EQUINE VETERINARY JOURNAL

Equine vet. J. (2010) **42** (Suppl. 38) 341-346
doi: 10.1111/j.2042-3306.2010.00283.x

Effects of crude protein intake from forage-only diets on muscle amino acids and glycogen levels in horses in training

B. ESSÉN-GUSTAVSSON*, **M. CONNYSSON**† and **A. JANSSON**†

Department of Clinical Sciences and †Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Keywords: horse; metabolism; treadmill; exercise test; muscle biopsy; leucine

Summary

Reasons for performing study: **There is little information about the influence of crude protein (CP) intake on glycogen and free pool amino acid concentrations in the muscle of horses in training. High energy forage-only diets may be an alternative to concentrate rich diets and may provide high levels of CP.**

Objective: **To study the effect of feeding 2 forage-only diets, containing either high or moderate CP concentrations on glycogen and free pool amino acid concentrations in the muscle.**

Materials and methods: **Two high energy forage-only diets based on high-energy grass forage were fed for 23 days in a crossover design to 6 Standardbred horses in racing condition. One forage diet provided a high (HP) CP (16.6%) intake and the other diet provided recommended intake (RP) of CP (12.5%). At Day 19 a standardised treadmill test was performed to mimic a race. Blood samples were taken before, during and after (up to 90 min) the treadmill test and muscle biopsies (*m. gluteus*) were taken before and after exercise and after 90 min. Amino acids were analysed with a HPLC-technique and glycogen with a fluorimetric method.**

Results: **A main effect of the HP diet was that muscle glycogen and leucine concentrations were higher compared to the RP diet. Branched chain amino acid concentrations in plasma remained higher during early recovery from exercise on the HP diet compared to the RP diet. Intense exercise caused a similar decrease in glycogen, aspartate and glutamate concentrations in muscle and increase in alanine concentration on both diets.**

Conclusion: **Feeding a forage-only diet with a high CP intake increases glycogen and leucine concentrations in muscle of horses in training. This may be beneficial for muscle recovery following intensive exercise.**

Introduction

It is well known that muscle glycogen plays an important role as a source of energy during intensive exercise. Several studies on Standardbred trotters have shown that degradation of glycogen in muscle is greater with increasing exercise intensity (Lindholm and Saltin 1974; Valberg 1986; Valberg *et al*. 1989). Protein degradation and amino acid metabolism are considered to be a minor energy source during exercise (Lemon and Nagle 1981; Gibala 2001). However, it has been shown that glutamate concentrations decrease and alanine concentrations increase in muscle after intense exercise in both man (Bergström *et al*. 1985; Essén-Gustavsson and Blomstrand 2002) and horses (Miller-Graber *et al*. 1990; Pösö *et al*. 1991), indicating that exercise increases amino acid metabolism and may serve other functions. For example, alanine can be released from muscle and used for gluconeogenesis in the liver (Felig and Wahren 1971).

In addition, human studies have shown that protein is utilised to a greater extent when glycogen stores are lowered (Lemon and Mullin 1980; Blomstrand and Saltin 1999). This finding is of special interest concerning horses in race training, as Standardbred trotters generally are trained intensively 2–3 times per week and it can take up to 72 h for glycogen to be restored in muscle after intense exercise (Snow *et al*. 1987; Hyyppä *et al*. 1997; Bröjer *et al*. 2006).

Chronic branched chain amino acid (BCAA) supplementation of the diet has been shown to elevate muscle and liver glycogen content in trained rats (De Araujo *et al*. 2006). Similar results have also been found when exercise trained rats were fed a whey protein diet compared to a casein diet (Morifuji *et al*. 2005). This indicates that the amount and source of protein and thus amino acids given in a diet may affect glycogen storage in muscle. Not much is known about the influence of different crude protein (CP) intake on glycogen and amino acid content in the muscle of horses in training.

Diets for athletic horses include large proportions of concentrates and are often high in crude protein content (Glade 1983; Gallagher *et al*. 1992). Feeding of concentrates has been associated with colics (Goncalves *et al*. 2002), rhabdomyolysis (MacLeay *et al*. 1999) and stereotypic behaviour (Kusunose 1992; Gillham *et al*. 1994). High-energy grass forage might be an alternative to concentrates (Connysson *et al*. 2006; Jansson and Lindberg 2008) and depending of the stage of maturity at harvest and species, these forages also are high in CP content. We have earlier shown that a high protein intake compared to a recommended CP intake from forage-only diets to horses in training did not affect plasma lactate and blood pH during exercise tests performed to mimic a race (Connysson *et al*. 2006).

*Corresponding author email: birgitta.essén-gustavsson@kv.slu.se
[Paper received for publication 10.01.10; Accepted 30.06.10]

The aim of the present study was to investigate if glycogen and amino acid concentrations in muscle of these same horses were affected by the high protein intake in the diet. It was hypothesised that Standardbred trotters fed a high CP forage-only diet increase muscle glycogen and free pool amino acid concentrations in the muscle compared to a recommended CP forage-only diet.

Materials and methods

The animal experiment was approved by the Umeå local ethics committee.

Horses

Six Standardbred geldings (7–10-years-old, 443–548 kg) in race condition and accustomed to treadmill exercise were used. The horses performed intensive exercise (4000 m slow trot warm-up, 2000 m at 10–11 m/s on the race track or five 500 m intervals at 9 m/s on a slope, slow trot downhill) twice a week and slow exercise (walk and slow trot [6–7 m/s] for approximately 45 min) 1–3 times every week. The horses were kept in individual stalls on sawdust and spent 5 h/day in a sand paddock.

Diets

The forage-only diets were offered as silages (40–50% DM) and fed for 23 days in a crossover design. Two forage-only diets, one high (HP) in CP (16.6%) and the other control diet providing recommended (Anon 1989) intake (RP) of CP (12.5%) for race training horses were fed. The forages (mainly timothy [*Phleum pratense L*] and meadow fescue [*Festuca pratensis L*]) were produced in the same area in the north of Sweden, but fertilised with different levels of N and cut early. From Day 18 of the second period, silage was replaced by hay in the RP diet, due to spoiled silage. The nutrient content of the hay (11.8% CP, metabolisable energy 10.3 MJ/kg DM) did not differ greatly from that of the silage. Feed refusals were taken away daily and weighed. More complete information on the nutrient intake can be found in Connysson *et al*. (2006).

Individual diets were calculated to fill the energy and mineral needs (Table 1). The energy intake corresponded to more than 'heavy exercise' but less than 'very heavy exercise', for 500 kg horses (Anon 2007). Horses had water *ad libitium* in buckets. Diets were supplemented with a commercial mineral supplement (Miner Vit)[1]. The horses were feed at 06.00, 12.00, 17.00 and 21.00 h.

Experimental design

The experimental periods started with a change of diet at 06.00 h the first day. Bodyweight (bwt) was measured daily. On Day 19 the horses performed a standardised exercise test (ST) on the treadmill[2]. The ST started with a warm-up of 5 min walk (1.8 m/s), 3 min trot (9 m/s), 45 s fast trot (11 m/s) and 4 min walk (1.8 m/s) that was designed to correspond to prerace occurrences. After the warm-up the horses trotted for 3 min 15 s at 10 m/s at 5% incline and the test ended with trot at 9.5 m/s for 1 min with no incline. Heart rate (HR) was measured (Polar S710i)[3] at rest and during exercise. No feed was given to the horses during 90 min of recovery after the exercise tests.

TABLE 1: Allowance of dry matter (g/100 kg bwt/day), metabolisable energy (MJ/100 kg bwt/day), crude protein (g/100 kg bwt/day), water-soluble carbohydrates[a] (g/100 kg bwt/day), and amino acids[b] (g/100 kg bwt/day) in 5 Standardbred trotters in training on 2 forage-only diets with recommended (12.5%) and high (16.6%) crude protein content

	Recommended diet	High protein diet
Dry matter	1.7 ± 0.1	1.9 ± 0.2
Metabolisable energy	19.9 ± 0.9	22.5 ± 2.4*
Crude protein	220 ± 19	319 ± 31*
Free glucose[a]	24.5 ± 8.1	46.3 ± 3.6*
Free fructose[a]	66.0 ± 46.1	110.9 ± 22.1*
Sucrose[a]	5.3 ± 3.2	4.1 ± 3.1*
Fructanes[a]	17.5 ± 1.5	15.4 ± 3.2*
Alanine[b]	13.0 ± 1.7	18.0 ± 2.1*
Arginine[b]	7.8 ± 0.9	11.4 ± 1.4*
Aspartate[b]	15.6 ± 2.0	22.3 ± 2.3*
Glutamate[b]	15.1 ± 2.0	20.4 ± 2.3*
Glycine[b]	8.8 ± 0.9	12.6 ± 1.3*
Histidine[b]	2.6 ± 0.3	3.7 ± 0.3*
Isoleucine[b]	8.6 ± 1.0	12.4 ± 1.3*
Leucine[b]	13.6 ± 1.6	19.9 ± 2.2*
Lysine[b]	7.2 ± 0.8	10.1 ± 1.1*
Methionine[b]	2.8 ± 0.2	3.7 ± 0.5*
Phenylalanine[b]	7.4 ± 0.8	11.3 ± 1.5*
Serine[b]	5.3 ± 0.5	9.2 ± 0.9*
Threonine[b]	6.6 ± 0.4	9.9 ± 1.0*
Tyrosine[b]	5.0 ± 0.1	7.8 ± 1.1*
Valine[b]	11.9 ± 1.6	16.7 ± 1.8*

*Significantly different from recommended diet ($P<0.05$).

Blood and muscle samples

Blood samples were taken from *vena jugularis* at rest, at end of exercise (after the 3 min 15 s trot at 10 m/s) on the inclined treadmill and at 15 and 90 min post exercise. The samples were kept on ice, then centrifuged and plasma samples stored at −20°C until analysed. Biopsies were obtained from the *gluteus medius* muscle using a biopsy needle (Lindholm and Piehl 1974). The muscle samples were taken at rest, after exercise and after 90 min of recovery and were frozen in liquid nitrogen, then stored at −80°C until analysed.

Free amino acids (AA) were measured in plasma and freeze-dried muscle (dissected free from blood, connective tissue and fat) after precipitation of proteins with a 1:10 dilution with 5% trichloroacetic acid followed by centrifugation at 2700 ***g***, in a Hettich Rotofix 32 centrifuge[4]. Supernatants were collected and stored at −80°C until the assays were performed. The amino acid content (μmol/l) was measured with reversed-phase HPLC using a 5 μm 150 × 3.9 mm C18 column (Resolve™ C18 90 Å)[5] according to the method of Pfeifer *et al*. (1983). Lactate was analysed on plasma using an ELISA[6].

For glycogen determination, muscle tissue (1–2 mg) was boiled for 2 h in 1 mol/l HCl and the glucose residues were determined fluorometrically (Lowry and Passonneau 1973).

Feed samples

The forage samples were prepared and analysed for forage properties as previously described (Connysson *et al*. 2006). In addition, samples of the forage from the 2 diets were sent to a laboratory[7] for analysis of the amino acid content. In brief, the sample was oxidised with performic acid for 16 h, hydrolysed in

6 mol/l HCl for 23 h, pH adjusted and filtered before amino acids were analysed by ion chromatography.

Statistical analysis

Data was analysed using the Mixed Procedure of SAS (version 9.2)[8]. The model included the fixed factors diet and sample time (before, after, 15 and 90 min). Horse within diet was the statistical unit and was treated as random. Interactions between the fixed factors were included in the model if P<0.05. Sample time was analysed with repeated statement in the Mixed Procedure. The model with the lowest Akaike's Information Criterion was chosen for each parameter. For comparisons, the Tukey test was performed and significance level set to 5%. Data is presented as least square mean ± s.e.

Results

Nutrient intake results are presented in Table 1. The daily crude protein intake was 3.2 g/kg bwt on HP and 2.2 g/kg bwt on RP diet which represent 60 and 10% above the recommended intake for intensely exercising horses, respectively (2 g/kg bwt, Anon 2007).

One horse had to be excluded from the study since it did not complete the ST according to the protocol. There were no differences between the 2 diets in HR at rest (RP, 34 ± 3 vs. HP, 38 ± 6 beats/min) and at end of exercise (RP, 212 ± 5 vs. HP, 215 ± 6 beats/min) or in plasma lactate at rest (RP, 0.9 ± 0.2 vs. HP, 0.5 ± 0.1 mmol/l) and at end of exercise (RP, 17.6 ± 2.8 vs. HP, 18.3 ± 2.2 mmol/l). At rest following 19 days, horses did not differ in bwt (RP: 480 ± 15 kg, HP: 483 ± 14 kg).

Muscle glycogen

Glycogen concentrations (LS means, s.e. = 28) on the HP diet were 630 mmol/kg DW at rest, 501 mmol/kg DW after exercise and 536 mmol/kg DW at 90 min of recovery, and the values on the RP diet were 552 mmol/kg DW at rest, 444 mmol/kg DW after exercise and 423 mmol/kg DW at 90 min of recovery. Muscle glycogen concentration was higher on the HP diet compared to the RP diet (P = 0.05) and there was a tendency for interaction between diet and sampling time (P = 0.06). Notably, for all horses all values for glycogen concentration were higher on the HP diet than on the RP diet at all time points. There was a significant effect of sampling time (P<0.0001) and glycogen concentration had decreased on both diets immediately after exercise (RP = 108, HP = 129 mmol/kg DW). The glycogen concentration remained low on diet RP whereas there was a tendency for an increase (P = 0.09) 90 min post exercise on diet HP.

Muscle amino acids

The mean leucine concentration of all time points was higher (0.69 vs. 0.60 mmol/kg DW, s.e. = 0.03) and the histidine concentration was lower (0.37 vs. 0.44 mmol/kg DW, s.e. = 0.02) on diet HP compared to diet RP.

There was a significant effect of diet on muscle leucine (P<0.05) and histidine concentrations (P<0.05) and a tendency for an effect on isoleucine concentration (P = 0.09) but there were no interaction between diet and sampling time in either of these parameters. There was an effect of exercise and recovery on all amino acids except serine, glutamine, methionine, isoleucine, valine, taurine and glycine (Table 2). Glutamate and aspartate concentrations decreased after exercise compared to rest and remained lower after 90 min of recovery. Alanine and leucine concentrations increased only after exercise compared to rest. Phenylalanine concentrations increased both after exercise and after 90 min of recovery compared to rest and asparagine, lysine and threonine concentrations increased only after 90 min of recovery.

Plasma amino acids

There was no effect of diet on plasma amino acids but there was an interaction between diet and sampling time for valine, lysine,

TABLE 2: Effects of exercise and recovery time (90 min without access to feed) on muscle amino acid concentrations (mmol/kg DW) in *m. gluteus* of 5 Standardbred trotters in training on 2 forage-only diets with recommended (12.5%) and high (16.6%) crude protein content (LS mean ± s.e.)

	Before	After	90 min	P value time
Alanine	3.62 ± 0.44^{a}	6.17 ± 0.42^{b}	4.00 ± 0.42^{a}	0.001
Asparagine	4.37 ± 0.64^{a}	4.87 ± 0.35^{a}	7.86 ± 0.94^{b}	0.009
Aspartate	1.03 ± 0.08^{a}	0.56 ± 0.04^{b}	0.68 ± 0.10^{b}	0.001
Glutamate	8.05 ± 0.43^{a}	3.34 ± 0.43^{b}	4.42 ± 0.43^{c}	0.001
Histidine	0.39 ± 0.04^{ab}	0.36 ± 0.02^{a}	0.47 ± 0.05^{b}	0.044
3MH	0.08 ± 0.01^{ab}	0.07 ± 0.01^{a}	0.09 ± 0.01^{b}	0.008
Leucine	0.58 ± 0.03^{a}	0.70 ± 0.03^{b}	0.66 ± 0.03^{ab}	0.046
Lysine	0.29 ± 0.07^{a}	0.39 ± 0.07^{ab}	0.53 ± 0.06^{c}	0.003
Phenylalanine	0.27 ± 0.01^{a}	0.31 ± 0.01^{b}	0.34 ± 0.01^{bc}	0.001
Threonine	0.84 ± 0.08^{a}	0.98 ± 0.06^{ab}	1.19 ± 0.11^{c}	0.013
Tryptophan	0.09 ± 0.01^{a}	0.10 ± 0.01^{ab}	0.11 ± 0.01^{b}	0.017
Tyrosine	1.07 ± 0.07^{ab}	1.15 ± 0.07^{a}	1.03 ± 0.07^{b}	0.019
BCAA	1.77 ± 0.10	1.91 ± 0.10	1.82 ± 0.10	ns
Glutamine	1.07 ± 0.09	1.12 ± 0.07	1.55 ± 0.36	ns
Glycine	2.57 ± 0.30	3.22 ± 0.43	3.68 ± 0.43	ns
Isoleucine	0.32 ± 0.02	0.36 ± 0.02	0.32 ± 0.02	ns
Methionine	0.15 ± 0.01	0.17 ± 0.01	0.17 ± 0.01	ns
Serine	0.21 ± 0.04	0.25 ± 0.02	0.33 ± 0.05	ns
Taurine	20.2 ± 3.0	18.6 ± 3.0	17.0 ± 3.0	ns
Valine	0.86 ± 0.06	0.85 ± 0.06	0.84 ± 0.06	ns

3MH = 3-methylhistidine; BCAA = branched chain amino acids; ^{a,b,c}Values with different superscripts differ within a row. There were no dietary effects and therefore the data were pooled from the 2 diets.

TABLE 3: Effects of exercise and recovery time (90 min without access to feed) on plasma amino acid concentrations (μmol/l) of 5 Standardbred trotters in training on 2 forage-only diets with recommended (RP = 12.5%) and high (HP = 16.6%) crude protein content (LS mean ± s.e.)

		Before	After exercise	15 min of recovery	90 min of recovery	P value interaction	P value time
BCAA	RP	286 ± 47^{a}	568 ± 47^{b}	385 ± 47^{a}	282 ± 47^{a}	0.049	<0.0001
	HP	361 ± 47^{a}	542 ± 47^{b}	506 ± 47^{b}	350 ± 47^{a}		
Glutamate	RP	46 ± 5^{ac}	88 ± 7^{b}	56 ± 7^{ac}	43 ± 5^{c}	0.02	<0.0001
	HP	45 ± 5^{abc}	73 ± 7^{b}	58 ± 7^{a}	37 ± 5^{c}		
Lysine	RP	51 ± 15^{ac}	118 ± 15^{b}	73 ± 11^{c}	49 ± 7^{a}	0.03	<0.0001
	HP	75 ± 15^{ac}	114 ± 15^{b}	95 ± 11^{a}	63 ± 7^{c}		
Methionine	RP	13 ± 3^{a}	25 ± 3^{b}	17 ± 3^{a}	15 ± 3^{a}	0.01	<0.0001
	HP	21 ± 3^{a}	24 ± 3^{a}	26 ± 3^{a}	20 ± 3^{a}		
Serine	RP	15 ± 4^{a}	25 ± 4^{b}	17 ± 3^{a}	15 ± 1^{a}	0.003	0.001
	HP	21 ± 4^{a}	25 ± 4^{a}	23 ± 3^{a}	18 ± 1^{a}		
Tryptophan	RP	19 ± 7^{a}	40 ± 7^{b}	27 ± 7^{ab}	36 ± 7^{ab}	0.042	0.07
	HP	39 ± 7^{a}	43 ± 7^{a}	46 ± 7^{a}	35 ± 7^{a}		
Tyrosine	RP	31 ± 6^{a}	61 ± 6^{b}	43 ± 6^{a}	42 ± 6^{a}	0.047	<0.0001
	HP	47 ± 6^{a}	61 ± 6^{b}	58 ± 6^{ab}	53 ± 6^{a}		
Valine	RP	168 ± 27^{a}	306 ± 27^{b}	200 ± 27^{a}	158 ± 27^{a}	0.047	<0.0001
	HP	217 ± 27^{ac}	297 ± 27^{b}	277 ± 27^{ab}	203 ± 27^{c}		
Alanine		164 ± 22^{a}	370 ± 25^{b}	305 ± 29^{c}	220 ± 18^{a}		<0.0001
Asparagine		238 ± 26^{a}	400 ± 26^{b}	299 ± 26^{c}	258 ± 26^{ac}		<0.0001
Aspartate		10 ± 1^{ab}	13 ± 1^{b}	8 ± 1^{c}	9 ± 1^{ac}		0.0002
Glycine		291 ± 27^{a}	466 ± 39^{b}	317 ± 29^{a}	295 ± 17^{a}		0.002
Glutamine		133 ± 14^{a}	212 ± 13^{b}	140 ± 14^{a}	115 ± 14^{a}		<0.0001
Histidine		62 ± 5^{a}	80 ± 5^{b}	60 ± 5^{a}	57 ± 5^{a}		<0.0001
Isoleucine		52 ± 6^{a}	90 ± 7^{b}	72 ± 6^{c}	46 ± 4^{a}		<0.0001
Leucine		79 ± 9^{a}	163 ± 9^{b}	135 ± 9^{c}	89 ± 9^{a}		<0.0001
Phenylalanine		40 ± 5^{a}	72 ± 5^{b}	61 ± 5^{cd}	53 ± 5^{d}		<0.0001
Taurine		28 ± 3^{a}	73 ± 6^{b}	49 ± 5^{c}	38 ± 4^{d}		<0.0001
Threonine		119 ± 13^{a}	172 ± 13^{b}	127 ± 13^{a}	113 ± 13^{a}		<0.0001

^{a,b,c,d} Values with different superscripts differ within a row. When there were no dietary effects the data were pooled from the 2 diets.

methionine, tyrosine, glutamate, serine and tryptophan, and a tendency for interaction (P = 0.06) in isoleucine and leucine (Table 3). The BCAA concentration increased with exercise and remained high until at least 15 min post exercise on diet HP, whereas it had started to decrease within 15 min on diet RP. The other amino acids were not affected by diet at all but were affected by sampling time (Table 3). Increased concentrations were seen for alanine, glycine, threonine, isoleucine, leucine, glutamine, asparagine and histidine after exercise compared to rest. After 90 min of recovery these amino acids reached resting concentrations again. Phenylalanine and taurine concentrations were increased both after exercise and after 15 and 90 min of recovery compared to rest.

Discussion

The major finding of this study was that horses in training had higher glycogen and free leucine concentrations in the muscle when fed a high CP forage-only diet compared to feeding a forage-only diet containing recommended CP concentration. The higher glycogen concentrations in muscle on the HP diet may either be due to a greater synthesis or to a lower degradation. If more amino acids were used for oxidation in muscle on the HP diet this could have had a sparing effect on glycogen utilisation. However, the utilisation of glycogen during exercise was similar on both diets. The intake of energy and sugars was higher on the HP diet which may theoretically have influenced the glycogen concentrations in muscle. It is known that feeding diets with higher amounts of soluble carbohydrates to horses will replenish glycogen faster after strenuous exercise (Lacombe *et al*. 2004). In the present study, the daily intake of sugars (glucose, fructose and sucrose) was calculated to be approximately 300 g higher on diet HP (800 g/day) compared to diet RP (500 g/day). This difference between the diets was much smaller than the difference in starch intake between a low and medium carbohydrate diet (1.35 kg/day) studied by Lacombe *et al*. (2004) that resulted in no difference between the diets in glycogen synthesis after exercise. Therefore, it is likely that the increase in glycogen content on the HP diet is related to the higher CP intake rather than the small increase in intake of water soluble carbohydrates. The daily energy intake was also somewhat higher on the HP diet but the horses had similar bodyweights on both diets. This could reflect that horses on the HP diet had a greater need for energy due to an increase in metabolism of N excretion as previously discussed (Blaxter 1989; Connysson *et al*. 2006). Since the diets differed in both CP intake and concentrations of carbohydrates it is difficult to state for certain which of these factors plays the greatest role in the increased muscle glycogen content. That the higher dietary supply of amino acids in the HP-diet may be related to increased muscle glycogen levels is supported by a study on trained rats (De Araujo *et al*. 2006). In that study, liver and muscle glycogen concentrations were higher after chronic BCAA supplementation to the diet. Liver and muscle glycogen levels were also shown to be higher when exercised trained rats were fed a whey protein diet compared to a casein diet (Morifuji *et al*. 2005).

The leucine content in muscle also increased on the HP diet. Whether this is related to the greater dietary supply of amino acids on this diet and a greater uptake of amino acids from the gut is not known. BCAAs are oxidised in muscle during exercise due to an increased delivery from plasma to muscle but also due to increased protein breakdown (Rennie and Tipton 2000).

Since tyrosine and phenylalanine are neither taken up nor metabolised by muscle the increase in their muscle concentrations indicates protein degradation during exercise (Blomstrand and Newsholme 1992). In the present study, phenylalanine concentrations increased in muscle after exercise but to a similar

extent on both diets, indicating some protein degradation which is in agreement with results from an earlier treadmill study (Pösö *et al*. 1991). A notable effect of intense exercise on both diets was that the highest muscle levels of asparagine, lysine and threonine were seen after 90 min of recovery. This indicates that these amino acids may play an important role in amino acid metabolism during recovery from exercise.

There was no dietary effect on the amino acid concentrations in plasma, but for some amino acids, there was an interaction between diet and sampling time after exercise. Concentrations of amino acids in plasma are not easy to interpret, as they depend not only on the dietary source, but also on the uptake and release from tissues such as kidney, liver and muscle. Furthermore, the red blood cells store and release amino acids. Another factor to consider when interpreting amino acid concentrations in plasma is the relationship to alterations that may occur in haemoconcentration. Part of the increase seen after intense exercise is due to plasma volume changes and total protein content has been shown to be around 20% higher after this type of exercise on both the RP and HP diets (Connysson *et al*. 2006). Most of the increase in plasma amino acid levels is, however, due to exercise and the alterations seen on both diets agree with earlier observations on horses performing intense exercise (Miller-Graber *et al*. 1990; Essén-Gustavsson *et al*. 1991; Pösö *et al*. 1991; Hackl *et al*. 2009; van den Hoven *et al*. 2009). Interestingly, the increase in BCAA concentration was maintained high until at least 15 min post exercise on diet HP, whereas it had started to decrease within 15 min on diet RP. It has recently been shown that plasma and muscle leucine and isoleucine concentrations are higher post exercise when horses during 6 weeks are given a protein amino acid mixture daily within 1 h after exercise (van den Hoven *et al*. 2009). This indicates that not only exercise but also the dietary intake of protein and amino acids can alter the amino acid profile in plasma and muscle which also is supported by the results in the present study. BCAA supplementation before and after exercise has been shown to be beneficial not only for promoting muscle protein synthesis but also for decreasing exercise induced muscle damage (Shimomura *et al*. 2006; Negro *et al*. 2008). A protein sparing effect especially during recovery after exercise has been shown in man after BCAA supplementation (Blomstrand and Saltin 2001). The increased leucine concentrations in muscle and the higher BCAA plasma concentrations post exercise on the HP diet may thus be beneficial for muscle recovery. It was notable that glycogen synthesis seemed to be faster on the HP diet post exercise as glycogen levels remained low on diet RP whereas there was a tendency for an increase 90 min post exercise on diet HP.

The question is what causes muscle glycogen concentrations to be higher on the HP compared to the RP diet? It is well known that insulin stimulates glucose uptake in muscle and that not only glucose but also protein and amino acids stimulate insulin secretion. It has been shown in man that intake of both carbohydrates and protein is more effective than carbohydrates alone for replenishment of muscle glycogen (Zawadski *et al*. 1992; van Loon *et al*. 2000).

Recently, from studies on rats it has been suggested that BCAA and, in particular leucine, would stimulate glucose uptake and glycogen synthesis through a mechanism that differs from that induced by insulin (Morifuji *et al*. 2010). This indicates that the dietary intake of protein and its amino acid content could play an important role for glycogen synthesis in muscle.

Glycogen degradation during the exercise test did not differ between the 2 diets, which shows the importance of carbohydrates as a substrate source for energy release during intense exercise. That anaerobic metabolism contributed to energy release was seen from the high lactate levels observed in blood after exercise. The increased alanine and decreased glutamate and aspartate concentrations in muscle after exercise indicates that amino acid metabolism played an important role during this type of work. These changes in amino acid concentrations in muscle agree with earlier studies on horses performing treadmill exercise (Miller-Graber *et al*. 1990; Pösö *et al*. 1991). The lowering of the aspartate concentrations seen after exercise may be related to its role in transamination reactions and in reamination of AMP to IMP in the purine nucleotide cycle. It is likely that IMP was produced in connection with exercise as increased IMP concentrations have been seen in horses after intense exercise and races (Essén-Gustavsson *et al*. 1997). The lowered glutamate concentrations in muscle after exercise and the high rate of glycolysis indicate that alanine was produced by the alanine aminotransferase reaction. In this reaction glutamate and pyruvate not only form alanine but also 2-oxoglutarate which is an intermediate in the citric acid cycle and therefore of importance for oxidative metabolism. Alanine concentrations in plasma increased after exercise and into the recovery period. This reflects that a release of alanine occurred from muscle, which could be used for gluconeogenesis in the liver. This study shows that there are no negative effects on the metabolic response to exercise in horses fed a high crude protein diet. This is also in accordance with observations on the effect of dietary protein level on metabolic response after treadmill exercise at an intensity of 4.5 m/s for 15 min (Miller and Lawrence 1988).

In conclusion, the present study shows that a high crude protein intake from forage-only diets increase the muscle glycogen and leucine concentrations in horses in training, which may be beneficial for recovery after intense exercise.

Acknowledgements

The authors thank veterinarian Katarina Nostell (SLU) and Johan Backman, Peter Buhlér, Ulf Hedenström, Katarina Olofsson and all personnel and students at Travskolan Wången. Special thanks also to Kristina Andersson (SLU) for statistical consultation. The research was supported by Stiftelsen Svensk Hästforskning and Travskolan Wången.

Conflicts of interest

The authors declare no potential conflicts.

Manufacturers' addresses

[1]Krafft, Falkenberg, Sweden.
[2]Säto, Uppsala, Sweden.
[3]Polar Electro Oy, Kempele, Finland.
[4]Hettich, Tuttlingen, Germany.
[5]Waters Corporation, Town, Massachusetts, USA.
[6]Roche Diagnostics, Basel, Switzerland.
[7]Eurofins, Lidköping, Sweden.
[8]SAS Institute Inc., Cary, North Carolina, USA.

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