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Insulin response to feeding forage with varying crude protein and amino acid content in horses at rest and after exercise

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RESEARCH PAPER

Abstract

This study assessed the insulin response to forage intake with varying crude protein (CP) content in horses at rest and after exercise. Six geldings were fed three grass haylage-only diets for 7 days according to a 3×3 Latin square design. On day 7, blood samples were collected before and for 120 min after feeding 15% of the daily allowance before exercise (feeding A) and after standardised exercise (feeding B). Feed samples were collected before each feeding. Dry matter (DM) and nutrient content varied (DM: 37-58%, water-soluble carbohydrates minus fructans (WSC-f): 3-12% of DM, CP: 10-15% of DM) which resulted in a variation in nutrient intake within haylage batches. Based on individual CP and WSC-f intakes, intake groups were therefore formed (low and high CP intake; ≤ and >180 g CP/100 kg body weight, respectively and low and high WSC-f intake; ≤ and >100 g/100 kg body weight, respectively). Amino acids were analysed and intakes were generally higher in the high CP group than in the low CP group. An ANOVA model including horse, CP group and WSC-f intake explained 95% of the variation in plasma insulin response compared to 87% using a model including horse and WSC-f group alone. The plasma insulin area under curve (AUC) following feeding A tended to be higher in the high CP group than in the low CP group (P=0.08), but there was no difference after feeding B. Plasma glucose AUC was not affected by CP group (P>0.05). The study indicates that the post-prandial plasma insulin response in horses fed a forage-only diet is increased by high WSC-f intake but may also be increased by high CP intake, at least at rest. However, due to the low number of observations further studies are needed.

Keywords: glucose, glycaemic response, intake, water-soluble carbohydrates

1. Introduction

Little is known about how forage sugar and crude protein (CP) content affect plasma insulin levels in horses. Low plasma insulin responses could limit muscle glycogen synthesis (Zawadzki *et al.*, 1992) and thus merit limited inclusion of large amounts of forage in the diet of athletic horses. In contrast, high plasma insulin concentrations could be a health risk in horses with equine metabolic syndrome and prone to developing laminitis (Asplin *et al.*, 2007).

It has been shown that a grass haylage diet results in lower basal plasma insulin concentrations than a diet including starch-rich concentrates (Connysson *et al.*, 2010; Jansson and Lindberg, 2012). Intake of grass forage temporarily elevates insulin levels (Borgia *et al.*, 2011; Muhonen *et al.*, 2008; Ragnarsson and Jansson, 2011; Vervuert *et al.*, 2004), although not as much as intake of concentrates (Connysson *et al.*, 2010). It is likely that the elevation is dependent on the content of glucose, fructose and sucrose in the forage. In horses, glucose appears to induce a larger insulin response than fructose (Borer *et al.*, 2012; Vervuert *et al.*, 2004). It has also been suggested that forage with a high CP content can elevate insulin levels in horses (Essén-Gustavsson *et al.*, 2010). It is well known that protein intake can stimulate insulin release. In particular, intake of leucine has been shown to increase the insulin response in man (Cleator *et*

al., 1975) and in horses (Urschel *et al.*, 2010). In addition, *in vitro* studies on islets of Langerhans from mice show that a cocktail of leucine, isoleucine, valine, lysine and threonine can induce higher insulin secretion than glucose (Salehi *et al.*, 2012). Protein intake in humans has also been shown to increase the levels of glucose-dependent insulinotropic polypeptide (GIP). Thus, when GIP was included in the amino acid cocktail in the study by Salehi *et al.* (2012), the insulin response was even higher. In grass forage, 22-27% of the CP content can be expected to consist of leucine, isoleucine, valine, lysine and threonine (Anonymous, 2003; Essén-Gustavsson *et al.*, 2010).

In humans, there is a well-documented additive effect on the plasma insulin response when amino acids or protein are ingested simultaneously with readily available carbohydrates (Rabinowitz *et al.*, 1966; Van Loon *et al.*, 2000a; Zawadski *et al.*, 1992). In pigs, a similar increase in insulin response has been observed with a diet containing 14% protein compared with a diet with 0% protein, although the latter diet included 25% more starch (Rérat *et al.*, 1985). Stull and Rodiek (1995) also found an increased insulin response in horses fed alfalfa together with maize compared with horses fed maize alone. Inclusion of alfalfa in the diet of horses also increased plasma isoleucine, leucine and phenylalanine levels compared with feeding only maize, leading those authors to suggest that the amino acid content of the alfalfa might have influenced GIP to mediate additional insulin secretion.

During exercise, plasma insulin concentrations are lowered (Thornton, 1985) and glucose enters muscle cells without insulin-mediated transport. Furthermore, glucose entry and glycogen synthesis have been suggested to be independent of insulin for up to 1 h post-exercise (Ivy and Kuo, 1998). After the insulin-independent effect of exercise on muscle glucose transport subsides, the sensitivity of glucose transport and glycogen synthesis to insulin is markedly increased (Ivy and Kuo, 1998). This sensitivity results in glucose uptake and glycogen synthesis at insulin concentrations that normally have no detectable effect on these processes (Cartee *et al.*, 1989). In horses, there is conflicting evidence on the effect of exercise on insulin sensitivity (Pratt *et al.*, 2007; Powell *et al.*, 2002) and a study by Urschel *et al.* (2010) showed that the plasma insulin response to intake of glucose, leucine and whey protein at rest and following exercise may be similar. The aim of the present study was thus to assess the insulin response to feeding in horses fed forage with varying CP content, both at rest and after exercise. The hypothesis was that CP content affects the plasma insulin response at rest but not after exercise.

2. Materials and methods

Horses and management

Six adult (age 5-11 years), trained Standardbred geldings were used. Body weight (BW) ranged from 469 to 623 kg (without restriction of feed and water) and body condition score (BCS) ranged from 5 to 6 according to Henneke *et al.* (1983). The horses were kept in individual free stalls on sawdust bedding at night and spent about 8 h in a sand paddock during daytime, except on days when blood samples were collected. The horses were exercised once a week (warm-up 4 km at heart rate <200 beats/min and 1,600 m exercise at heart rate >200 beats/min). The experiment was performed in May-June 2010 at a training camp for Standardbred horses close to Uppsala, Sweden. The experiment was approved by the Uppsala local ethics committee.

Experimental diets and design

The horses were fed one of three grass haylage diets according to a 3×3 Latin square design (2 horses per diet in each period). Each diet was fed for 7 days and on day 7 sample collection and exercise were performed. The three haylage batches (H1, H2 and H3) had similar estimated metabolisable energy (ME) content (Anonymous, 2011; Lindgren, 1979) but varying CP content based on analyses performed on samples collected prior to the study in connection with the harvest. Haylage was fed restrictedly to result in a daily ME intake corresponding to 19 MJ/100 kg BW. The daily amount of haylage offered was 6.36-9.45 kg dry matter (DM). To meet the mineral and vitamin requirements (NRC, 2007), diets H1 and H3 were supplemented individually (60-150 g/d) with a commercial mineral product (Krafft, Falkenberg, Sweden), providing per kg of mineral feed: Ca 55 g; P 65 g; Mg 60 g; NaCl 125 g; Cu 900 mg; Se 15 mg; vitamin A 100,000 IU; vitamin D_3 10,000 IU; and vitamin E 5,000 mg. The supplement used with the H2 diet provided (per kg mineral feed): Ca 110 g; P 17 g; Mg 60 g; NaCl 125 g; Cu 1 200 mg; Se 15 mg; vitamin A 200,000 IU; vitamin D_3 10,000 IU; and vitamin E 15,000 mg. All three diets were supplemented with calcium carbonate (30-40 g/d). The horses were fed approximately 33% of the daily feed allowance at 04:00, 16:00 and 23:00 h respectively, except on the day before sampling days, when 23% of the daily feed allowance was fed at 18:00 and 23:00 h and the rest was divided on the 04:00 and 16:00 meals, and on sampling days, when 15% of the daily feed allowance was fed at 04:00, 09:00 and 45 min after exercise and the rest was divided between the remaining meals. The meal sizes were reduced prior to sampling to ensure that all feed would be consumed without pause and thereby make the insulin responses more 'standardised'. Water was offered *ad libitum* from water bowls on days 1-6 and from water bowls and buckets during sampling days.

The grass haylage (mainly timothy and meadow fescue) was first-cut material (from a site at 59°37.8'N, 17°04.5'E) wilted to approximately 50% of DM and conserved in bales (approximately 350 kg/bale) wrapped in plastic. H1 was cut on 8 June from a sward sown 8 years earlier with a mixture of 45% timothy, 30% meadow fescue and 25% ryegrass, although most of the ryegrass had disappeared by the time of the H1 cut. H2 was cut on 21 June from a sward sown 4 years earlier with the same mixture. They were both fertilised in spring with 124 kg N. H3 was cut on 1 June from a sward sown 3 years earlier (65% timothy and 35% meadow fescue) and fertilised with 75 kg N in April. Haylage samples were collected every sampling day from the same part (in several places) of the bale that were used that day and immediately frozen (-20 °C) for later analysis.

Design of sampling days

A permanent catheter (Intranule, Vygon, Ecouen, France) was inserted under local anaesthesia (Carbocain, 20 mg/ml, Astra Zeneca AB, Södertälje, Sweden) into the jugular vein of all horses between 07:00 and 08:30. Blood samples were collected before the feeding at 09:00 (15% of the daily feed amount, feeding A) and 20, 40, 60, 90 and 120 min post-feeding (Figure 1). The horses were fed in the stalls and kept loose during the sampling period. A standardised exercise was then performed in a jog cart (two sessions, three horses/session) on a field track (0.6% slope). The exercise included 15 min of warm-up (5 min walk, 10 min trot at 7 m/s), a 1,600 m bout in trot at 11 m/s and ended with a slow-down trot back to the stable (3 min 30 sec at 6 m/s). A blood sample was collected post-exercise (PE), within 1 min after the slow-down trot and plasma lactate concentration in this was 12.1±5.5 mmol/l (mean ± standard deviation (SD)). Heart rate 15 min after end of slow-down trot was 105±6 beats/min (mean ± SD, measured by phonendoscope). The horses were then showered, returned to their stalls and 45 min post-exercise fed 15% of the daily amount (feeding B). Blood samples were collected before and at 20, 40, 60, 90 and 120 min after this feeding (Figure 1).

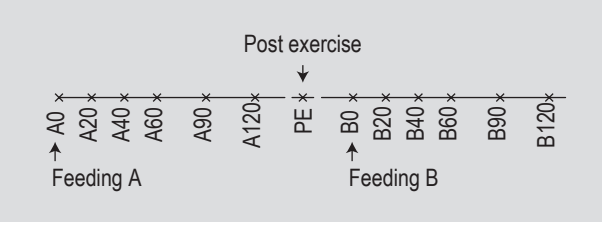

Figure 1. Time-axis illustrating the design of sampling days. Blood samples were collected at each cross on the line: 0, 20, 40, 60, 90 and 120 min after feeding A, 1 min post-exercise (PE) and 0, 20, 40, 60, 90 and 120 min after feeding B. The exercise was performed 45-120 min after sample A120 and feeding B occurred 45 min after exercise was finished.

Collection and analyses

Blood was collected in EDTA tubes (7 ml) and stored cool until centrifuged. After centrifugation (8 min, 1,157×*g*) the plasma was frozen (-20 °C) until later analysis. Plasma was analysed for insulin using Mercodia Equine Insulin ELISA (Mercodia AB, Uppsala, Sweden) and glucose using an enzymatic method (Boehringer Mannheim D-glucose, r-Biopharm, Food-diagnostics, Darmstadt, Germany). The samples taken immediately after exercise were analysed for lactate with an enzymatic method in a LM5 Lactate Analyser (Analox Instruments, London, UK). Feed samples were dried, milled and analysed for DM content. Analysis of water-soluble carbohydrates (WSC), glucose, fructose, sucrose and fructans was carried out according to Larsson and Bengtsson (1983) using enzymes from Roche Diagnostics GmbH (Mannheim, Germany). Analysis of CP was performed by the Kjeldahl method (Kjeldahl, 1883), where ammonia N concentration was determined by direct distillation with a Kjeltec 2460 (Foss, Hilleröd, Denmark). Nitrogen content was then multiplied by 6.25 to give the CP content. A separate analysis of amino acid content in forage was performed for each CP intake group (see below) by Eurofins Food and Agro Testing Sweden with high performance liquid chromatochaphy according to the ISO standard 13903:2005. The analysis was performed on a pooled sample where each forage and period sample represented in each CP intake group was used proportionally to the number of observations within the group.

Calculations and statistical analysis

Intake of CP, WSC and single sugar fractions was calculated on individual basis for each sampling occasion. There was a variation in DM, WSC and CP content within each haylage batch during the three periods, resulting in variations in total intake. Thus based on individual CP intake, two CP intake groups were formed, with ≤180 g/100 kg BW and >180 g/100 kg BW (n=5 respectively). One horse had all observations within the higher CP intake group and was therefore excluded from the analysis, while for individuals with two observations within the same intake group, the data were pooled into one mean. All 3 periods are represented in the high CP group and period 1 and 3 are represented in the low CP group. In addition, two groups were formed based on the WSC intake excluding fructan (WSC-f) (≤100 g/kg BW and >100 g/kg BW, n=6) and duplicate observations were pooled using the same strategy as for the CP groups.

For insulin and glucose concentrations, area under curve (AUC) was calculated in GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Curve AUC_A included the six samples (0, 20, 40, 60, 90 and 120 min) collected in connection with feeding A and curve AUC_B the six samples

collected post-exercise with feeding B (0, 20, 40, 60, 90 and 120 min). Curve AUC_{A+B} included both curves.

Data were subjected to analysis of variance (GLM procedure in SAS package 9.3) (SAS Institute Inc., Cary, NC, USA). When assessing the effect of CP and WSC-f group on plasma insulin and glucose, the effect of individual WSC-f (≤ and >100 g/100 kg BW) and CP (≤ and >180 g/100 kg BW) intake, respectively was included as a class in the model. More specifically the following models were used:

$$Y_{i\,j\,k\,l\,m} = \mu + \alpha_i + \beta_j + \varepsilon_k + \eta_l + e_{i\,j\,k\,l\,m}$$

and

$$Y_{i\,j\,k\,l\,m} = \mu + \alpha_i + \beta_j + \varepsilon_k + \eta_l + \gamma_m + (\beta\gamma)_{j\,m} + e_{i\,j\,k\,l\,m\,n}$$

where $Y_{i\,j\,k\,l\,m}$ is the observation, μ the mean value, α_i the effect of animal, β_j the effect of intake group, ε_k the effect of exercise, η_l the effect of CP or WSC-f intake level, γ_m the effect of sample and $e_{i\,j\,k\,l\,m\,n}$ the residuals; $e_{i\,j\,k\,l\,m\,n}$ ~IND (0, δ^2). For analysis of differences of nutrient intake between intake groups, the following model was used:

$$Y_{i\,j\,k} = \mu + \alpha_i + \beta_j + e_{i\,j\,k}$$

where $Y_{i\,j\,k}$ is the observation, μ the mean value, α_i the effect of animal, β_j the effect of intake group and $e_{i\,j\,k}$ the residuals; $e_{i\,j\,k}$ ~IND (0, δ^2).

To assess differences before and after exercise, within and between intake groups, a Tukey's test was also used. Differences were considered significant at $P<0.05$. A test for correlations between nutrient intake and plasma insulin and glucose AUC was performed using Pearson's Correlation test. Values are presented as means ± SD or least square means (LS Means) ± standard error (SE).

3. Results

Nutrient intake

All horses consumed their ration completely throughout the study. The DM of the forage samples collected during the sampling days varied between 37-58% and WSC and CP content between 3-12% and 10-15% of DM, respectively. This resulted in a variation in nutrient intake (Table 1). The CP intake was significantly higher ($P<0.05$) in the high CP group than in the low CP group, but there was no difference in WSC-f intake between CP groups ($P=0.17$) (Table 1). WSC intakes were higher in the high WSC-f group compared to the low WSC-f group but there was no

Table 1. Daily intake of dry matter (DM, kg/100 kg BW), crude protein (CP), water-soluble carbohydrates (WSC), glucose, fructose, sucrose, WSC minus fructans (WSC-f) and fructans (g/100 kg bodyweight (BW)) in six horses fed haylage one (H1), two (H2) and three (H3) in three periods (two horses in each period, mean ± standard deviation and corresponding intakes when observations were divided into either two CP intake groups (≤180 (n=5)[1] and >180 (n=5)[1] g/100 kg BW) or two WSC-f intake groups (≤100 (n=6) and >100 (n=6) g/100 kg BW). Forage CP and WSC-f content (% of DM) are also shown.

Diet	Period	DM	CP	WSC	Glucose	Fructose	Sucrose	WSC-f[2]	Fructans	CP%	WSC-f[2]%
H1	1	1.15±0.01	115±1	34±0	13±0	16±0	8±0	33±0	0±0	10.0	3.0
	2	1.61±0.05	210±6	82±2	44±1	32±1	1±0	75±2	7±0	13.1	5.1
	3	1.52±0.03	159±4	71±2	39±1	23±1	3±0	67±1	4±0	10.4	4.7
H2	1	1.54±0.01	205±2	150±1	66±1	79±1	0±0	147±1	2±0	13.3	9.7
	2	1.48±0.05	202±7	149±5	64±2	73±2	4±0	148±5	1±0	13.7	10.0
	3	1.53±0.04	225±6	112±3	53±1	55±1	0±0	110±3	3±0	14.7	7.3
H3	1	1.28±0.02	171±2	154±2	62±1	75±1	2±0	146±2	7±0	13.3	12.0
	2	1.47±0.02	216±3	133±2	56±1	64±1	2±0	126±2	7±0	14.7	9.1
	3	1.44±0.05	204±8	106±4	44±2	54±2	0±0	100±4	5±0	14.2	7.3
CP intake[3] groups											
≤180	-	1.30±0.06	145±10	81±20	35±7	36±11	5±1	78±19	3±1	11.2±1.6	6.5±4.3
>180	-	1.50±0.06	210±10*	127±20	56±7	62±11	1±1	124±19	4±1	14.0±0.7	8.0±1.8
WSC-f[3] groups											
≤100	-	1.41±0.07	160±14	64±5	32±3	25±2	4±1	60±5	4±1	11.3±1.7	4.4±1.3
>100	-	1.47±0.07	204±14	138±5*	59±3*	69±2*	0±1*	134±5*	4±1	13.9±0.5	9.4±1.0

[1] One horse had all observations within the highest CP intake group and was therefore excluded.

[2] Calculated from total water-soluble carbohydrate fraction minus fructans.

[3] LS Means ± standard error except for CP% and WSC-f%, which are means ± standard deviation.

* Indicates significant difference between CP or WSC-f intake groups ($P<0.05$, Tukey's test).

difference in CP intake (Table 1). The amino acid intakes were higher in the high CP group compared to the low CP group, except for arginine intake that was highest in the low CP intake group (Table 2). Intakes of aspartate, glutamate, leucine, alanine valine and proline were numerically highest on both diets. Ornithine and hydroxyproline contents were below the detection limit and therefore this data is not shown.

Effect of feeding occasion and intake group

There were no overall effects of feeding occasion on insulin AUC (AUC_A: 29±2 vs. AUC_B: 30±2) or glucose AUC (AUC_A: 627±14 vs. AUC_B: 623±14) (P>0.05). The ANOVA model including horse, CP group and WSC-f intake explained 95% of the variation in plasma insulin. The insulin AUC response to feed intake tended to be higher in the high CP intake group than in the low CP intake group before but not after exercise (Table 3). There was a positive correlation between individual CP intake and insulin AUC (r=0.50, P=0.04; r=0.45, P=0.06 and r=0.45, P=0.03 for AUC_A, AUC_B and AUC_{A+B}, respectively). The glucose AUC response was not affected by CP intake group (Table 3).

The plasma insulin levels was elevated compared to before feeding (0 min) in the low CP intake group at 20, 40, 60, 90 and 120 min after feeding A and at 20, 40, 60 and 90 min after feeding B and in the high CP intake group in all sampling occasions after both feeding A and B (Figure 2). Peak insulin levels were observed after 90 min in both intake groups after feeding A, and after 40 and 60 min in the low and high CP intake groups, respectively, after feeding B (Figure 2). However, there was no interaction between sample and intake group. Plasma glucose was elevated only at 90 min after feeding A in the both CP intake groups and at 120 min after feeding B in the high CP intake group. There was no difference in the plasma insulin or glucose response at PE between CP intake groups (Figure 2).

The creation of two groups based on WSC-f intake resulted in higher insulin AUC in the high intake group than in the low (AUC_A: 32±3 vs. 22±2, AUC_B: 34±5 vs. 22±4, AUC_{A+B}: 66±5 vs. 43±4), but no differences in glucose AUC.

Table 2. Daily intake (LS means ± standard error) of amino acids (g/100 kg body weight) in five horses on forage-only diets providing two levels of crude protein (CP) intake (≤180 (n=5) and >180 (n=5) g/100 kg body weight).

	CP intake ≤180	CP intake >180
Alanine	8.7±0.4	11.1±0.4*
Arginine	4.3±0.2	3.3±0.2*
Aspartate	10.4±0.5	14.3±0.5*
Cysteine	0.9±0.0	1.4±0.0*
Glutamate	9.8±0.4	13.2±0.4*
Glycine	5.9±0.3	8.1±0.3*
Histidine	1.8±0.1	2.4±0.1*
Isoleucine	5.7±0.3	7.8±0.3*
Leucine	9.0±0.4	12.9±0.4*
Lysine	5.2±0.2	6.5±0.2*
Methionine	1.8±0.1	2.6±0.1*
Proline	7.7±0.3	9.9±0.3*
Phenylalanine	5.1±0.2	7.8±0.2*
Serine	4.6±0.2	6.3±0.2*
Threonine	4.8±0.2	6.6±0.2*
Tyrosine	4.4±0.2	5.4±0.2*
Valine	7.7±0.3	10.4±0.3*

* Indicates significant difference between CP intake groups (P<0.05, Tukey's test).

Table 3. Glucose and insulin areas under curves (LS Mean ± standard error) for feeding A (before exercise, AUC_A) and B (after exercise, AUC_B) in five horses on forage-only diets providing two levels of total daily crude protein intake (≤180 and >180 g/100 kg body weight).

	CP intake ≤180	CP intake >180	*P*-value
Insulin			
AUC_A	23±2	32±3	0.08
AUC_B	26±3	32±4	0.32
AUC_{A+B}[1]	49±5	65±5	0.12
Glucose			
AUC_A	618±27	638±30	0.67
AUC_B	596±17	655±19	0.11
AUC_{A+B}[1]	1,214±42	1,292±47	0.32

[1]Total area of curves AUC_A and AUC_B.

4. Discussion

Although this study includes a low number of observations the results indicate that the post-prandial plasma insulin response in horses fed a forage-only diet can be increased by high CP and amino acid intake. An ANOVA model including horse, CP group and WSC-f intake explained 95% of the variation in plasma insulin response compared to 87% using a model including horse and WSC-f group alone.

The elevation in insulin response brought about by simultaneous ingestion of readily available carbohydrates and protein or amino acid confirms previous findings in horses (Stull and Rodiek, 1988; Urschel *et al.*, 2010) and other mammals (Rabinowitz *et al.*, 1966; Rérat *et al.*, 1985; Van Loon *et al.*, 2000b). The increase in insulin response observed with higher CP and amino acid intakes in the present study could explain the high muscle glycogen content and enhanced post-exercise glycogen synthesis

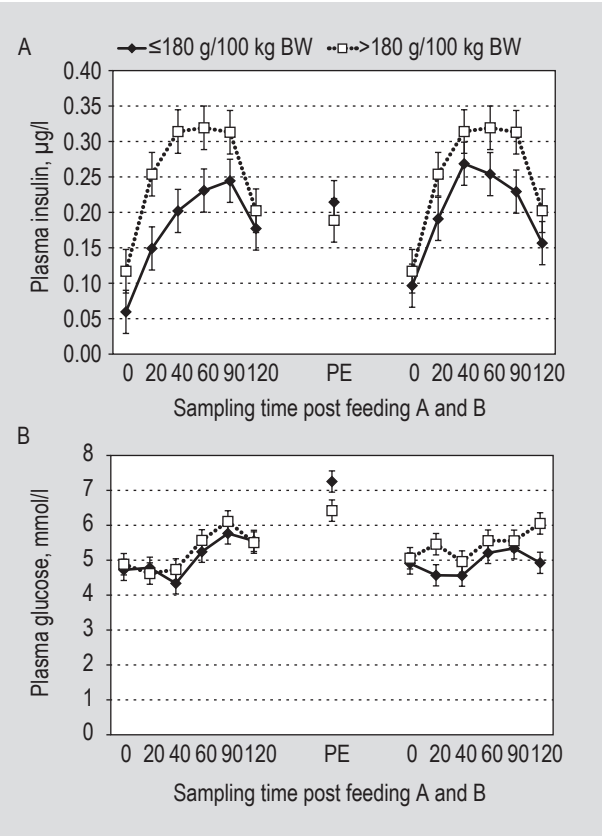

Figure 2. (A) Plasma insulin and (B) plasma glucose response 0, 20, 40, 60, 90 and 120 min after feeding A (rest), 1 min post exercise (PE) and after feeding B (after exercise) in five horses fed two forage-only diets resulting in different crude protein (CP), but not water soluble carbohydrate intakes (LSmean ± standard error).

reported previously in horses in training fed forage with a high CP content (Essén-Gustavsson *et al.*, 2010), since insulin stimulates glycogen synthase. However in the present study, the effect of feeding high CP forage on insulin response tended to be elevated only before exercise and not after, indicating that the effect of different CP intakes on the insulin response in the immediate post exercise period might be less important. Accordingly, the increase in muscle glycogen content observed by Essén-Gustavsson *et al.* (2010) within 90 min post-exercise may not have been insulin-dependent. Ivy and Kuo (1998) suggest that glucose entrance and glycogen synthesis are both independent of insulin for up to 1 h post-exercise in humans and that early post-exercise muscle glycogen recovery is enhanced with a carbohydrate-protein supplement, without any clear effects on the plasma insulin response (Ivy *et al.*, 2002). In the present study, the second highest amino acid intake observed was for leucine and as for most of the amino acids, it was numerically higher on the high CP diet compared to the low CP diet. Studies in rats indicate that branched amino acids and in particular leucine can also stimulate glucose uptake and glycogen synthesis through insulin-independent mechanisms (Morifuji *et al.*, 2010; Nishitani *et al.*, 2002). In the study by Essén-Gustavsson *et al.* (2010), the plasma BCAA and muscle leucine content was also significantly elevated. However, the lack of insulin response to forage CP intake post-exercise contradicts findings in a study on horses by Urschel *et al.* (2010), where there was a significant increase in the post-exercise insulin response to gastric gavage including both glucose and leucine compared with glucose alone. However, intake (gastric gavage) of glucose and leucine was higher in their study, 1 and 0.3 g/kg BW, respectively, which corresponds to 530% and 230% of the intake of WSC-f and leucine in the high CP group in the present study. In general, when corrected for differences in DM intake, the amino acid intake in the present study was similar or little lower than the intakes observed by Essén-Gustavsson *et al.* (2010) on forage with higher CP contents.

Although the present study indicated an effect of CP intake on the insulin response, including horse and WSC-f intake group alone in the ANOVA model resulted in a higher R^2 than a model including horse and CP intake group alone (0.87 vs. 0.82). As expected, this indicates that intake of soluble carbohydrates explains more of the variation in plasma insulin than CP intake. An effect of forage WSC intake on plasma insulin response has previously been shown by Borgia *et al.* (2011) and Ragnarsson and Jansson (2011). In the former study, on healthy but unfit Quarter horses (BCS 4.5-6), peak plasma insulin levels were 3.4-fold higher with hay containing 12% WSC (glucose, fructose and sucrose) and 12% CP than with hay containing only 2% WSC and 10% CP (2.5 vs. 0.7 µg/l). However, even on the hay with the extremely low WSC content, there was still a marked increase in the response, 4-fold the baseline and >2.5-fold higher than observed in the low intake group in the present study. In the study by Ragnarsson and Jansson (2011), where two groups of horses (Icelandic horses with BCS 7.5 and Standardbred horses with BCS 4.5) were fed two haylages, one containing (DM basis) 11% CP and 8% WSC (fructans included) and another containing 6% CP and 15% WSC (fructans included), the post-prandial plasma insulin response in the Standardbred horses was significantly higher (1.0 vs. 0.6 µg/l) on the haylage with the higher WSC content. Interestingly, in the fatter Icelandic horses, the overall insulin response was significantly higher than in the Standardbred horses (>2-fold) but similar (.2 µg/l) on the two haylages. This indicates that horses likely to be less sensitive to insulin may respond markedly either to quite low WSC intake and/or increased CP intake.

It is probable that horses with insulin resistance could benefit from a diet resulting in low plasma insulin response, since high insulin levels can induce laminitis (Asplin *et al.*, 2007). It is currently recommended that such horses be fed hay containing less than 10-12% non-structural carbohydrates (Frank, 2007, 2009), corresponding to approximately 12-13% of DM. However, the basis for this

recommendation is unclear and based on the response observed in the fat and unfit horses studied by Ragnarsson and Jansson (2011) and Borgia *et al.* (2011), it could be suggested that even forage with 2-8% WSC per kg DM (including fructans) is an unnecessary challenge. The lowest insulin response to forage feeding reported so far appears to be in the study by Connysson *et al.* (2010), where the insulin concentration was elevated to only 0.1 µg/l within 30 min in fit Standardbred horses after ingestion of 1.4 kg DM of grass forage containing (DM basis) 7% WSC (glucose, fructose and sucrose) and 12% CP.

Obese, insulin-resistant horses would benefit from forage that is not only low in WSC, but also has low digestible energy content. Forage with low energy content can easily be produced by harvesting when grasses have matured, but unfortunately the WSC content of grass at harvest is difficult to predict using indirect methods. However, in mature grasses the CP content is generally low and in many cases below dietary requirements, even for adult horses. The CP requirement of an adult horse is approximately 1.3 g per kg BW and day (NRC, 2007) and during all feedings but two in the present study (where the intake was 1.1 and 1.2 g CP/kg BW), this requirement was estimated to be met. To avoid exceeding the insulin response observed in the low CP intake group in the present study without undersupplying the recommended intake of CP, a maximum WSC-f concentration in forage of 6.5% would be needed (Table 1). This is slightly lower than the previous suggestion. However, even in the low CP group there was a marked increase in the insulin response and it could be expected that in a group of insulin-resistant horses, the response would have been even higher, as in the study by Ragnarsson and Jansson (2011). Altogether, it could therefore be suggested that forage intended for feeding to insulin-resistant horses should have a WSC-f content of less than 6% of DM.

The present study also sheds some light on the occurrence of pasture-associated laminitis. In fact, most laminitis cases tend to occur in horses kept at pasture (Geor, 2009), which tend to spend most of their feeding time on short grass (≤4 cm; Fleurance *et al.*, 2001). At this botanical stage, grasses have very high digestibility and high CP content. We have observed CP contents of 20-25% of DM in Swedish pastures grazed by horses and alpaca (which also graze very short grass) (unpublished). We therefore suggest that the high CP content of the short grasses preferentially grazed by horses might augment the diurnal insulin response and therefore may pose a risk factor for horses prone to laminitis.

In this study the insulin response was related to the intake of WSC excluding fructans. Fructans are typically not digested before the hind gut (Oku *et al.*, 1984), resulting in no glucose uptake, although Bailey *et al.* (2007) showed that fructans can also induce some insulin response. However, the intake of fructans, and of sucrose, was small compared with the intake of fructose and glucose and simple correlation analysis revealed no positive correlations between insulin response and fructans and sucrose intake (data not shown).

This study examined AUC responses rather than peak responses, although the latter are easier to compare between different studies. However, peak glucose and insulin levels may be reached quite quickly after feeding and the same peak values have been observed in horses fed meals with 2 and 4 g of starch/kg BW (Radicke *et al.*, 1994) and in horses fed 20 and 50% of their daily concentrate allowance in one meal (Jansson *et al.*, 2006). Therefore, it appears that above a certain level of starch intake, the plasma insulin response does not respond further to increases in starch intake, as reported previously by Vervuert *et al.* (2009) for intake of >1.1 g/kg BW. Using AUC may therefore reflect the impact of insulin in a better way.

This study also highlights the variation that can be expected within forage batches and the importance of collecting forage samples of the feed ration that is actually going to be fed in experiments rather than relying on average batch or bale analyses. A forage batch analysis should be used in practical feeding to estimate average daily intake over a longer period, but it cannot be used with any precision to assess intake on a daily basis. In this study, preliminary batch analyses were made on samples collected by hand before the crop was compressed and wrapped in plastic. During the experimental days, 3 samples from each batch were collected by hand and the coefficient of variation in CP content was 15% in the batch with the lowest CP content. Another important factor in obtaining a good estimate of long-term nutrient intake is to collect a representative forage sample. Analysis of a non-representative sample is of no value and using the data for formulation of feed ratios could of course cause long-term nutrient deficiency.

In conclusion, this study indicates that the post-prandial plasma insulin response in horses at rest fed a forage-only diet can be increased not only by a high WSC content, but also by high CP intake, and that the effect of different forage CP intakes on the response post exercise might be less important. However, due to the low number of observations further studies are needed.

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