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The impact of dietary protein levels on nutrient digestibility and water and nitrogen balances in eventing horses[1]

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ABSTRACT: This study was performed to evaluate the impact of dietary protein levels on nutrient digestibility and water and nitrogen balances in conditioning eventing horses. Twenty-four Brazilian Sport Horses, male and female (8.0 to 15.0 yr; 488 ± 32 kg BW), were used in a randomized design with 4 levels of CP diets: 7.5%, 9.0%, 11.0%, and 13.0%. A digestion assay was performed with partial feces collection over 4 d, followed by 1 d of total urine collection. Data were submitted to regression analysis and adjusted to linear and quadratic models ($P < 0.05$). No differences were observed in the intake of DM, OM, EE, ADF, and NDF as a function of dietary protein levels. Dry matter intake average was 1.7% of BW. CP and N intake showed a linear increase as a function of increasing protein level in diets. A quadratic response ($P < 0.05$) was observed on the CP and NDF digestibility coefficients, with the maximum estimated level of digestibility at 11.6% and 11.4% CP in the diet, respectively. There was a linear effect on ADF digestibility coefficients, digestible DM and protein intake, and CP/DE ratio according to dietary protein levels. There was no impact of dietary protein levels on daily water intake, total water intake, or fecal water excretion. Urinary excretion values showed a linear increase in response to increased dietary protein levels, but no impact was observed on water balance, with an average of 8.4 L/d. Nitrogen intake (NI), N absorption (NA), and urinary N increased linearly as a function of increasing dietary protein levels. There was no impact of dietary protein levels on N retention (NR), with an average of 7.5 g N/d. Nitrogen retention as a percentage of NI or NA showed no significant changes in the function of dietary protein levels. There was an impact of dietary protein levels on the digestibility coefficient of CP, NDF, ADF, and digestible protein intake on conditioning eventing horses. The 11.6% CP level in the diet provided an intake of 2.25 g CP/kg BW and 0.37 g N/kg BW, and this intake was the most appropriate for the conditioning of intensely exercised horses, considering the responses related to NI, NA, and the estimated NR to NA ratio. The NDF and ADF responses indicated that dietary fiber was more digested with an increased amount of N in the digestive tract.

Key words: conditioning, crude protein, equine, exercise, intake, NDF

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INTRODUCTION

Horse diets usually contain more protein than necessary to meet nutritional requirements (Hintz, 1994; Graham-Thiers and Kronfeld, 2005). Just a few controlled studies have evaluated the effects of nutrients on water intake and water excretion in horses, but DM and intake level are known to modify the water balance in horses (Hintz, 1994). Protein digestibility is related to the protein source, concen-

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tration of protein in the diet, the ingredients, and the forage:concentrate ratio of the diet (NRC, 2007).

The majority of digestibility studies already published have used horses at maintenance level (Julliand et al., 1993; Almeida et al., 1998; Drogoul et al., 2001; Oliveira et al., 2003a, 2003b; Graham-; Graham- Thiers and Kronfeld, 2005) or horses at maintenance submitted to a short conditioning period. Additionally, few studies related to horses performing long-term exercise as a routine exist.

Previous studies reported that the nitrogen balance increased in exercising horses during training and remained at greater levels even after training was discontinued, implying that more dietary protein is required for the maintenance of muscle mass gained during training (Freeman et al., 1988; Graham-Thiers and Bowen, 2011a). The amount and profile of dietary protein for exercising horses may have particular importance in maintaining muscle integrity and improving postexercise recovery in competition horse (Hackl et al., 2009). In addition, the protein balance in exercising horses can be adjusted by reducing or increasing protein excretion, indicating that protein could be used for muscle mass repositioning or indicating the adaptation of the animal to the levels of protein intake for the exercise intensity (Graham-Thiers and Bowen, 2011b).

The aim of the present study was to evaluate the impact of 4 different dietary protein levels on nutrient digestibility and water and nitrogen balances in conditioning eventing horses.

MATERIALS AND METHODS

This experiment was approved by the Ethics in Research Board of Universidade Federal Rural do Rio de Janeiro, number 029/2009.

Horse Management

Twenty-four Brazilian Sport Horses, 16 males and 8 females, with ages ranging from 8 to 15 yr, BW ranging from 432 to 562 kg, and BCS ranging from 5.0 to 5.5 (Henneke et al., 1983), were used. During the study, horses were randomly distributed in individual 4 × 4 m box stalls, with a water dispenser, a feeder, and wood shaving bedding. The horses were used for the 2010 Brazilian Army Cavalry School instructors' course and continued their training classes and competition schedule during the experimental assay (150 d), except for the digestibility assay (6 d). The weekly workload consisted of 6 d per week with 60 min of daily training, including 30% walking, 30% trotting, 10% galloping, and 30% jumps on dirt and grass tracks, corresponding to intense physical activity (NRC, 2007) targeting eventing modality. During the digestibility assay, the animals were kept in their stalls and walked on an automated exerciser for 30 min twice a day. The trial was conducted in July (winter), when the temperature ranged between 18°C and 25°C.

Table 1. Feed nutrient composition on a DM basis

Nutrient	Soybean meal	Rolled oat	Concentrate[1]	Soy oil	Coast cross hay
DM, %	87.43	89.15	91.86	88.95	86.83
CP,[2] %	40.5	10.0	13.1	—	5.3
GE,[3] Mcal	35.6	46.4	36.2	93.3	37.5
EE,[4] %	0.9	3.9	4.8	99.6	1.0
NDF,[5] %	15.3	26.2	28.5	—	66.4
ADF,[5] %	10.7	13.6	11.1	—	45.0

[1]Nutriequi BR extruded (Guabi Alimentos) (Guabi Alimentos, Campinas-SP, Brazil): CP, 130 g; ether extract, 70 g; Ca, 12 g; P, 7 g; Lys, 9 g.

[2]Kjeldahl method.

[3]Parr adiabatic bomb calorimeter.

[4]Goldfisch fat extraction with petroleum ether.

[5]Van Soest et al. (1991).

Experimental Design

A digestibility assay was conducted using a randomized design with 4 dietary protein levels (diets) and 6 repetitions (horses). Horses were assigned to 1 group, depending on their sex and experience in competitions to balance the 4 different groups. Diet adaptation lasted 21 d and was followed by partial feces collection over 4 d (Oliveira et al., 2003b) and then 1 d of total urine collection (Cymbaluk, 1989).

Diets

The experimental diets were formulated on the basis of the nutritional requirements for mature horses in intense physical activity, according to the NRC (2007). The intake was controlled as 1.8% of BW, corresponding to 90% of the recommended NRC (2007) daily intake, to minimize refusals. The diets were composed of soybean meal, rolled oats (*Avena sativa*), concentrate, soybean oil, and coast cross hay (*Cynodon dactylon*; Table 1). Diets were formulated with 4 different levels of CP: 7.5%, 9.0%, 11.0%, and 13.0%, defined as diets 1, 2, 3, and 4, respectively (Table 2), at a concentrate:roughage ratio of 50:50 on a DM basis. L-Lysine was included in diet 1 to ensure proper requirements, as described in NRC (2007). Diets were formulated with an average of 42.8% NDF and 27.5% ADF, according to NRC (2007).

The concentrate was fed 3 times daily in equal amounts at 0400, 1300, and 2000 h, and the roughage

Table 2. Diet ingredients and nutrient composition on a DM basis

	Diet			
Item	1	2	3	4
Ingredient				
Soybean meal, %	1.5	7.0	12.5	17.5
Rolled oat, %	28.3	23.0	19.5	15.8
Concentrate,[1] %	10.0	10.0	8.0	7.0
Soybean oil, %	7.0	7.0	7.0	6.7
Mineral supplement,[2] %	2.0	2.0	2.0	2.0
Calcium carbonate, %	1.0	1.0	1.0	1.0
L-lysine,[3] %	0.15	—	—	—
Coast cross hay, %	50	50	50	50
Total	100	100	100	100
Composition				
DM, %	88.1	88.0	87.9	87.9
CP,[4] %	7.5	9.0	11.0	13.0
EE,[5] %	9.0	8.9	8.7	8.3
NDF,[6] %	43.7	43.1	42.5	42.0
ADF,[7] %	27.6	27.5	27.3	27.2
DE,[7] Mcal	27.6	28.1	29.2	29.4
DE,[8] Mcal/kg DM	2.8	2.8	2.8	2.8

[1]Nutriequi BR extruded (Guabi Alimentos, Campinas-SP, Brazil): CP, 130 g; ether extract, 70 g; Ca, 12 g; P, 7 g; Lys, 9 g.

[2]Omolene-phos (Purina, Paulínia_SP, Brazil): Ca (max), 150 g; P, (min), 70 g; S, 10 g; Mg, 10 g; Na, 150 g; Fe, 2.500 mg; Cu, 820 mg; Zn, 2,500 mg; Mn, 2,124 mg; I, 20 mg; Se, 12.5 mg; Co, 20 mg; Cr, 6 mg.

[3]L-lysine was included in diet 1 to ensure that it was not a limiting factor in the performance.

[4]Kjeldahl method.

[5]Goldfisch fat extraction with petroleum ether. EE = ether extract.

[6]Van Soest et al. (1991).

[7]Calculated: (GE intake × digestibility coefficient of GE) × 100.

[8]Calculated: [(GE intake × digestibility coefficient of GE) × 100]/DMI.

was fed 2 times daily in equal amounts at 1100 and 1600 h. Soybean oil, previously weighed and calculated individually, was added directly to the concentrate 3 times daily in equal amounts.

Feed and Fecal Sample Collection

Samples of feed and eventual refusals were collected every day during the digestibility assay and were weighed, placed into plastic bags, and frozen. Fecal samples were collected from the rectum (200 g) twice a day at 0800 and 1700 h for 4 d. Samples were immediately placed into plastic bags and frozen at −18°C. At the end of the collection period, composite samples for analyses were made from daily samples of feed, from daily samples of refusals, and from daily samples of feces for the whole 4-d collection period for each animal. At the end of the experimental period, feeds, refusals, and feces were thawed at room temperature and oven-dried through forced ventilation at 55°C until constant weight. After drying, samples were ground into 1-mm pieces, for chemical analyses.

Laboratory Analyses

Feed and feces sample analyses were performed to determine DM, OM, and CP using the Kjeldahl method, ether extract (**EE**) using a Goldfisch fat extraction apparatus with petroleum ether, and GE using the Parr adiabatic bomb calorimeter. Neutral detergent fiber and ADF were determined according to Van Soest et al. (1991) Urine samples were analyzed to determine the amounts of DM and CP (Kjeldahl method). The analyses were performed according to the methodology described by Silva and Queiroz (2002).

The digestibility coefficients were indirectly estimated using the internal marker of indigestible NDF (**iNDF**) according to Oliveira et al. (2003). The levels of the iNDF were obtained after in vitro incubation of samples from feed, refusals, and feces for anaerobic fermentation at 39°C for 144 h, according to the methodology described by Cochran et al. (1986). In vitro incubation was conducted in 100-mL bottles in 2 repetitions, with 0.5 g of sample in each bottle. A buffer solution was used, as described by Theodorou et al. (1994). The final solution consisted of a mixture of ruminal fluid (inoculum) and buffer solution at a 1:4 ratio, and carbon dioxide was injected into the bottles to ensure anaerobic conditions. Ruminal fluid was collected through a nasogastric tube from 2 cattle fed strictly roughage. Each bottle contained samples moistened with 2 mL of 100°C distilled water and, 40 mL of the final solution were added to each bottle. Bottles were then placed in a water bath at 39°C for 144 h. Twice daily, the bottles were gently stirred (Cochran et al., 1986). At the end of the digestion process, the contents of the bottles were transferred to 600-mL beakers, in which the NDF analysis was performed for the determination of iNDF.

The equations for estimating the coefficients of the apparent digestibility of the diets and of the recovery marker were proposed by Church (1979):

$$\text{Digestibility coefficient of DM } (\%) = 100 - \frac{100 \times (\%\text{ diet iNDF})}{\%\text{ feces iNDF}},$$

$$\text{Digestibility coefficient } (\%) = 100 - \frac{100 \times (\%\text{ diet iNDF}) \times (\%\text{ feces nutrient})}{(\%\text{ feces iNDF}) \times (\%\text{ diet nutrient})},$$

$$\text{Recovery marker } (\%) = 100 \times \frac{(\text{feces iNDF, g})}{(\text{intake iNDF, g})},$$

$$\text{Total fecal DM output } (\text{kg}) = \frac{\text{Intake iNDF } (\text{kg})}{\%\text{ iNDF on feces}}.$$

Water and Nitrogen Balances

To determine the water and nitrogen balances, urine sampling was performed in 2 periods of 12 h for 2 consecutive days (from 0000 to 1200 h on the first day and from 1200 to 0000 h on the second day), totaling 24 h. For the urine collection, only males were used (16 horses, 4 males in each treatment group). Horses wore rubber collectors, similar to those described by Tasker (1966). The urine samples were placed in plastic containers containing 25 mL of 5 *M* hydrochloric acid to avoid the volatilization of ammonia. During the 24-h period, 2 urine samples (200 mL each) were collected and immediately stored at −18°C for nitrogen analysis. Water intake was measured during the digestion assay and the urine collection period to determine the water balance. Water was offered in 100-L containers that were measured and cleaned 4 times a day. The water balance was evaluated using the following equations (Cymbaluk, 1989):

Total water intake (L/d) = Drinking water (L/d) + Diet intake water (L/d),

Total water excretion (L/d) = Urine water (L/d) + Fecal water (L/d),

Water balance (L/d) = Total water intake (L/d) − Total water excretion (L/d).

The nitrogen balance was evaluated according to the amount of nitrogen compounds taken in through the diet and excreted in feces and urine. The amounts of nitrogen compounds absorbed (**NA**) and retained (**NR**) were calculated by the equations

NA (g/d) = N intake (g/d) − fecal N (g/d),

NR (g/d) = N intake (g/d) − fecal N (g/d) – urinary N (g/d).

Statistical Analysis

Data from the digestion assay and water and nitrogen balances were input to a regression analysis testing the effect of the diet (protein levels) and adjusted to linear and quadratic models using Statistical and Genetics Analyses System (SAEG 9.1) software (Fundação Arthur Bernardes - Universidade Federal de Viçosa, Viçosa-MG, Brazil). The individual horse effect was considered a random effect. The significance threshold for all tests was set at $P < 0.05$. The variable response (X) was analyzed according to the following statistical model: $X_{ij} = \mu + \alpha_i + C_j + \varepsilon_{ij}$, where X_{ij} is the studied variable, μ is the overall mean, α_i is the fixed effect of the ith diet, C_j is the random effect of the jth animal, and ε_{ij} is the normally distributed error.

RESULTS

Diets and Nutrient Intake

In the present study, the horses' average intake was 8.3 kg DM/d (4.2 kg hay/d and 4.1 kg concentrate/d). No differences were observed in the DMI among horses fed diets with different protein levels. There were no differences in the intake values for OM, EE, ADF, or NDF (Table 3). Linearly increasing responses were observed for CP intake ($\hat{Y} = 100.8x - 19.7$, $r^2 = 0.73$) and N intake ($\hat{Y} = 1.6x - 3.1$, $r^2 = 0.73$) with increasing dietary protein levels. Crude protein intake, expressed either in grams per kilogram of DM or in grams of CP per kilogram of BW, showed the same linear response with increasing levels of protein in the diet. There was no horse effect. Horses' BCS and BW did not change during digestibility assay, with an average of 5.0 ± 0.49 and 488.2 ± 32 kg, respectively.

Coefficients of Digestibility

The NDF recovery marker was 102%. No differences were observed in the apparent digestibility coefficient values for DM, OM, GE, and EE among the diets with different protein levels. The mean values were 52.2%, 51.8%, 83.7%, and 86.5% for DM, OM, GE, and EE digestibility, respectively (Table 3).

A trend for a quadratic response was observed for DM digestibility ($P = 0.055$) with increasing dietary protein level, with the maximum estimated response of CP digestibility at 10.9% CP in the diet. Also, a quadratic response was observed for OM digestibility ($P = 0.019$) with increasing dietary protein level, with the maximum estimated response at 10.9% CP.

For the CP digestibility coefficient, a quadratic response was observed with increasing dietary protein level, with the maximum estimated response ($P < 0.05$) of CP digestibility at 11.6% CP in the diet (Table 4), which supplied an intake of 2.25 g CP/kg BW (Fig. 1).

A quadratic response ($P = 0.016$) of the NDF digestibility coefficient and a linearly increasing response ($P = 0.029$) of the ADF digestibility coefficient were observed with increasing levels of protein in the diets. The maximum estimated response of NDF digestibility was at 11.4% CP in the diet, which supplied an intake of 2.2 g CP/kg BW. No differences were observed in the apparent digestibility coefficient values

Table 3. Average of BW and intake of nutrients in horses fed diets with different protein levels[1]

	Diet[2]					
Intake	1	2	3	4	SEM	Linear P-value[3]
BW, kg	488.5 ± 24	482.3 ± 38	489.0 ± 23	493.6 ± 42	2.9	0.368
DMI, % BW	1.6 ± 0.2	1.7 ± 0.2	1.7 ± 0.3	1.7 ± 0.2	0.02	0.194
DMI, kg/d	8.0 ± 0.9	8.3 ± 0.9	8.4 ± 1.3	8.5 ± 0.7	0.8	0.174
OM, kg/d	6.7 ± 0.8	6.8 ± 0.7	6.8 ± 1.0	7.0 ± 0.5	0.9	0.226
EE, kg/d	0.12 ± 0.9	0.10 ± 0.7	0.11 ± 0.2	0.10 ± 0.3	72.9	0.089
NDF, kg/d	3.8 ± 0.5	3.8 ± 0.5	3.8 ± 0.7	4.0 ± 0.3	1.0	0.275
ADF, kg/d	1.7 ± 0.3	1.8 ± 0.2	1.7 ± 0.3	1.8 ± 1.0	50.7	0.318
CP, g/d	754.9 ± 0.8	884.6 ± 1.0	1,039.8 ± 1.3	1,208.3 ± 1.2	24.8	<0.001[4]
CP, g/kg DM	94.4 ± 4.4	107.3 ± 3.8	124.7 ± 4.4	141.9 ± 3.3	1.9	<0.001[5]
CP, g/kg BW	1.6 ± 0.2	1.8 ± 0.2	2.0 ± 0.3	2.5 ± 0.9	20.4	<0.001[6]
N, g/d	120.8 ± 13.5	141.5 ± 16.4	166.3 ± 20.4	193.3 ± 20.1	0.04	<0.001[7]

[1]Intake of DM, OM, ether extract (EE), NDF, ADF, CP, and N is on a DM basis.

[2]Diets: 1 = 7.5% CP, 2 = 9.0% CP, 3 = 11.0% CP, and 4 = 13.0% CP.

[3]Linear relationships were analyzed between each item and increasing CP level of the diet ($P < 0.05$).

[4]$\hat{Y} = -19.65 + 100.87x$, $r^2 = 0.73$.

[5] $\hat{Y} = 12.55 + 1.063x$, $r^2 = 0.94$.

[6] $\hat{Y} = 0.218 + 0.20x$, $r^2 = 0.80$.

[7]$\hat{Y} = -3.14 + 1.61x$, $r^2 = 0.72$.

for GE and EE among the diets, with mean values of 83.7% and 86.5%, respectively.

Regarding the digestible nutrients, there was a linear response in the digestible DMI ($P = 0.016$), digestible protein intake ($P < 0.05$), and CP:DE ratio ($P < 0.05$), expressed in grams per megacalorie, with increasing levels of protein in the diets. The values of DE and the DE:BW ratio (kcal/kg) showed no differences among diets with different protein levels (Table 5).

Water and Nitrogen Balances

No differences among dietary treatment groups were observed for water intake per kilogram of DMI (3.0 ± 0.6 L/kg DM) or total water consumption (24.8 ± 2.1 L/d). No differences were observed in daily fecal water excretion, total water excretion, or water balance among diets. Urinary excretion values showed a linearly increasing response ($P = 0.019$) with increasing dietary protein levels. Horses showed positive water balance, with an average of 8.4 L water/d for all diets (Table 6).

With regard to the nitrogen balance, a linearly increasing response in nitrogen intake (**NI**), NA ($P <$

Table 4. Mean values of apparent digestibility coefficients of nutrients in horses fed diets with different protein levels

Digestibility	Diet[1]					P-value[2]	
coefficient	1	2	3	4	SEM	Linear	Q
DM,[3] %	48.9 ± 0.4	51.1 ± 0.6	56.4 ± 0.5	52.3 ± 0.1	0.51	0.079	0.055[4]
OM,[5] %	48.2 ± 0.4	51.4 ± 0.6	56.1 ± 0.5	51.8 ± 0.1	0.51	0.096	0.019[6]
CP,[5] %	59.9 ± 0.5	67.9 ± 0.2	73.1 ± 0.6	72.8 ± 0.2	0.69	<0.001	<0.001[7]
GE,[5] %	82.9 ± 0.1	83.4 ± 0.3	84.9 ± 0.2	83.7 ± 0.1	0.69	0.139	0.129
EE,[5] %	85.9 ± 0.2	86.4 ± 0.2	87.7 ± 0.2	86.1 ± 0.1	0.71	0.265	0.072
NDF,[5] %	32.5 ± 0.3	35.6 ± 0.8	43.8 ± 0.6	39.3 ± 0.2	0.21	0.016	0.016[8]
ADF,[5] %	22.8 ± 0.2	26.5 ± 1.1	32.3 ± 0.4	30.1 ± 0.2	0.19	0.029[9]	0.096

[1]Diets: 1 = 7.5% CP, 2 = 9.0% CP, 3 = 11.0% CP, and 4 = 13.0% CP.

[2]Linear and quadratic (Q) relationships were analyzed between each item and increasing CP level of the diet ($P < 0.05$).

[3]Calculated: $100 - \{[100 \times (\% \text{ diet iNDF})]/(\% \text{ feces iNDF})\}$.

[4]$\hat{Y} = -12.23 + 1.22x - 0.005x^2$, $R^2 = 0.16$.

[5]Calculated: $100 - \{[100 \times (\% \text{ diet iNDF}) \times (\% \text{ feces nutrient})]/[(\% \text{ feces iNDF}) \times (\% \text{ diet nutrient})]\}$. EE = ether extract.

[6]$\hat{Y} = 20.7 + 1.38x - 0.0063x^2$, $R^2 = 0.31$.

[7]$\hat{Y} = -34.76 + 18.64x - 0.80x^2$, $R^2 = 0.60$.

[8]$\hat{Y} = -43.31 + 1.569x - 0.0069x^2$, $R^2 = 0.32$.

[9]$\hat{Y} = 9.66 + 18.561x$, $r^2 = 0.20$.

Table 5. Mean values of digestible nutrient intake, relationship between CP intake and DE, and DE in relation to BW in horses fed diets with different protein levels

Digestible nutrient intake	Diet[1] 1	2	3	4	SEM	Linear P-value[2]
DM,[3] kg/d	3.9 ± 0.02	4.2 ± 0.06	4.6 ± 0.03	4.4 ± 0.04	0.05	0.016[4]
CP,[3] g/d	448.6 ± 70.0	600.9 ± 74.5	753.3 ± 68.8	876.8 ± 89.8	17.94	<0.001[5]
DE,[3] Mcal/d	27.6 ± 2.8	28.1 ± 3.3	29.0 ± 3.4	29.3 ± 2.3	0.28	0.444
CP:DE,[3] g/Mcal	27.4 ± 1.2	31.6 ± 2.2	35.8 ± 1.0	41.2 ± 1.5	0.55	<0.001[6]
DE:BW,[3] kcal/kg	56.5 ± 0.5	58.4 ± 0.4	59.4 ± 0.6	59.5 ± 0.1	0.48	0.120

[1]Diets: 1 = 7.5% CP, 2 = 9.0% CP, 3 = 11.0% CP, and 4 = 13.0% CP.

[2]Linear relationships were analyzed between each item and increasing CP level of the diet ($P < 0.05$).

[3]Calculated: (nutrient intake × digestibility coefficient) × 100.

[4]$\hat{Y} = 3.2 + 0.109x$, $r^2 = 0.22$.

[5]$\hat{Y} = -267.9 + 9.3x$, $r^2 = 0.85$.

[6]$\hat{Y} = 7.45 + 26.463x$, $r^2 = 0.93$.

0.05), and urinary nitrogen (**UN**; $P < 0.05$) was observed with increasing dietary protein levels (Table 7). The level of protein in the diets did not affect the fecal N excretion. The percentages of NR in relation to NI (%) or NR in relation to NA (%) were not significantly affected by the level of protein in the diets, with mean values of 4.7% and 7.3%, respectively.

DISCUSSION

The average daily DMI values observed in this study (1.7% of BW) were in agreement with DMI values reported by Miller and Lawrence (1988; 1.6% to 1.8% of BW), NRC (2007; 1.5% to 2.0% of BW), and Graham-Thiers and Bowen (2011a; 1.4% to 1.7% of BW), evaluated in exercising horses.

In the intense exercise category, the recommendations for mature horses are 1.7 g CP/kg BW and 0.27 g N/kg BW (NRC, 2007). In the present study, diet 1 provided 1.6 g CP/kg BW and 0.24 g N/kg BW; however, no symptoms of protein deficiency were observed. This result is in agreement with a previous study reporting that for efficient retention of N in 500-kg BW horses undergoing moderate to intense exercise, the protein intake should be equivalent to 1.6 g CP/kg BW (Graham-Thiers and Bowen, 2011c).

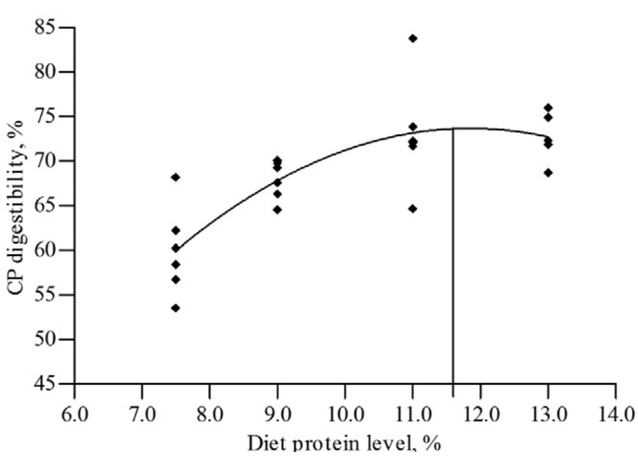

Figure 1. Coefficient of CP digestibility response as a function of protein levels (diamonds). Diets: 7.5%, 9.0%, 11.0%, and 13.0% CP. Quadratic relationships ($P = 0.001$) were analyzed between each item and increasing CP concentration of the diet, with the equation $\hat{Y} = -34.76 + 18.64x - 0.80x^2$ ($R^2 = 0.60$) and the maximum estimated response of CP digestibility at 11.6% CP in the diet, with an intake of 2.25 g CP/kg BW and 0.37 g N/kg BW.

The increasing dietary protein levels in the present study provided intakes of 1.8, 2.0, and 2.5 g CP/kg BW and 0.29, 0.35, and 0.41 g N/kg BW in diets 2, 3, and 4, respectively, which are greater than the NRC (2007) recommendations.

In the present study increasing dietary protein levels had an impact on the digestibility coefficients of CP, NDF, and ADF and tended to have an impact on the digestibility coefficients of DM and OM for horses subjected to intense exercise. Goachet et al. (2010) reported higher values for the DM digestibility coefficient, 67.7% and 69.2%, for intensely exercising horses with DMI of 1.7% of BW. According to Goachet et al., conditioning had an impact on digestibility, and this impact improved DM, OM, and NDF digestibility. We measured no significant differences in DMI or DM digestibility coefficients with increasing dietary protein levels in our eventing horses, but there was a trend in both variables, especially in DM digestibility coefficients. These variables tendency have an impact on digestible DM variable, that increased with increasing dietary protein levels.

Responses of CP digestibility coefficients in the present study were similar to those reported by Pereira et al. (1995) and Almeida et al. (1998), who observed a quadratically increasing response in CP digestibility coefficients with similar increasing CP levels in maintenance horses and foals. Higher CP digestibility values of 72% and 76% CP digestibility in 8.7% and 10% CP diets, respectively, were reported by Karlsson et al. (2000), who evaluated diets fed to lightly exercising horses. These observations confirmed that protein inclusion in horse diets

Table 6. Mean values of water balance in horses fed diets with different protein levels

Water balance	Diet[1] 1	2	3	4	SEM	Linear P-value[2]
Drink water, L/kg DM	2.6 ± 0.9	3.1 ± 0.3	2.9 ± 0.6	3.3 ± 0.5	0.25	0.063
Total water intake,[3] L/d	24.9 ± 1.1	23.8 ± 3.3	22.9 ± 1.8	27.1 ± 2.1	0.25	0.070
Urine water, L/d	6.3 ± 2.2	6.9 ± 1.0	7.6 ± 3.3	10.6 ± 2.3	0.26	0.019[4]
Fecal water, L/d	9.1 ± 1.2	8.2 ± 2.3	7.9 ± 3.0	8.4 ± 0.6	0.17	0.277
Total water excretion,[5] L/d	15.4 ± 1.8	15.1 ± 1.9	15.5 ± 4.5	19.0 ± 1.9	0.29	0.087
Water balance,[6] L/d	9.5 ± 1.5	8.6 ± 4.3	7.4 ± 4.0	8.1 ± 0.4	0.27	0.194

[1]Diets: 1 = 7.5% CP, 2 = 9.0% CP, 3 = 11.0% CP, and 4 = 13.0% CP.

[2]Linear relationships were analyzed between each item and increasing CP level of the diet ($P < 0.05$).

[3]Calculated: drinking water (L/d) + diet intake water (L/d).

[4]$\hat{Y} = -1.21 + 0.95x$, $r^2 = 0.30$.

[5]Calculated: urine water (L/d) + fecal water (L/d).

[6]Calculated: total water intake (L/d) − total water excretion (L/d).

improved CP digestibility; however, there was a maximum digestibility response for each requirement level (Farley et al., 1995). In the present study the maximum response of CP digestibility was expected at 11.6% CP in the diet, corresponding to an intake of 2.25 g CP/kg BW. The effect of CP level observed on digestible nutrients as DM and protein was related to the intake and digestibility coefficients. A linear increase in digestible protein was expected on the basis of the linear increase of CP intake and linearly increasing response of the CP digestibility coefficient. Anywise, all the positive digestible effects are beneficial for exercising horses, which need an efficient and highly digestible diet (Goachet et al., 2010).

The quadratic response of the NDF digestibility coefficient and the linear response of the ADF digestibility coefficient with increasing dietary protein levels indicated a response to fiber digestion. These responses suggested a positive effect of the protein in the cecum and colon, improving the cellulolytic bacteria (Julliand et al., 1993). Values of NDF digestibility higher than those observed in the present study were verified by Godoi et al. (2009), who reported an NDF digestibility value of 48.8% in oil-added diets with 13% CP in horses undergoing moderate conditioning. Also, Goachet et al. (2010) measured an average NDF digestibility value of 51.9% in conditioning horses fed diets with 11% CP.

In this study, the inclusion of soybean meal in the diets to increase dietary protein levels was responsible for the increasing CP, NDF, and ADF digestibility. The quality of the soybean meal protein is responsible for the high digestion and absorption of N observed in the nitrogen balance with increasing NI and UN and the constant N excretion in feces.

A greater amount of protein in the cecum and colon acts positively on the bacterial population and is beneficial for fiber digestion. However, protein excess in the hindgut can increase ammonia production and thus exceed the capacity of bacteria to utilize it. Con-

Table 7. Mean values of nitrogen balance in horses fed diets with different protein levels

Nitrogen balance[1]	Diet[2] 1	2	3	4	SEM	Linear P-value[3]
NI, g/d	119.4 ± 2.4	142.1 ± 13.5	171.6 ± 23.2	204.1 ± 19.9	30.0	<0.001[4]
FN, g/d	50.2 ± 5.6	51.9 ± 5.8	55.9 ± 14.5	62.5 ± 8.1	4.1	0.077
NA,[5] g/d	69.2 ± 5.5	90.1 ± 10.8	115.8 ± 13.7	141.6 ± 12.7	26.1	<0.001[6]
UN, g/d	62.3 ± 5.6	85.1 ± 7.6	108.3 ± 14.2	131.0 ± 8.1	31.3	<0.001[7]
NR,[8] g/d	6.8 ± 2.1	5.1 ± 3.3	7.5 ± 2.6	10.6 ± 8.2	3.1	0.198
NR,[9] % NI	5.7 ± 1.6	3.6 ± 1.9	4.4 ± 1.6	4.9 ± 3.6	2.5	0.149
NR,[10] % NA	9.8 ± 3.2	5.6 ± 2.7	6.6 ± 2.4	7.1 ± 5.2	4.7	0.119

[1]NI = nitrogen intake, FN = fecal nitrogen, NA = nitrogen absorbed, UN = urinary nitrogen, and NR = nitrogen retained.

[2]Diets: 1 = 7.5% CP, 2 = 9.0% CP, 3 = 11.0% CP, and 4 = 13.0% CP.

[3]Linear relationships were analyzed between each item and increasing CP level of the diet ($P < 0.05$).

[4]$\hat{Y} = -3.14 + 1.61x$, $r^2 = 0.72$.

[5]Calculated: NI (g/d) − FN (g/d).

[6]$\hat{Y} = -22.63 + 1.26x$, $r^2 = 0.83$.

[7]$\hat{Y} = -33.71 + 1.28x$, $r^2 = 0.87$.

[8]Calculated: NI (g/d) − FN (g/d) − UN (g/d).

[9]Calculated: (NR/NI) × 100.

[10]Calculated: (NR/NA) × 100.

sequently, this can result in an increase of ammonia blood levels and urea plasma levels. Ammonia plasma levels are regulated by hepatic metabolism, with the conversion of ammonia to urea that is excreted by the kidneys, with a potential increase in water excretion in urine (Cymbaluk, 1989). In the present study, the water excretion in urine increased as dietary CP increased, but water balance was not affected. These observations confirmed that the soybean meal had higher digestibility and absorption, although the N excess is excreted by urine in horses.

The efficiency of the cecum and colon fermentation activity in horses depends on substrate availability, and it is influenced by feed intake, diet composition, and prececal digestibility (Santos et al., 2012). Santos et al. (2012), studying in vitro fermentative responses of the cecum microbial population to N sources (casein and urea), observed that the protein N source influenced and promoted microbial growth and fermentative activity. Furthermore, according to Julliand and Tisserand (1992), supplementing soybean meal can stimulate the growth of the proteolitic microbial population in the cecum and colon. These observations could support the positive impact of dietary protein level on NDF and ADF digestibility, which is affected by greater microbial growth and fermentative activity. The NDF and ADF responses indicated that dietary fiber was more digested with the increase in the amount of protein N in the digestive tract.

Water intake is related to DMI and to the diet's composition (Cymbaluk, 1989). According to NRC (2007), horses fed hay and concentrate diets have an intake of 2.9 L water/kg DM, with the recommended intake ranging from 2 to 3 L/kg DM. In the present study the water intake was 2.6, 3.1, 2.9, and 3.3 L/kg DM in diets 1, 2, 3, and 4, respectively. Drinking water, fecal water, and total excretion water were not affected by dietary protein levels. Fecal water levels were positively correlated with DMI (Cymbaluk, 1989; Oliveira et al., 2003b, suggesting that the controlled DMI in the present study may explain no diferences for the total excretion water. In the present study, the sweat water losses were not taken into account because during the digestibility assay the horses were not in training and could not have had significant water loss from sweat.

Our observations confirmed a positive increasing response in N absorption as a function of the dietary protein level. This response was indeed reported earlier by Almeida et al. (1997), who evaluated the nitrogen balance in maintenance horses fed diets with increasing CP levels of 7.5%, 10.3%, and 13.2% and related N absorption values of 97.8, 203.6, and 254.8 g $N \cdot animal^{-1} \cdot d^{-1}$, respectively. In the present study, the average value of fecal N was 55.1 g $N \cdot animal^{-1} \cdot d^{-1}$, and N absorption values were 69.2, 90.1, 115.8, and 141.6 g $N \cdot animal^{-1} \cdot d^{-1}$ in response to the dietary protein levels of diets 1, 2, 3, and 4, respectively. In a previous study evaluating moderately exercising horses fed 12% CP in intermediate training conditions, Graham-Thiers and Bowen (2011b) found an NA average of 70 g $N \cdot animal^{-1} \cdot d^{-1}$.

Results similar to those obtained in the present study for urinary N excretion were observed by Freeman et al. (1988), Almeida et al. (1998), Antilley et al. (2007), Malesky et al. (2011), and Graham-Thiers and Bowen (2011b, 2011c). Those authors evaluated increasing levels of dietary protein and observed a positive linear response in the urinary N excretion as a function of the dietary protein levels, indicating urine was the main pathway for the excretion of nitrogenous compounds consumed in excess.

In a previous study, Graham-Thiers and Bowen (2011c) evaluated the nitrogen balance in conditioning horses and reported better NR values during training, which varied from 20.5 to 30.7 g $N \cdot animal^{-1} \cdot d^{-1}$. In the present study, the 11.6% CP diet appeared to be the most adequate to maximize the digestibility of dietary protein and the responses related to NR or the relation of NR to NA, although an increase in urine excretion did occur. Similar responses were observed by Graham-Thiers and Bowen (2011b) for conditioning horses with diets containing 11% CP.

An intake of 2.25 g CP/kg BW and 0.37 g N/kg BW appears to be the most adequate to maximize the digestibility of dietary protein and the responses related to NR or the relation of NR to NA. More research must be done to confirm the use of this knowledge in the field, with perspectives for future research with elite eventing horses.

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