-moderate intensity exercise. But if the glycogen stores are severely depleted prior to

exercise, a decrease in exercise capacity in both endurance type and short high-intensity exercise will occur. Topliff and co-workers (1985) reported a decrease in the relative work capacity of a horse pulling a sled when pre-exercise muscle glycogen concentration was reduced by approximately 41%. Lacombe and co-workers (1999) described a 55% reduction in muscle glycogen concentration (~55 mmol/kg, wet weight, before trial) to result in impairment of anaerobic, but not aerobic metabolism, in horses running at a speed requiring 120% VO_{2max}.

2.3.7 Muscle glycogen concentration and carbohydrate loading

In human athletes, an effective muscle glycogen loading programme may increase muscle glycogen concentration up to 1.9-fold higher than normal resting levels (Ahlborg et al. 1967; Bergström et al. 1967). Glycogen supercompensation programmes, which are used for performance enhancement in prolonged exercise, classically consist of significant depletion of muscle glycogen, usually by exercise, followed by consumption of a high-carbohydrate diet for several days (Bergström 1967; Ahlborg et al. 1967). Successful glycogen supercompensation will not enable an athlete to exercise at a faster pace, but may improve the athlete's ability to maintain an optimal pace for longer (Bergström et al. 1967; Costill 1972; Madsen et al. 1990). For muscle glycogen loading to be effective, the exercise should last longer than 90 min (Sherman et al. 1981).

In the period after prolonged and heavy exercise, glycogen synthesis is of high priority for the previously exercised muscles. The repletion process is biphasic, exhibiting a rapid early phase (< 24 h), during which muscle glycogen returns to normal levels, and a slow phase lasting for several days during which the supercompensated levels can be reached (Price et al. 1994a). Glycogen resynthesis is first generated in proglycogen, which is the predominant form until glycogen returns to normal resting levels, and the later increase, and ultimately the supercompensation in total muscle glycogen, occurs predominantly in macroglycogen (Adamo et al. 1998).

Generally, this glycogen supercompensation response has been attributed to an increase in glycogen synthase activity in the post-exercise period and an exercise-induced increase in muscle insulin sensitivity that persists for a variable time after exercise (Bergström and Hultman 1966; Richter et al. 2001). The activation of glycogen synthase that is present in the glycogen-depleted state after exercise reverses before glycogen supercompensation occurs, approximately when muscle glycogen returns to normal levels at the end of the rapid early phase (Bergström et al. 1972; Conlee et al. 1978). Burstein and coworkers (1985) have shown that the exercise-induced increase in muscle insulin sensitivity could be associated with insulin receptor changes. Naka-

tani and co-workers (1997) reported that exercise training-induced increases in muscle GLUT-4 and hexokinase play an important role in the enhancement of glycogen supercompensation in a trained state.

In horses, the results regarding muscle glycogen supercompensation are not conclusive. Toppliff et al. (1983, 1985) found an increase of approximately 50% in muscle glycogen concentration by feeding a low-carbohydrate diet during exhaustive, high-intensity exercise for 5 days, followed by a high-carbohydrate diet during a 3-day repletion period. However, Snow (1992) failed to demonstrate a glycogen overload in horses after a 40-50% depletion of glycogen stores and a 3- to 4-day carbohydrate-rich diet. Even after a high-carbohydrate diet for 5 weeks, Essén-Gustavsson and coworkers (1991) reported only a modest 12% increase in muscle glycogen concentration, which agrees with the findings of Kline and Albert (1981). Although these results are somewhat conflicting, in horses muscle glycogen supercompensation appears not to be possible to the same extent as in humans.

Furthermore, in horses, attempts at carbohydrate loading have the potential to create serious problems. In addition to intestinal problems with attendant disorders, such as laminitis and colic, muscle problems can occur. Early research by Carlström (1932) implicated excessive glycogen storage with a high-carbohydrate diet during a period of respite from exercise, and when exercise was recommenced, subsequent rapid glycogenolysis, resulting in lactic acidosis and thereby triggering exertional rhabdomyolysis in draught horses. Although there is often an increase in the incidence of exercise-associated myopathy in race and performance horses following a period of rest when fed full rations, the mechanism for induction of myopathy remains elusive (Harris and Snow 1986; Valberg et al. 1999).

MacLeay and co-workers (1999b) found no significant differences in muscle glycogen concentrations in Thoroughbred horses fed a high-carbohydrate diet compared with a low-carbohydrate diet, but they did observe increased nervousness and excitability of horses consuming the high-carbohydrate diet compared with the low-carbohydrate and high-fat diets. The increased excitability and anxiety could have contributed to the onset of recurrent exertional rhabdomyolysis in these horses. Lindholm and Piehl (1974) have suggested that hypoxia could be an important contributer to the problem. Anxiety could contribute to hypoxia by increasing muscle tension so that muscles are not properly relaxed between the contractions, and therefore, venous return from the muscle decreases. Another potential cause for vasoconstriction could be hypokalaemia (Stuck and Reinertson 1987). An abnormality in intracellular calcium regulation and malignant hyperthermia have also been suggested as possible aetiologies for exertional rhabdomyolysis (MacLeay et al. 1999a; Valberg et al. 1999).

Polysaccharide storage myopathy is an inherited glycogen storage disorder that predisposes horses to muscle problems (Valberg et al. 1993; 1999). In addition to muscle glycogen concentration being 150 mmol/kg dry weight higher than normal in affected horses, the structure of glycogen is abnormal (Valberg et al. 1993; 1999). These horses have also shown enhanced cellular uptake of glucose that may be, in part, caused by an increased sensitivity to insulin (De La Corte et al. 1999). This inherited glycogen storage disorder has been found to be specific to Quarter Horses, draft breeds and related breeds, with only incidental findings of accumulation of abnormal glycogen-related polysaccharide inclusions within the skeletal muscle fibres of other breeds (Valberg et al. 1999; Valentine et al. 2000).

2.3.8 Muscle glycogen concentration and a fat-supplemented diet

The basic horse diet of grass and the modified diets of oats and hay are normally very low (1-5%) in fat, although some fat is essential for absorption of fat-soluble vitamins and as a source of the essential fatty acid linoleic acid (Meyer 1987). Added fat, primarily in the form of vegetable oils, may be used in a horse´s diet to improve performance, maintain weight, improve coat and skin condition or increase the energy density of the diet (Meyers et al. 1989; Clarke et al. 1990; Oldham et al. 1990; Kronfeld 1996). However, the fat-rich diets fed to horses are never as extreme as those provided to human or rat subjects.

Vegetable oils are both highly palatable and digestible, provided the added fat is introduced gradually (Kronfeld et al. 2004). Following rapid introduction of fat, signs of impaired fermentation in the equine large intestine may appear, including greasy and abundant stools that can become greyish, loose and deteriorate into frank steatorrhoea (Kronfeld et al. 2004). Dietary fat is digested primarily in the small intestine. Hepatic bile promotes emulsification, which increases the fat-to-water interface so that lipase can more readily hydrolyse fats to fatty acids and monoglycerides, which are readily absorbed. Adaptation to fat could be due to increased bile production and to exocrine pancreatic secretion of lipase to enhance the capacity for emulsification, hydrolysis, micelle formation and absorption (Manas et al. 1996). However, feeding a high-fat ration (8%) to horses resulted in no changes in the activity of pancreatic lipase compared with a low-fat ration (Landes and Meyer 1998).

Fats can be metabolized aerobically through a relatively slow process to produce ATP, or if they are excessive with regard to the horse´s immediate energy requirements they are stored in adipose tissues. During exercise, triglycerides stored in adipose tissue are hydrolysed, and in blood free fatty acids are bound to albumin for transport to working muscles (Coyle 1995).

Fat may be added to the diet of athletic horses because of the reputed benefits to horses suffering from chronic muscle problems. However, findings of the effect on muscle glycogen have not been consistent. In most studies, a significant increase in muscle glycogen content (up to 55%) has been reported after feeding dry matter with 5-12% fat (Meyers et al. 1989; Oldham et al. 1990; Harkins et al. 1992; Jones et al. 1992; Scott et al. 1992; Hughes et al. 1995; Julen et al. 1995). When more than 15% fat was added to the total diet, muscle glycogen stores have been reported to decrease by almost 15% (Pagan et al. 1987). Since most diets are supplemented at a rate no more than 10% by weight or vegetable oil up to 600 ml/day, a significant decrease in muscle glycogen is not likely to occur (McKenzie et al. 2002). Other potential mechanisms for the reputed benefits of decreasing starch and increasing fat for horses with chronic muscle problems, such as neurohormonal changes that result in a calmer demeanor, have therefore been suggested (Holland et al. 1996).

2.3.9 Muscle glycogen concentration and water intake

Even though branching is designed to make the glycogen molecule compact, it is still a polar molecule and thus must be stored with associated water. Every gram of muscle glycogen is stored with 3-5 g of water (Bridge and Bridges 1932; Olsson and Saltin 1969; 1970). Total muscle glycogen stores have been estimated to be 4.7 kg in a 500-kg horse (McMiken 1983). This would mean that 14-23 litres of water is needed for the storage of total muscle glycogen.

During exercise, a significant amount of energy is lost from each chemical reaction in the form of heat. For the horse to remain healthy and continue to exercise, excess heat must be dissipated from the body. The main route of heat dissipation in the horse is evaporation of sweat, and therefore, prolonged exercise, especially when performed in hot and humid conditions, will lead to substantial water and electrolyte losses in addition to the depletion of muscle glycogen stores.

Activation of hypothalamic osmoreceptors stimulates the thirst mechanism and, given sufficient time, drinking water and consumption of adequate amounts of electrolytes will restore exercise-induced losses. In addition, changes in post-exertional renal function will help to restore the fluid and electrolyte balances.

Antidiuretic hormone (ADH) is released in response to an increase in blood osmolality, which increases as a result of dehydration. ADH is the most important determinant of water excretion, inducing the kidney to retain water and not returning to prehydration levels until osmolality returns to normal (Houpt et al. 1989; Sneddon et al. 1993).

Aldosterone, the principal mineralocorticoid from the adrenal cortex, is released in response to a decrease in renal blood flow, an increase in plasma potassium concentration, a decrease in plasma sodium concentration and an increase in plasma hydrogen ion concentration (McKeever and Hinchcliff 1995). Aldosterone causes sodium to be conserved in the extracellular fluid, while more potassium is excreted into the urine (Jansson et al. 2002). With receptors also in the colonic epithelial cells, aldosterone may enhance sodium and chloride absorption across the equine colon wall, thereby facilitating colonic fluid absorption (Turnamian and Binder 1989; Clarke et al. 1992).

2.3.10 Muscle glycogen concentration and catabolic/ anabolic balance

Body metabolism is the balance of multiple hormone regulation systems in response to such varying metabolic states as post-exercise glycogen repletion, muscle damage repair and adaptation so that future bouts of exercise of a similar type, intensity and duration will cause less injury to the muscle.

Insulin, besides controlling glucose uptake into skeletal muscle, is one of the most potent anabolic hormones and enhances the uptake of amino acids by the muscles as well as their incorporation into protein (Jefferson et al. 1972). The concentration of insulin is slightly decreased at the end of exercise, mainly because catecholamines inhibit its secretion from the pancreas. But the plasma half-life of catecholamines is short, less than 30 s, and within a few minutes of cessation of exercise concentrations have returned to their resting levels (Snow et al. 1992; Kokkonen et al. 2002), and within 1 h the insulin concentrations rise above resting levels (Pagan and Harris 1999).

Testosterone increases the deposition of protein in tissues throughout the body, including an increase in the contractile proteins of muscles and an increase in lipolytic activity (Kendrick and Ellis 1991). It is secreted from the adrenal cortex, and, in stallions, from the testicles. It exerts its effect through an intracellular receptor which, when bound to testosterone, acts as a regulator of gene activity. In geldings, Golland et al. (1999) found an increase in plasma testosterone in response to maximal exercise, and observed that exercise disrupted the basal plasma concentrations of testosterone for 26-32 h post-exercise. In rats castration has been shown to lead to decreased glycogen stores accompanied by diminished glycogen synthase activity and increased glycogen phosphorylase activity (Ramamani et al. 1999), and testosterone potentiates a training-induced increase in muscle glycogen stores (van Breda et al. 2003).

Anabolic steroids, including nandrolone, are synthetic derivatives of testosterone that possess the anabolic effects of testosterone, while having less

androgenic effects. They have been used in the treatment of various catabolic conditions, but also by athletes hoping to enhance their physical performance.

Growth hormone, also known as somatotropin, increases the rate of protein synthesis, conserves carbohydrate, and increases the use of fatty acids for energy in most tissues of the body. It is secreted by the anterior pituitary gland. Growth hormone exerts most of its effects through intermediate substances called insulin-like growth factors (IGF) or somatomedins. Plasma growth hormone concentration can increase 8- to 10-fold after 5 min of acute physical exercise (Thompson et al. 1992). Furthermore, maximal exercise can disrupt basal plasma concentrations of growth hormone for up to 24 h after exercise (Golland et al. 1999).

Normal levels of thyroid hormones increase metabolism in almost all cells of the body. They may also enhance the release of growth hormone and insulin-like growth factors. If adequate substrates for energy are available, thyroid hormones can enhance the rate of protein synthesis. If, however, carbohydrates and fats are insufficient for energy, thyroid hormones cause rapid degradation of protein for energy. Furthermore, hyperthyroidism may be catabolic to proteins and accelerate bone loss in adults. According to Gonzáles et al. (1998), the levels of triiodothyronine (T3), but not thyroxin (T4), increased 5 min after a maximal speed race for 1200 m.

Cortisol has catabolic effects on muscle. During exercise, the main metabolic effect of cortisol is to increase hepatic gluconeogenesis and to promote lipolysis, which provides fuel for prolonged submaximal exercise. The degree of increase in plasma cortisol concentration appears to better reflect duration of workload rather than work intensity. For example, Linden and co-workers (1991) found elevated levels of plasma cortisol in endurance riding, and a lesser increase in show-jumping than in 3-day cross-country eventing, galloping or trotting. During brief exertion, cortisol was unrelated to work intensity, or to blood lactate concentration, and maximum plasma concentration was observed 5-30 min after the end of a short high-intensity exercise (Jimenez et al. 1998; Nagata et al. 1999). Normal basal cortisol secretion was restored within 24 h after an exercise-induced increase (Golland et al. 1999).

Moreover, glucagon can have catabolic effects by increasing hepatic amino acid uptake and protein degradation for gluconeogenesis. It is likely that, by this diversion of amino acids, glucagon diminishes the precursor pool of amino acids available for protein synthesis, thus inhibiting the amino acid-induced stimulation of protein synthesis (Charlton et al. 1996).

Overtraining

An elite sport requires a high commitment to training and overtraining is, therefore, a frequent concern. The physiological features of overtraining are, however, poorly understood. Because both anabolic and catabolic hormones

have a significant effect on protein synthesis and degradation, mistakes in exercise regimes can result in greater catabolic effects. Disorders in hormonal regulation at the pituitary-hypothalamus level, adrenal exhaustion, with consequent reduction in blood cortisol and down-regulation of peripheral and, perhaps, central beta-adrenergic receptors, have all been attributed to overtraining (Urhausen et al. 1995; Budgett 1998; Lehmann et al. 1998). A catabolic state due to excessive training may also be a cause of hypothyroidism (Sojka 1993). Nevertheless, no single hormonal or metabolic change occurres with sufficient consistency to identify individual horses, which have been overtrained; for example, both an increase and a decrease in adrenocortical response to ACTH administration, have been reported (Bruin et al. 1994; Golland et al. 1999; Tyler-McGowan et al. 1999).

3 Aims of the study

The objectives of this study were as follows:

- to determine the rate of glycogen resynthesis and measure changes in plasma metabolite concentrations in Standardbred trotters before and after repeated bouts of exercise (Study I).
- to investigate the effects of insulin and a glucose precursor on repletion of muscle glycogen (Study II).
- to compare the effects of a glucose-electrolyte solution and water on rehydration and muscle glycogen repletion after exercise (Studies II and III).
- to determine whether fat supplementation improves post-exercise repletion of muscle glycogen (Study IV).
- to evaluate whether increased anabolic hormonal balance of the athlete affects post-exercise repletion of muscle glycogen (Study V).

4 Materials and methods

The study protocols were approved by the Ethics Committee for Animal Experiments at the Agricultural Research Centre, Jokioinen, Finland.

For a more detailed account of Materials and Methods, see Studies I-V.

4.1 Horses

Study I

Six Standardbred trotters (2 stallions, 3 geldings and 1 mare) 3-10 years old were used. All horses had been in regular training for several months, and at the time of the study all were in condition to participate in a trotting race.

Studies II, III and V

Nine half-bred riding horses (4 geldings and 5 mares), 6-12 years old were used in Studies II and III. Six of these horses (3 geldings and 3 mares) continued in Study V. All horses were trained for at least 1 year to perform in a 3-day event.

Study IV

Twelve Finnhorse mares 4-14 years old were used. All horses had a 3-month adjustment period with regular exercise before the trial period.

4.2 Work tests, muscle and blood samples

During the treadmill tests, heart rate was monitored continuously with the use of a pulse metre (Horse Tester PEH200, Polar Electro Co., Kempele, Finland). Heart rate was recorded when a steady state was reached within the last 20 s of each level of exercise.

All biopsies were taken at indicated time points by using the method of Lindholm and Piehl (1974) from the middle gluteal muscle under local anaesthesia using a biopsy needle (diameter 5 mm). Samples were immediately frozen in liquid nitrogen and stored at -70°C until analysed.

Blood samples were taken at indicated time points through a catheter placed in the jugular vein prior to the test.

Submaximal exercise test (SET) on treadmill (Studies II, IV and V)

The SET consisted of a 10-min warm-up period at 1.7 m/s, followed by 4 exercise intervals, 2 min each, at speeds of 5, 6, 7 and 8 m/s on a high-speed treadmill with a 3.5^{o} incline.

Blood samples were collected before the test, during the last 10 s of each exercise speed.

Competition exercise test (CET) on treadmill (Studies II, III and V)

The CET was designed to simulate the Endurance Test of a 3-day event (Marlin et al. 1994). The horses first walked for 10 min (1.7 m/s) and then trotted for 10 min (3.7 m/s). After this, there was a 2-min gallop (10.0 m/s), followed by a 20-min trot (3.7 m/s) and a 10-min walk (1.7 m/s). This was followed by an 8-min canter (8.0 m/s) and a 30-min walk (1.7 m/s). The incline of the treadmill was 3°, except for the last 30-min walk, which was performed at a 0° incline.

Horses were weighed before and immediately after the CET and on the following day 22.5 h after the CET.

Muscle samples were taken before the test, immediately after the test and 22.5 h after the CET. Blood samples were collected before the test, during the last 30 s of each exercise speed and 0.5, 1, 2, 4, 6 and 22.5 h after the CET.

Horses were given the test solutions [isotonic glucose-electrolyte (GE) solution, GE supplemented with leucine, GE supplemented with propionic acid or water] via a nasogastric tube 30 min after the CET.

Exercise test (ET) on the treadmill (Study IV)

In the ET, the horses walked for 5 min (1.7 m/s) and then trotted for 11 min at 4 m/s and 4 min at 7 m/s. This was repeated 3 times and followed by a 10-min final walk (1.7 m/s). The incline of the treadmill was 3°.

Blood samples were collected before the ET, during the last 10 s of the first and third trot at 7 m/s and 1, 2, 3, 4, 5, 6 and 23 h after the ET. Muscle biopsies were taken before, immediately after and 23 h after the ET.

Race track exercise (Study I)

On day one of the study, horses trotted 3 times on a race track with 1-hour rest periods between exercise bouts. During the rest period, horses walked for 15 min and then stood in a shelter. Distances trotted were 3000, 3000 and 2000 m and the speed was increased for each successive heat. Exercise was

repeated on day 4 of the study, when the distances were 2100, 2100 and 1600 m. Speed was adjusted so that it was equivalent for horses on the basis of training records. Trotting speed was kept steady throughout each exertion. In the last exercise bout of 1600 m on day 4, the horses trotted as fast as possible.

Blood samples and muscle biopsies were obtained before each trial and 0, 4, 24, 48 and 72 h after the third exercise bout. Blood samples were also taken between exercise bouts.

4.3 Analysis of muscle samples

Histochemical analysis of fibre area and relative glycogen content (Studies I and II)

Transverse 20-µm-thick slices were cut in a cryostat at -20°C from the muscle sample taken immediately after CET, and stained with periodic acid-Schiff dye for glycogen (Pearse 1961). The fibre area and the relative glycogen content were assayed with a computer-directed image analyser (ScanBeam, Hadsund, Denmark).

Muscle glycogen content (Studies I, II, IV and V)

For the analysis of muscle glycogen content, muscle samples were freeze-dried and then dissected free of visible blood, fat and connective tissue; 1-2 mg of muscle tissue was homogenized, and the glycogen was hydrolysed in 1 M HCL for 2 h at 100°C (Essén and Henriksson 1974). The concentration of glucose was analysed by use of a commercially available kit (Glu MPR2 1442449, Boehringer Mannheim, Mannheim, Germany).

Muscle triglycerides (Study IV)

Muscle triglycerides were measured from 1-2 mg of freeze-dried muscle as described by Essén et al. (1975).

Citrate synthase and 3-hydroxyacyl-Coa dehydrogenase (Studies II and V)

The activity of citrate synthase (CS) as a marker for oxidative capacity and 3-hydroxyacyl-CoA dehydrogenase (HAD) as a marker for ß-oxidation were determined from muscle homogenates according to Essén et al. (1975).

4.4 Analysis of blood and plasma samples

For lactate analysis, blood was transferred to tubes containing EDTA and sodium fluoride. For the analysis of insulin and glucagon, blood samples were taken into EDTA tubes containing 1 TIU of a protease inhibitor (Aprotinin, Sigma Chemical Co., St. Louis, MO, USA). For other analyses, heparinized tubes were used. Plasma was separated by centrifugation and stored at –70°C until analysis.

Analysis of metabolites from blood and plasma

Blood lactate concentrations were measured with an enzymatic lactate analyser (YSI 2300 STAT, Yellow Springs Instrument Co., Yellow Springs, OH, USA).

Exercise heart rate causing a blood lactate level of 4 mmol/l (P_{La4}) was calculated from the velocity of the treadmill, heart rate and blood lactate concentration in the SET (Persson 1983).

Concentrations of plasma glucose, non-esterified fatty acids (NEFA), triglycerides, cholesterol and β-OH-butyrate were analysed with an automatic analyser (Kone Specific, Espoo, Finland) by standard methods (Trinder 1969; Wahlefeld 1974; Shimizu et al. 1980; Soveri et al. 1992). For the analysis of glycerol, a plasma sample was first deproteinized with 0.6 M perchloric acid, and the concentration of glycerol was analysed from the neutralized supernatant by using an enzymatic method (Eggstein and Kreutz 1966).

Analysis of hormone concentrations (Studies II, III and V)

A radioimmunoassay was used to measure plasma erytropoietin, cortisol, insulin, glucagon, aldosterone and arginine vasopressin concentrations (Erythropoietin, Diagnostic Systems Laboratories Inc., Webster, TX, USA; Cortisol Radio Immunoassay Kit, Orion Diagnostica, Espoo, Finland; Coat-A-Count Insulin, Diagnostic Products Corporation, Los Angeles, CA, USA; Double Antibody Glucagon, Diagnostic Products Corporation, Los Angeles, CA, USA; Coat-A-Count Aldosterone, Diagnostic Products Corporation, Los Angeles, CA, USA; Vasopressin Rapid, Bahlmann Laboratories AG, Allschwil, Switzerland).

Analysis of electrolyte concentrations (Study III)

Plasma concentration of sodium, potassium and chloride were analysed using ion selective electrodes (Microlyte Ion Selective Analyzer, Kone Ltd., Espoo, Finland). Calcium and magnesium were determined by atomic absorption

(Perkin-Elmer 2380 Atomic Absorption Spectrophotometer, Perkin-Elmer Co., Norwalk, CT, USA).

Plasma osmolality and plasma protein concentration (Study III)

Plasma osmolality was measured by the freezing point depression method with an osmometer (Osmette A, Model 5002, Precision Systems, Inc., Sudbury, MA, USA). Plasma protein was analysed using the biuret method (Weichselbaum 1946).

Nandrolone (Study V)

Plasma concentrations of nandrolone laurate were measured with an ELISA assay (19-nortestosterone ELISA Kit, Eurokit S.r.l., Gorizia, Italy).

Plasma volume and red cell volume (Studies III, IV and V)

Plasma volume (PV) and red cell volume (CV) were determined using a dye dilution method (Evans blue; Persson 1967).

4.5 Statistical analysis

All results are expressed as mean ± standard error. Significance of differences within each group was tested with repeated measures analysis of variance. When any overall effect was noted, comparisons between the groups were performed using Student's *t* test, and comparisons between trials were tested by paired *t* test. In Study V, to compare the curves of the plasma parameters measured during and after exercise, areas under the curve were calculated. Correlations between variables were tested using linear regression analysis. Differences were regarded as significant at $P<0.05$.

5 Results

The main results of Studies I-V are presented in the following section. More detailed data are available in the original publications.

Rate of glycogen resynthesis and changes in plasma metabolite concentrations in Standardbred trotters before and after repeated bouts of exercise (Study I)

Muscle glycogen content in biopsy specimens obtained immediately after the third exercise bout of trials A and B was significantly lower than values before exercise, and a further decrease was found in biopsy specimens obtained 4 h after the third exercise bout. Resynthesis of glycogen was negligible during the first 24 h after exercise in both trials. In trial A, muscle glycogen content on day 4 had returned to before-exercise values. In trial B, however, muscle glycogen content on day 4 continued to be significantly lower than before-exercise content.

Plasma glucose concentration increased slightly after each exercise bout during trial A, and somewhat higher increases were observed during trial B, when the intensity of exercise was greater, but no significant difference was found between the two trials. Within 1 h after exercise in both trials, plasma glucose concentrations had returned to before-exercise values.

Concentrations of plasma NEFA after exercise were significantly higher during trial A than during trial B ($P<0.01$). During trial A, the increases in NEFA concentrations after all 3 exercise bouts were significantly ($P<0.01$) higher than concentrations before exercise, and concentrations returned to before-exercise values between exercise bouts. During trial B, the intensity of exercise was greater and the increase in plasma NEFA concentrations was not significantly different from before-exercise values. Low NEFA concentration was measured 2-48 h after exercise in trial A and 2-72 h after exercise in trial B.

In trials A and B, a significant difference was present between plasma glycerol concentrations before exercise and after each exercise bout ($P<0.01$, except $P<0.001$ for the last heat of trial B). Glycerol concentrations also remained increased during the 1-h rest periods between exercise bouts. Two hours after the third exercise bout of trial B, plasma glycerol concentration decreased to less than before-exercise values ($P<0.05$) and remained low ($P<0.05$) until the end of the study. In trial A, changes in plasma glycerol concentrations were similar, but not statistically significant.

Significant increases in plasma TG concentrations were measured after each exercise bout during trial B ($P<0.05$ for the first 2 exercise bouts, and $P<0.01$

for the third exercise bout). In both trials, plasma TG concentrations at 2-24 h after exercise were significantly ($P<0.01$, except $p<0.05$ for trial B, 24 h after exercise) lower than the values before exercise.

Effects of glucose and a glucose precursor on repletion of muscle glycogen (Study II)

During CET, concentrations of insulin were not significantly changed in any of the groups. During the recovery period, 1-4 h after CET, insulin concentration was increased in the glucose-electrolyte (GE) and GE-leucine (GEL) groups ($P<0.05$ and $P<0.001$), whereas no increases were observed in the GE-propionic acid (GEP) group. Administration of leucine together with GE caused a higher ($P<0.001$) increase than GE alone.

Concentration of glucagon increased during CET in all groups ($P<0.01$), and decreased rapidly to the basal level in all groups. In all groups, glucagon concentration was again above the respective resting level 6 and 22.5 h after CET ($P<0.01$).

Within each group, plasma glucose concentration increased ($P<0.001$) during CET, and remained high during the first 2 h of recovery. Administration of GE solutions caused no significant increase in plasma glucose concentration. In the GEL group, glucose concentration at 6 h was lower ($P<0.01$) than in the GE group, and also the disappearance of glucose, calculated from the concentration difference between 0.5 h after solutions (1-h sample) and the 6-h sample, was faster in this group than in the other two groups.

During CET, plasma NEFA concentrations were elevated at low-intensity phases and decreased during high-intensity phases in all groups. Basal concentration was reached in 2 h after exercise. During recovery, 6 h after CET, plasma NEFA concentrations were below resting level in GE and GEL groups, but not in the GEP group. In all groups, NEFA concentrations were higher ($P<0.05$) than pre-exercise values measured 22.5 h after CET.

In all groups, plasma glycerol concentrations increased during CET ($P<0.001$), and decreased linearly afterwards. After administration of the rehydration solutions in the GEL group, the decrease in the glycerol concentration, i.e. the difference between 0.5- and 1.0-h samples, was greater ($P<0.05$) than in the other two groups.

Plasma TG concentrations increased in all groups during CET ($P<0.05$) and decreased to the pre-exercise level within 1 h after CET.

In all trials, glycogen content 22.5 h after CET was still significantly lower ($P<0.001$) than the respective concentrations before CET, but no statistically significant differences appeared between the groups.

Effects of glucose-electrolyte solution and water on rehydration after exercise (Study III)

Mean body weight loss during the CET was -3.2% at the first trial, and similar values were recorded throughout the study. The horses did not regain their body weight during the 22.5 h after CET, and body weight loss at 22.5 h after the CET was -1.8% in the first trial, decreasing linearly with time ($P<0.05$), and was -0.8% in the fourth trial. In the fifth trial, when only water was given, body weight loss at 22.5 h after the CET was -2.5%, which was significantly greater than in the fourth trial ($P<0.01$).

The lowest values for voluntary water intake were recorded in the last trial, but differences between trials were not significant due to large individual variations.

Concentrations of magnesium and calcium remained unchanged during exercise and recovery. The exercise-induced increase in the concentration of potassium after the gallop ($P<0.001$) and canter ($P<0.001$) as well as the transient decrease ($P<0.001$) after the CET were similar at all trials. Concentrations of sodium increased slightly ($P<0.001$) during the gallop phase of the CET, whereas a significant decrease ($P<0.01$) was observed in samples taken after the low-intensity phases of the CET and during recovery. The decrease was significantly larger in the first trial than in the fourth and fifth trials ($P<0.05$). Concentrations of sodium were still below the resting value at 22.5 h after the CET ($P<0.01$). Water caused a significant decrease in sodium concentration ($P<0.001$), whereas no change was observed when GE solution was given. Changes in plasma chloride concentration resembled those in sodium. The exercise-induced decrease was attenuated from the changes measured in the first trial ($P<0.01$), and recovery was incomplete at 22.5 h after the CET. Water caused a significant decrease in chloride concentration ($P<0.01$), whereas a significant increase ($P<0.05$ in the 2nd and 3rd trials; $P<0.001$ in the 4th trial) was observed when GE solution was given.

A significant increase was found in plasma osmolality at the end of the gallop ($P<0.001$) and canter ($P<0.001$) during the CET. A decrease ($P<0.001$) was seen 30 min after water was given, but no change occured after providing GE solution.

Exercise increased ($P<0.001$) plasma protein concentrations, which returned to resting values in 22.5 h. A positive correlation was observed between plasma protein concentrations immediately after the CET and body weight loss during the CET ($r=0.722$, $P<0.001$), whereas no correlation was found between plasma protein concentration before the SET and body weight loss at 22.5 h after the CET. Exercise also increased aldosterone concentrations significantly ($P<0.001$), and concentrations remained elevated during the recovery period. The highest aldosterone concentrations were found 22.5 h

after the CET. A positive correlation was present between plasma protein and aldosterone concentrations 22.5 h after the CET (r=0.812, P<0.001), but no correlation was seen between body weight loss at 22.5 h after the CET and aldosterone concentration.

AVP increased significantly during the gallop (P<0.05) and canter (P<0.001) phases of the CET, and a positive linear correlation (r=0.509, P<0.001) between AVP and trial number was found at the end of the canter. In comparison with resting values, AVP concentrations were significantly (P<0.01) elevated 1.5 h after GE solution, but not after water.

A significant linear correlation (r=0.490, P<0.001) was observed between P_{La4} and BWL_2 when body weight loss at 22.5 h after the CET was greater than -1.2%.

Dehydration and muscle glycogen repletion (Studies II and III)

A significant linear correlation (r = -0.4495, P<0.01) was present between weight change and glycogen repletion (0 h vs. 22.5 h).

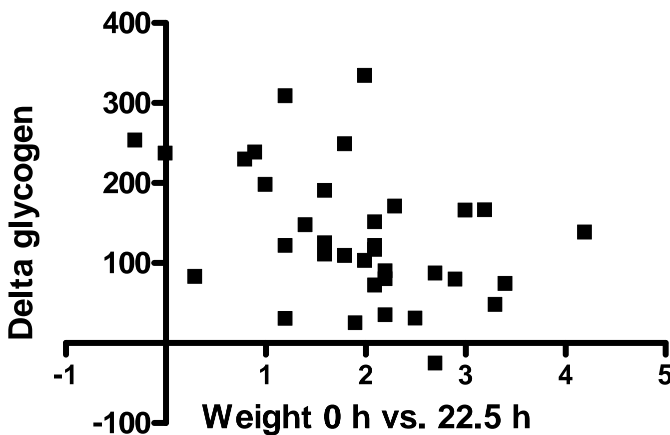


Figure 2. Relationship between weight difference (weight change relative to the pre-exercise weight expressed as a percentage) immediately and 22.5 h after CET (weight 0 h vs. 22.5 h), and the difference in muscle glycogen content (mmol/kg dry weight) immediately and 22.5 h after CET (delta glycogen).

Effects of fat supplementation on post-exercise repletion of muscle glycogen (Study IV)

Exercise test (ET) on the treadmill consisted of warm-up and cooling-down walks, and trotting at speeds of 4 and 7 m/s. At the end of each ET, plasma

insulin concentration was decreased (P<0.05), but within 1 h after the ET it had increased above the resting level (P<0.001). High insulin levels were measured 1 h after the first portion of the CHO-rich feed (trials B and C), but no further increase occurred after the second portion. In the fat groups (trials B and C), plasma insulin concentrations were lower than after CHO supplementation (P<0.01 and P<0.001, respectively). In trial B, plasma insulin was significantly lower 1 h after the first fat dose than at the same time point in trial C (P<0.001).

Plasma glucagon concentration was elevated by exercise (P<0.001). After fat adaptation, the plasma glucagon concentration stayed above the resting level throughout the recovery period (P<0.05).

The concentration of plasma glucose increased significantly during the ET (P<0.01), except in the fat-adapted horses (trial C), and returned to resting levels within 1 h after ET. In the fat-adapted group, plasma glucose was significantly lower (P<0.01) 1-4 h after feeding than in the respective CHO group.

At the end of ET, plasma NEFA, glycerol and TG concentrations were all elevated (P<0.05; P<0.001; P<0.001, respectively). Neither fat nor CHO feeding increased plasma concentrations of NEFA, glycerol or TG during the recovery period. In trial C, the concentrations of NEFA were lower than in the other trials in both the CHO and fat groups (P<0.001). After feeding, plasma glycerol and TG were the lowest in the fat group in trial C (P<0.05; P<0.05, respectively).

Exercise did not affect plasma cholesterol concentrations, but cholesterol concentrations were higher (P<0.001) after fat adaptation. Plasma ß-OH-butyrate concentration was affected neither by feeding nor exercise.

In all trials, muscle glycogen content after ET was 60.1 ± 1.9% of that before exercise. Fat adaptation affected neither muscle glycogen content at rest nor the use of muscle glycogen during ET, but a significant correlation appeared between muscle glycogen content before ET and the use of muscle glycogen during ET(r =0.607, P<0.01). On average, muscle glycogen 23 h after ET was 78.1 ± 2.6% of content before ET. The lowest 23-h post-exercise content of muscle glycogen concentrations was found in trial B in the fat group as compared with the other 23-h values in this group (P<0.05). When horses were adapted to fat, no difference was seen from trial A.

Muscle TG content before, immediately after and 23 h after ET varied widely (range 7-128 mmol/kg dry weight), with no significant changes present in any of the trials or in any of the groups.

Effect of increased anabolic hormonal balance of the equine athlete on post-exercise repletion of muscle glycogen (Study V)

The two weekly intramuscular injections of 1 mg/kg nandrolone laurate resulted in 0.60 ± 0.07 µg/l plasma nandrolone concentrations.

The CET, the simulated Endurance Test of a 3-day event, was used in this study.

During exercise, insulin concentrations tended to decrease, but returned to resting values at the end of exercise. In the AS trial ($P<0.01$), but not in the C trial, values of insulin were elevated as compared with those at rest for 2 h after exercise.

Glucagon was significantly higher during exercise in both trials ($P<0.01$). During recovery, in the AS trial, glucagon values stayed significantly elevated compared with values in the C trial (AUC, $P<0.05$).

In the AS trial, cortisol was lower than in the C trial during and 30 min after exercise in the CET (AUC, $P<0.05$).

Intensive exercise increased ($P<0.05$) plasma glucose concentrations, and in both trials they returned to resting levels 4 h after exercise.

In the CET, plasma NEFA concentrations were significantly higher at the end of the second 10-min walk and after the final 30-min walk ($P<0.001$ and $P<0.01$, respectively). The gallop and canter caused a decrease in plasma NEFA values. NEFA concentrations returned to resting values within 2 h after exercise.

In both trials, exercise increased plasma glycerol values ($P<0.001$), which returned to resting values within 2 h. Differences between the trials were not significant.

Triglyceride concentrations increased significantly more in the C trial than in the AS trial during exercise (AUC, $P<0.05$), in both trials returning to resting values within 30 min.

After the CET, muscle glycogen content in the AS trial did not differ from that in the C trial. However, repletion of muscle glycogen content during the 22.5 h following the CET was significantly ($P<0.01$) faster in the AS trial.

6 Discussion

6.1 Muscle glycogen stores over the training period

Several studies have described muscle glycogen depletion after different types/intensities of single exercise protocols (Essén-Gustavsson et al. 1984; Hodgson et al. 1984; Valberg 1986; Snow et al. 1987; Essén-Gustavsson et al. 1991), but less is known of muscle glycogen stores over the training period. Snow and Harris (1991) investigated muscle glycogen content of racing Thoroughbreds over a typical 11-day training period. To my knowledge, Study I is the first to describe muscle glycogen content in Standardbred trotters over a typical intensive training week.

The major finding of Study I was the slow rate of resynthesis of glycogen, indicating that Standardbred trotters may be progressively depleted in muscle glycogen stores during an intensive training period.

During the first 4 h after exercise, muscle glycogen content actually decreased, and the increase in glycogen content during the first 24 h after exercise was negligible. Several factors, such as animal species, intensity of exercise, gait and feeding during the recovery period, should be considered when comparing these results with the results of others. The lack of glycogen synthesis during the first 4 h of the recovery period is consistent with findings of earlier studies in horses (Snow et al. 1982; Simmon and Ford 1991; Davie et al. 1995), but contradicts results of studies in human beings and rats (Bergström and Hultman 1966; Fell et al. 1980; Casey et al. 1995), where glycogen resynthesis began immediately after exercise, especially when carbohydrate supplementation was provided. This species difference could be related to differences in the digestion of carbohydrates when administered as a glucose polymer by nasogastric tube or perhaps carbohydrate content of the diet does not influence the rate of glycogen repletion in the horse (Snow et al. 1987; Davie et al. 1994). The latter view is supported by the recent study by Jose-Cunilleras and co-workers (2006), where oral glucose availability had only a minimal effect on muscle glycogen replenishment after exercise, despite marked differences in availability of glucose to skeletal muscle.

Results of other studies in horses (Snow et al. 1982; Snow and Harris 1991; Davie et al. 1995) indicate that partial or full repletion of glycogen takes place within 24 h of an endurance ride, daily exercise for 1000-1600 m or high-intensity exercise on a treadmill. In Study I, however, repletion of glycogen was not observed. The results of studies in human beings (Bonen et al. 1985; Choi et al. 1994) indicate that the resynthesis of glycogen may be prevented by physical activity during the recovery period. Thus, in Study I the

repeated exercise bouts with 1-h intervals can be speculated to have delayed the onset of glycogen resynthesis. The pattern of glycogen repletion up to 48 h after exercise was similar for trials A and B, indicating that the repetition of exercise bouts rather than the exercise intensity may have been the dominant factor in the rate of glycogen resynthesis and could, at least partly, explain the discrepancy between this study and the other equine studies.

Signs of structural damage to the muscle fibre increase over three days following exercise, after which signs of muscle repair and regeneration are observed (Armstrong et al. 1983; Frieden et al. 1983). In humans, exercise-induced muscle damage has been shown to impair post-exercise glycogen resynthesis (Costill et al. 1990; Doule et al. 1993). Possibly exercise-induced alterations and damage to muscles affected the rate of muscle glycogen repletion also in Study I. This may partly explain the low glycogen content on day 3 after the second intensive exercise day, when the last exercise bout was performed at maximal speed.

In Studies II and III, the largest depletion of glycogen appeared in type I fibres. Studies in human athletes have shown that synthesis of glycogen may vary between different fibre types (Casey et al. 1995). The rate of post-exercise synthesis of glycogen should therefore be related to the intensity and duration of the exercise. In our studies muscle glycogen repletion was measured in whole muscle samples, not in individual muscle fibres.

Adamo and co-workers (1998) have demonstrated that in humans proglycogen was resynthesized far faster than macroglycogen. In horses, glycogen resynthesis seems to start in the macroglycogen pool (Bröjer et al. 2006). However, pro- and macroglycogen concentrations were not measured in our studies.

6.2 Muscle glycogen repletion and hormones

Intensity of exercise affects the post-exercise hormonal milieu, and strenuous or exhaustive exercise may result in more prolonged post-exercise catabolic hormone concentrations. For example, high cortisol levels after exercise may attenuate the anabolic responses of growth hormone, testosterone and insulin. Moreover, glucagon can have catabolic effects by increasing hepatic amino acid uptake and protein degradation for gluconeogenesis.

Study V provided evidence of the importance of athletes' maintaining a positive anabolic hormonal balance of the athlete. The results of Study I suggest that overreaching and negative hormonal balance may be explanations for the further depletion of glycogen stores during the 4-h post-exercise period.

Besides controlling glucose uptake into skeletal muscle, insulin is one of the most potent anabolic hormones and enhances the uptake of amino acids by the muscles, as well as their incorporation into protein (Jefferson et al. 1972). The other major anabolic hormones, i.e. growth hormone, insulin-like growth factor-1, and testosterone, were not measured in this study.

The concentration of insulin is slightly decreased at the end of exercise, mainly because catecholamines inhibit its secretion from the pancreas (Hunt and Ivy 2002). Within a few minutes after exercise, concentrations of catecholamines have returned to their resting levels (Snow et al. 1992; Kokkonen et al. 2002). This allows insulin concentrations to increase above resting levels within 1 h of exercise, as shown in Studies II-V.

Insulin concentration may be further increased by post-exercise feeding. Any increase in plasma insulin concentration may be beneficial for glucose uptake. However, an attempt to increase glucose uptake by muscles by raising plasma insulin concentration was not successful despite a significant leucine-stimulated insulin release and enhanced post-exercise blood glucose disappearance (Studies II and III). There was also no difference in these studies in glycogen repletion between GEL and GEP groups despite a significant difference in insulin concentration within 1-4 h of recovery. In Studies II and III, insulin levels were back to control values by 6 h after exercise. These results are consistent with the findings of Jose-Cunilleras and co-workers (2006).

Davie and co-workers (1995) were able to enhance the rate of glycogen resynthesis when they gave horses a large dose of dextrose intravenously over approximately 8 h starting 30 min after exercise. During the dextrose infusion, insulin levels and plasma glucose were significantly elevated. Furthermore, those horses were fed after completion of the infusion.

Taken together, these studies suggest that the duration of insulin elevation may be a crucial factor for glycogen repletion.

Both cortisol and glugacon have catabolic effects. In Study V, there was a significant increase in cortisol concentrations during and up to 6 h after CET. The restoration of normal basal cortisol after an exercise-induced increase may take up to 24 h (Golland et al. 1999). Glucagon concentration may be increased for more than 23 h after exercise, as shown in Studies II, III and V.

In Study V, short treatment with pharmacological doses of nandrolone was used to induce a better anabolic hormonal balance in the AS trial than in the C trial. The results show that this improved repletion of muscle glycogen stores after exercise, indicating that it was not the availability of glucose but the hormonal balance that favoured resynthesis of muscle glycogen. Cohen and Hickman (1987) found increased insulin resistance in humans using ana-

bolic steroids. There was no indication of this in the present experiment because no differences appeared in plasma glucose levels during the CET, and insulin levels rose significantly more than in the C trial only during the brief 2-h recovery period. In the AS trial, cortisol was the same or even lower than in the C trial during and 30 min after exercise in the CET. Glucagon increased during exercise in both trials, but remained significantly elevated during recovery in the AS trial only, suggesting an increased glucose output by the liver. Thus, insulin-independent glucose transport into the muscle cells may have been activated by the AS treatment because insulin was only transiently elevated after the CET, but glucagon was elevated during the whole 23-h recovery period.

Improved repletion of muscle glycogen stores after exercise in anabolic steroid-treated horses may enhance the ability of these individuals, to train more than would otherwise be possible. However, anabolic steroids also have harmful side-effects, and their use in horses is illegal in many countries. The present results emphasize the need for an adequate work:rest ratio in athletes' training programmes, to maintain a positive anabolic hormonal balance. Because elite sports require high training volumes, trainers should pay particular attention to signs of overreaching and overtraining.

6.3 Muscle glycogen repletion, glucose and glucose precursor

In human athletes, post-exercise repletion of muscle glycogen is accelerated by consumption of carbohydrates (Bergström and Hultman 1966; Costill et al. 1981). Human studies have also shown that the rate of glycogen resynthesis is affected by such factors as dose of glucose administered after exercise (Blom et al. 1986, 1987), timing of the administration (Ivy et al. 1988), activity of glycogen synthase and initial muscle glycogen concentration (Danforth 1965; Larner et al. 1967; Bergström et al. 1972; Kochan et al. 1979). Also in sled dogs immediate post-exercise carbohydrate supplementation promoted more rapid and complete muscle glycogen repletion (Reynolds et al. 1997). In horses, however, dietary carbohydrate appears to have only a minimal effect on the rate of glycogen repletion.

Snow and co-workers (1987) investigated repletion of muscle glycogen stores after a moderate (~40%) muscle glycogen depletion. The first feeding was 2 h after the intense exercise and 1 h after walking. The horses were then given either a low-carbohydrate diet of hay, a normal diet of hay and cubes or a high-carbohydrate diet; the normal diet was supplemented with 450 g of glucose as a 5% solution intravenously over ~6 h starting 60 min after exercise. Glucose infusion tended to increase plasma glucose levels for about 2 h; insulin levels were not reported. After a 20-h recovery, glycogen contents

with the high-carbohydrate and normal diets were 81% of pre-exercise levels, compared with 70% for the low-carbohydrate diet.

In Study I, Standardbreds had a 28% depletion of the glycogen content of the middle gluteal muscle immediately after exercise and 47% depletion 4 h after exercise. The horses received on a normal diet of hay, oats, molasses and commercial pelleted concentrate. Feed was withheld for at least 4 h after exercise. After 24 h, no repletion was observed, and after 48 h muscle glycogen content was 78% of the pre-exercise level.

Also in Study V with half-bred riding horses, no repletion was observed after 23 h during the control trial. Although resting muscle glycogen samples were not obtained in this study, they were presumably around 500 mmol/kg dry weight. This would mean an approximate 47% depletion of the glycogen content of the middle gluteal muscle. The first feeding was 2 h after exercise.

In Studies II and III, the amount of glucose in the isotonic GE solution given 30 min after exercise was very small (about 34 g of glucose per 500 kg of horse), and thus, was not likely to have a significant effect on muscle glycogen content.

The horses in Studies II and III also received 200 ml of propionic acid together with the GE solution. Propionic acid is an efficient precursor of glucose, but this amount would produce no more than 100 g of glucose (Ford and Simmons 1985; Simmons and Ford 1991). Moreover, this was not sufficient to significantly increase the rate of glycogen repletion.

In Study IV on Finnhorses with about 39 % depletion of the glycogen content of the middle gluteal muscle, the diet supplemented with 800 g of molasses per os did not enhance post-exercise repletion of muscle glycogen compared with the basal diet. Molasses plus first feeding were given in two equal portions at 2 and 4 h after exercise. After a 23-h recovery, glycogen contents with high CHO and normal diets were ~73% of the pre-exercise amount.

Nout and co-workers (2003) gave 2 g of glucose polymer per kg bodyweight (1000 g/500 kg horse), and Davie and co-workers (1994) 3 g of glucose polymer per kg body weight (1500 g/500 kg horse) by nasogastric tube to horses 30 min after exercise. In both studies, the glucose/glucose polymer did not influence the rate of glycogen repletion in the 24-h (Nout et al. 2003) / the 12-h (Davie et al. 1994) recovery period after exercise, although blood glucose and insulin concentration increased. The authors concluded that either the blood glucose concentration was insufficient or blood glucose per se is not an important factor in glycogen repletion of horses.

Only by provision of large doses (~2700 g) of dextrose as a 20% solution intravenously during ~8 h starting 30 min after exercise, producing ~47%

depletion of the glycogen content of the middle gluteal muscle, were Davie and co-workers (1995) able to enhance the rate of glycogen resynthesis. In their experiment, the feeding was withheld until 9 h after exercise. During the dextrose infusion, plasma glucose and insulin levels were significantly elevated. After a 9-h recovery, glycogen content with dextrose infusion was 79% of the pre-exercise level and after 24 h 85%. In the control group receiving equivalent volume (~13.5 l) of polyanionic, isotonic fluid infusion, muscle glycogen repletion was negligible during the first 9 h after exercise, and attained a level of only 63% of the pre-exercise value after 24 h. These findings are supported by those of Lacombe and co-workers (2003), who administered 6 g/kg glucose as 13.5% intravenous infusion for 12 h (~3000 g/500 kg) or a similar amount (~25 L) of isotonic saline solution.

Taken together, these studies suggest that increasing oral glucose availability appears to have little influence on the rate of glycogen repletion in adequately fed horses, and an attempt to do so only puts the horse at unnecessary risk of intestinal disturbances and laminitis. On the other hand, these results suggest that in normal horses on a normal diet muscle glycogen stores will not be excessively loaded over one or even two days of rest, and therefore this is not likely to be the primary cause of exercise-induced muscle damage. The exact reason for a significantly slower rate of muscle glycogen repletion compared with man remains unknown. The limiting factor does not appear to be glucose availability, but it could be glucose transport capacity into the muscle cells and/or glycogen synthase activity.

6.4 Muscle glycogen repletion and fats

In the horse, glucose, volatile fatty acids and long-chain fatty acids are the main sources of energy for muscles at rest. In Study I, plasma concentration of lipid substrates, both triglycerides and fatty acids, were less than the before-exercise concentrations 2-24 h after exercise. Uptake of lipids into muscle cells depends on their concentration in plasma (Ahlborg et al. 1974). Results of Study I therefore indicate that during the late recovery period less fatty acids were available to enter muscle tissue than before and during exercise. Lack of lipid substrates may lead to the oxidation of carbohydrates for energy production in muscle cells, and may partially explain the slow rate of resynthesis of glycogen in Study I. The benefits of high insulin may have been diminished by insulin being a potent inhibitor of lipolysis, as indicated by the changes in glycerol concentrations in Studies II and III, and therefore causing a further depletion of lipids. These findings suggest that during recovery energy is produced by the oxidation of carbohydrates, resulting in less carbohydrate available for the resynthesis of glycogen.

Equine muscle has a high aerobic capacity, and thus, a significant ability to use fatty acids as an energy source (Kronfeld et al. 1994). Therefore, a fat-

supplemented diet might produce a substantial flux of fatty acids, which could increase their use as a fuel for aerobic metabolism, leading to more carbohydrates available for resynthesis of glycogen.

In Study IV, after the exercise test, 400 g of rape seed oil was first given to horses on a normal low-fat diet. The horses had a lower-than-normal insulin concentration 1 h after the feeding, repletion of muscle glycogen was slowed down during the 23-h recovery period and the horses had softer-than-normal faeces on the second day of the trial. The low plasma insulin concentration suggests that absorption of carbohydrates in the small intestine was decreased. This implies that an increased amount of soluble carbohydrates was delivered to the large intestine, altering the microbial fermentation. In addition, fats that pass the small intestine undigested may have a direct effect on the microbial fermentation of the caecum and colon; although fats are not subject to microbial fermentation, they may disturb fermentation by coating the fibre, thereby decreasing fibre digestion (Coppock and Wilks 1991: Frape 1998**;** Jansen et al. 2002). These findings support those of Hughes and co-workers (1995), who reported that in horses an adaptation period is needed for fat feeding. Adaptation to fat could be due to increased bile production and exocrine pancreatic secretion of lipase to enhance the capacity for emulsification, hydrolysis, micelle formation and absorption (Manas et al. 1996). However, feeding a high-fat ration (8%) to horses showed no changes in the activity of pancreatic lipase when compared with a low-fat ration (Landes and Meyer 1998).

In Study IV, 400 g of rape seed oil (7.5% fat of dry matter in the total ration) was given to fat-adapted horses during recovery. This time, muscle glycogen repletion was enhanced compared with the fat-supplemented unadapted state, but glycogen repletion equalled muscle glycogen repletion during the basal diet without supplements. Furthermore, adaptation to a fat diet increased plasma cholesterol concentration, but did not increase plasma TG, NEFA or glycerol concentrations during the recovery period. This agrees with the study of Eaton and co-workers (1995), where the pre-exercise muscle glycogen concentration was not raised by the addition of 390 ml of corn oil to the diet.

However, this result disagrees with other studies, where a significant increase (up to 55%) in muscle glycogen content occurred after feeding fat comprising 5-12% of dry matter (Meyers et al. 1989; Oldham et al. 1990; Harkins et al. 1992; Jones et al. 1992; Scott et al. 1992; Hughes et al. 1995; Julen et al. 1995). The variability in results between studies could be due to many factors, e.g. breed, age and body condition of horses and the small number of horses in studies. Kronfeld et al. (1994) reported that 10-13% of dietary fat yields the maximum muscle glycogen content, indicating that the results of Study IV may be partly explained by the smaller doses of fat used.

6.5 Muscle glycogen repletion and dehydration

Physical exercise leads to considerable heat production, and evaporation of sweat is the most efficient means of heat loss during exercise. Horses may sweat up to 15 L/h when activity is maintained at a high rate. As indicated by the correlation between body weight loss during the CET and plasma protein concentration in Studies II and III, during sweating water is initially mainly lost from the extracellular fluid. The consequent decreases in blood and plasma volumes can reduce perfusion in skeletal muscle and in other vital organs.

If significant amounts of water and electrolytes are lost through sweating, the recovery of fluid balance is slowed down. ADH, which is released in response to changes in blood osmolality, increases in response to dehydration and does not return to predehydration levels until osmolality normalizes (Houpt et al. 1989; Sneddon et al. 1993). After CET, ADH was elevated for at least 6 h (Studies II and III). However, horses did not restore the body weight losses incurred during the CET (Studies II and III) in the next 22.5 h, agreeing with the results of Andrews et al. (1994, 1995). Aldosterone is released to increase blood volume and therefore blood pressure. Schott et al. (1997) found the greatest plasma aldosterone concentration after a 12-h recovery from endurance exercise. In Studies II and III, aldosterone remained significantly elevated 22.5 h after a modified 3-day event endurance test. This agrees with the finding in endurance horses in which aldosterone peaked after overnight recovery (Schott et al. 1997).

Weight loss and elevated aldosterone concentrations indicate that 22.5 h after CET horses were still dehydrated. At this stage, plasma protein values had returned to basal level and did not correlate with body weight, suggesting that dehydration was due to a reduction in the intracellular or interstitial fluid space. This could affect muscle glycogen repletion. During recovery, water is needed for glycogen resynthesis, as studies have shown that muscle glycogen is complexed with water, presumably 3-5 g per gram of glycogen (Bridge and Bridges 1932; Olsson and Saltin 1969; 1970). For these reasons, dehydration could delay post-exercise glycogen repletion. In studies II and III, a significant linear correlation was observed between weight recovery and glycogen repletion, suggesting that rehydration has some positive effect on repletion of glycogen stores.

This view is supported by Vervuert (1998), who compared the effects of no treatment and administration of ~ 15 L of plain water, hypotonic (25 g glucose/L water) glucose solution, isotonic glucose solution, hypotonic (4.5 g NaCl/L water) NaCl solution or isotonic NaCl solution via nasogastric tubes 17 min after exercise on repletion of glycogen stores during a 2-h recovery period; 44% depletion of glycogen stores and ~ 3,5% bodyweight loss occurred. On a whole, muscle glycogen resynthesis was negligible in these

horses. Only administration of ~ 15 L of hypotonic (4.5 g NaCl/L water) and isotonic NaCl solutions showed some positive effect on glycogen repletion during the short recovery period.

The findings of Davie and co-workers (1995) contradict the results of these two studies. In their study, muscle repletion was negligible during the first 9 h after exercise, increasing from the 53% post-exercise content to 63% of the pre-exercise content after 24 h with ~13.5 L of polyionic, isotonic fluid infusion. However, the fluid was given over a fairly long period (8 h) and changes in body weight were not reported.

From these studies, one can conclude that despite an apparent rapid return of plasma volume and ionic composition to near-normal values, substantial depletion of body fluid and electrolyte stores may persist after an overnight recovery period in both endurance and 3-day event horses. Because a 2% reduction in body weight the day after a modified 3-day event endurance test was associated with reduced performance and incomplete recovery (Studies II and III), changes in body weight may serve as useful indicators of recovery of muscle glycogen stores and fluid and electrolyte balance, whereas measurements of plasma electrolyte and protein concentrations may be of limited value.

7 Summary of findings

The major finding was the slow rate of resynthesis of glycogen, indicating that horses may be progressively depleted in their muscle glycogen stores during an intensive training period.

The results emphasize the need for an adequate rest:work ratio over the training period to maintain a positive anabolic hormonal balance and to provide sufficient time for repletion of muscle glycogen stores.

For horses on a normal basal diet, neither extra carbohydrate nor extra fat will enhance the repletion of muscle glycogen stores; instead these may, especially in unadapted horses, produce undesirable effects.

Maintaining horses in a good state of hydration seems to have a moderate positive effect on repletion of muscle glycogen stores.

Providing the horse with the opportunity to drink isotonic glucose-electrolyte rehydration solution soon after exercise helps to overcome dehydration significantly better than providing plain water.

In practical situations, changes in body weight serve as useful indicators of recovery.

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