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Repeated measurements of P retention in ponies fed rations with various Ca:P ratios[1]

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ABSTRACT: This study addresses the question of whether feeding rations rich in P for a period of up to 42 d induces a positive P balance in adult ponies. Biochemical bone markers and parathyroid hormone (PTH; intact as well as whole PTH) were measured to obtain clues as to the effect of P loading on bone metabolism. The experiment had a Latin square design. Each feeding period lasted 42 d, and there were 2 balance trials (ECP1 and ECP2) within each feeding period. Each balance trial lasted 10 d (ECP1: d 11 to 21; ECP2: d 33 to 42). Six ponies aged 2.5 to 7 yr were fed a control diet that provided P and Ca according to the requirement (Control diet: 54 mg Ca·kg $BW^{-1}·d^{-1}$; 36 mg P·kg $BW^{-1}·d^{-1}$), a diet high in Ca and P (HCaHP diet: 146 mg Ca·kg $BW^{-1}·d^{-1}$; 121 mg P·kg $BW^{-1}·d^{-1}$), and a diet with a high P level only and Ca fed to the requirement (HP diet: 54 mg Ca·kg $BW^{-1}·d^{-1}$; 122 mg P·kg $BW^{-1}·d^{-1}$). When fed the Control diet, the ponies showed a zero P and Ca balance over the 42-d period. The HCaHP diet resulted in both P and Ca retention (about 2 g Ca and P/d; $P < 0.05$). Phosphorus retention (about 2 g P/d) alone was observed when ponies were fed the HP diet, but P retention was only different ($P < 0.05$) from the Control diet in ECP1. The excretion of P in urine was reduced by greater Ca intake ($P < 0.05$), and Mg absorption was reduced by high P intake ($P < 0.05$). Plasma P concentration was raised by high P intake. Plasma Ca levels were not affected by dietary treatment. The greater ($P < 0.05$) P retentions observed for the HCaHP diet during ECP1 and ECP2 and HP diet during ECP1 could not be explained by processes that could have been indicated by the bone markers or PTH values. It was concluded that dietary-P-induced retention of P in ponies does not seem to be associated with altered bone metabolism in this study.

Key words: balance, bone, calcium, magnesium, phosphorus, retention

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INTRODUCTION

Nonpregnant, nonlactating mature animals with constant BW and body composition are expected to have a zero phosphorus (P) balance, but this is difficult to measure because of methodological problems related to balance trials (Duncan, 1967). In our earlier studies (Van Doorn, 2003; Van Doorn et al., 2004a,b) mature horses and ponies retained 11 to 25 mg P·kg $BW^{-1}·d^{-1}$. This phenomenon has also been observed by others (Stillions et al., 1968; Schryver et al., 1971a,b; Argenzio et al., 1974; Buchholz-Bryant et al., 2001; Nielsen et al., 1998). P retention cannot be explained by P losses in sweat (Schryver and Hintz, 1972), P storage in hair (Wells et al., 1990) or hoofs (Ley et al., 1998), or P recycling by saliva as equine salivary P concentrations are low (Van Doorn et al., 2011) and the amounts do not significantly contribute to P balance. Methodological errors, such as a short collection period, or analytical errors can cause an overestimation of the retention. Bone analysis did not confirm the hypothesis that retained P may have been temporarily incorporated into hydroxyapatite crystals at the expense of carbonate (Schryver et al., 1971b, 1974). If increased bone formation occurs during a positive P balance, bone markers reflecting the activity of bone cells should show

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a response. Argenzio et al. (1974) suggested that a diet with a relatively low Ca and high P induces a parathyroid hormone (**PTH**) response. Such an increase in PTH can lead to the release of P from bone and an increase in urinary P excretion instead of P deposition in bone. The observed P retention in our earlier studies may be a consequence of a high dietary P level, possibly in combination with an imbalanced Ca:P ratio of the diet (Van Doorn et al., 2004b). This study addresses the hypothesis that long-term supply of P with or without additional Ca will not lead to enforced P retention in mature ponies. The objective of this study is to investigate P retention by measuring 2 consecutive mineral balances for each treatment during a 42-d period. To assess bone metabolism, bone markers and PTH were measured repeatedly. In addition, balances of Ca and magnesium (Mg) were also determined.

MATERIALS AND METHODS

The experimental protocol was approved by the Animal Experiments Committee of the Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands.

Animals and Diets

Six castrated male Shetland ponies (mean BW 138 kg, age 2.5 to 7 yr) were used. Two ponies were assigned to each of 3 diets in a 3 × 3 replicated Latin square design. After each 42-d feeding period, diets were switched, allowing 6 observations for each of the 3 diets. The ponies were fed 3 similar rations, but rations contained different mineral concentrations. Rations consisted of hay and concentrates (Research Diet Services, Wijk bij Duurstede, the Netherlands). The Control diet (**Control**; 0.7% Ca, 0.5% P) provided Ca and P levels at maintenance requirements (NRC, 2007). The high-Ca, high-P diet (**HCaHP;** 2% Ca, 1.65% P) was formulated so that it would induce P retention as seen in earlier experiments (Van Doorn, 2003; Van Doorn et al., 2004a,b). A high-P diet (**HP;** 0.7% Ca, 1.65% P) containing adequate Ca was formulated in an attempt to enhance P retention (Van Doorn, 2003; Van Doorn et al., 2004a,b). The HCaHP and HP diets resulted in a 3.3 times higher P intake and a 2.4 times higher Ca intake than set by the requirements (NRC, 2007).

Each animal received 2.2 kg of hay daily, supplemented with either 0.38 kg of the Control concentrate, 0.44 kg of the HCaHP concentrate, or 0.40 kg of the HP concentrate. The ponies were fed twice a day at 0900 and 1700 h and received half the daily amount of concentrate and half the hay per meal. The hay was supplied 1 h after the concentrate. The ponies were fed according to the energy and protein requirements set for horses (Centraal Veevoederbureau, 1996). Trace elements and vitamins were provided according to the NRC (2007) requirements for horses. The amount of feed supplied was based on the amount required by a pony with a BW of 138 kg.

Table 1. Ingredient composition of the concentrates and analyzed macronutrient composition of the semipurified diets and hay[1]

	Diet[2]			
Item	Control diet	HCaHP diet	HP diet	Hay
Concentrate composition, % (as fed)				
Molasses, cane	14.3	12.5	13.4	—
Grass meal	3.53	3.08	3.32	—
Oats grain	9.53	8.33	8.96	—
Soya oil	4.77	4.17	4.48	—
Potato protein	4.77	4.17	4.48	—
Salt	0.95	0.83	0.9	—
Corn starch	57.2	50.0	53.76	—
Monosodium phosphate	0	9.68	10.4	—
Sodium carbonate	4.67	0	0	—
Limestone	0	6.99	0	—
Premix[3]	0.29	2.5	0.27	—
Macronutrient composition				
DM, %	90.88	92.03	90.6	94.14
CP, % DM	7.05	6.43	6.61	7.43
Crude fat, % DM	5.78	5.65	6.17	1.30
Crude fiber, % DM	2.01	1.78	1.92	37.7
Ash, % DM	8.9	17.41	13.19	8.35
P, % DM	0.08	2.89	3.19	0.24
Ca, % DM	0.15	3.28	0.16	0.33
Mg, % DM	0.07	0.10	0.07	0.14

[1]Research Diet Services, Wijk bij Duurstede, the Netherlands.

[2]Control = ration with Ca and P fed to requirement; HCaHP = high-Ca, high-P ration; HP = ration containing adequate Ca but a high P level; Hay = mean of all hay samples taken (n = 6).

[3]The premix had the following composition (per kilogram of premix): $Na_2SeO_3 \cdot 5H_2O$, 0.97 g; KI, 0.33 g: $MnSO_4 \cdot H_2O$, 33.33 g; $FeSO_4 \cdot 7H_2O$, 466.7 g; $CuSO_4 \cdot 5H_2O$, 26.7 g; $ZnSO_4 \cdot H_2O$, 26.67 g; $Na_2MoO_4 \cdot 2H_2O$, 0.083 g; retinyl acetate (500 IU/g), 25 g; cholecalciferol (500,000 IU/g), 0.033 g; DL-α-tocopheryl acetate (500 IU/g), 333.3 g; thiamin (purity 100%), 9 g; riboflavin (purity 80%), 7.33 g; cyanocobalamin (purity 0.1%), 3.33 g; biotin (purity 100%), 0.47 g; maize feed meal to 1 kg.

Table 1 indicates the ingredient composition of the concentrates and analyzed macronutrient composition of the semipurified diets and the hay. Monosodium phosphate and limestone were used as Ca and P sources because they are highly available to horses (Hintz and Schryver, 1972a). Sodium carbonate was used to correct for differences in sodium intake between treatments. The concentrate intake was corrected for the exchange of limestone and sodium carbonate.

The analyzed P, Ca, and Mg contents (g/kg) of the concentrates and hay are shown in Table 1. During the experiment, actual feed intakes were measured; orts, if any, were weighed and subtracted from the amount supplied. During the second feeding period some ponies showed coprophagy. To prevent coprophagy, the ponies were muzzled during collection periods as soon as they had ingested the concentrate and hay.

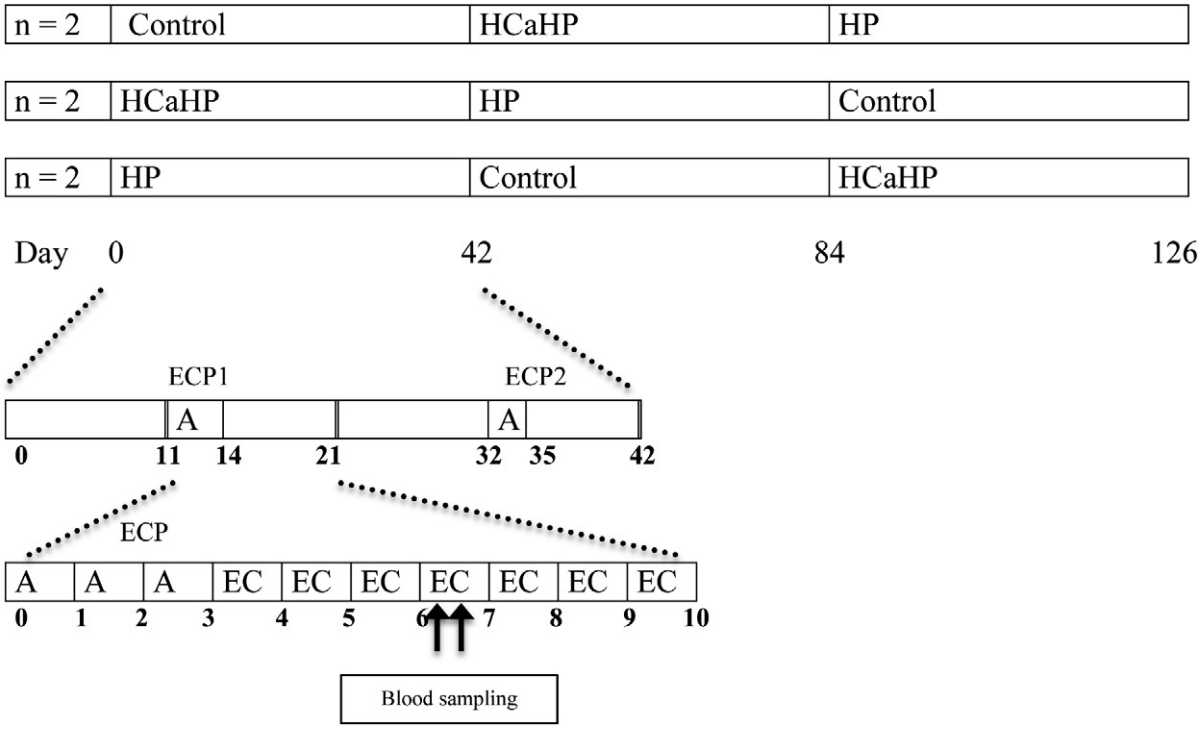

Figure 1. Experimental design. Control = ration with Ca and P fed to requirement; HCaHP = high-Ca, high-P ration; HP = ration containing adequate Ca but a high P level; ECP1 = excreta collection period 1; ECP2 = excreta collection period 2. There were 2 ponies per sequence of treatments. Every 10-d, the excreta collection period (ECP) started with a 3 d adaptation (A) to the housing and was followed by 7 d of collection. During this period urine and feces were collected quantitatively for 4 and 7 d, respectively. Arrows indicate hour of blood sampling (d 6 of ECP), which was before (0900 h) and after (1200 h) the supply of concentrate in the morning (0900 h).

Each feeding period lasted 42 d (Fig. 1). The first 11 d of a period were followed by a 10-d excreta collection period **(ECP1**), and after another 11 d there was a second collection period of 10 d (**ECP2**). During the first 3 d of each feeding period, the ponies were gradually transferred to the new concentrate. The ponies were individually housed in boxes (2.85 × 1.95 m). The indoor boxes contained sawdust except during the collection periods, when the ponies were kept on rubber mats. The ponies were given a half-hour walk per day on a treadmill, but during the collection periods, they received two 10-min walks per day. The ponies were housed together in a paddock for 2.5 h/d, except during the collection periods. The ponies were vaccinated against influenza. In addition, ponies were dewormed and had their hooves trimmed routinely. The ponies were weighed weekly during the experiment. Water was presented in buckets, and the intake was recorded twice daily.

Urine and Feces Collection

Every 10-d excreta collection period started with a 3-d adaptation to the housing and was followed by 7 d of collection (Fig. 1). Urine and feces were collected quantitatively for 4 and 7 d, respectively. Feces and urine were collected continuously as students monitored the ponies 24 h/d during the 7 d of collection. Feces were collected from the rubber mats. Urine was collected with urine quivers (Genap B.V., 's Heerenberg, the Netherlands) designed according to Tasker (1965). Before the experiment, all ponies were accustomed to the urine quivers. Each day at 0900 h, urine samples were taken from the total amount of urine collected and stored (-20°C) until further analysis. Urine samples were acidified directly after urine sampling with 6 *M* HCl (urine:acid = 9:1, vol/vol) to prevent the complex formation of Mg and P (Quinlan and DeSesa, 1955). The feces collected each day were frozen (-20°C) until further analysis.

Blood Collection

Blood samples were collected from the external jugular vein at 0900 h (prefeeding) and 1200 h (postfeeding) on d 6 of each collection period and were put directly on ice. Plasma for mineral analysis was collected in heparin-containing tubes and, after centrifugation 10 min at 3000 g at 20°C, was stored at −20°C. Serum for the measurement of biochemical bone markers and PTH was collected in glass tubes and put on ice immediately after venipuncture, 20°C centrifuged for 10 min at 3,000 × *g*, and stored in 2 mL, 12.5/38-mm, round-bottom, sterile cryogenic vials with an internal screw cap (Greiner bio-one, Frickenhausen, Germany) at −71°C. The fixed time of blood sampling for bone-specific al-

kaline phosphatase and osteocalcin determination was based on the circadian pattern described for these bone markers (Lepage et al., 1991; Black et al., 1999).

Chemical Analysis

Before analysis, feces were defrosted, pooled per pony per collection period, and mixed in a concrete mixer. Three homogenous samples of 1 kg were obtained per pony per collection period. The samples were dried at 60°C for 72 h, ground, and stored at room temperature before mineral and macronutrient analysis. A 0.25-mm sieve was used to grind samples subjected for mineral analyses, and a 1-mm sieve was used to prepare samples for macronutrient analysis (Retsch Brinkmann ZM-1 Centrifugal Mill, Retsch GmbH, Haan, Germany). Dry matter content was determined by overnight drying at 105°C. Nitrogen contents were determined by the macro-Kjeldahl method (International Dairy Federation, 1986); a factor of 6.25 was used to convert grams of N into CP. Ether extract of the feedstuffs was prepared according to AOAC (1984); the solvent was evaporated, and the crude fat residue was weighed. The crude fiber content of the feedstuffs was estimated using Fibertec System M2 (Tecator, Stockholm, Sweden). Before the determination of the selected minerals in feedstuffs and feces, the samples were ashed (500°C for 6 h) and dissolved in 15 mL of 4 mol/L HCl.

Phosphorus in feed, feces, plasma, and water was analyzed by stannous chloride-hydrazine reduction of phosphomolybdic acid (vanadate yellow method; Quinlan and DeSesa, 1955). Plasma samples for mineral analysis were deproteinized with trichloric acid. Phosphorus in urine and plasma was analyzed by the molybdenum blue method (Quinlan and DeSesa, 1955). Calcium and Mg in feed, feces, urine, and water were measured using atomic absorption spectroscopy (PerkinElmer 3110 PC, Norwalk CT). A commercial reference sample (hay powder, CRM 129, Community Bureau of Reference, Brussels Belgium) was used to control the accuracy of the determinations.

The bicarbonate content of urine and feces was determined using the titrimetric method of Lin and Chan (1973). The pH was measured using an Accumet Basic AB15 pH meter (Fisher Scientific, Landsmeer, The Netherlands). The pH of the feces was measured after dissolving 100 g of fecal sample in 10 mL of demineralized water (AOAC, 1984).

Serum samples for analysis of bone-specific alkaline phosphatase were subjected to a wheat germ lectin precipitation assay (Hank et al., 1993), and the activity was expressed as U/L. The intra-assay coefficient of variation was 1.9%. Osteocalcin in serum samples was analyzed with a Metra Osteocalcin immunoassay (Quidel Corp., San Diego, CA), according to the method described by Hoekstra et al. (1999). Because of the high osteocalcin concentration (ng/mL), the serum samples were diluted twice (100:1) before analysis. The intra-assay coefficient of variation was 8.6%, which was in line with the provided performance characteristics of this immunoassay. As PTH quantifications can be assessed by different assays (Pineda et al., 2012; Vieira, 2012), this study analyzed PTH (pg/mL) in serum for whole (**W-PTH**) and intact (**I-PTH**) PTH according to the method of Estepa et al. (2003). The intra-assay coefficients of variation were 2.5% (I-PTH) and 5.3% (W-PTH).

Calculations and Statistical Analysis

Phosphorus balance was calculated as P intake (feed + water) − (P excretion with urine + P excretion with feces). Apparent absorption of minerals and DM was calculated as (intake by feed and water − fecal excretion)/intake × 100%. During the experiment, actual feed intakes were measured; leftovers, if any, were weighed and subtracted from the amounts supplied. Urinary mineral excretion is expressed as percentage of the mineral intake (**%P_u, %Ca_u,** and **%Mg_u**). The results from each collection period (ECP1 or ECP2) were separately analyzed with ANOVA for a Latin square design. Differences between dietary treatments were tested with a paired *t* test (Genstat procedure RPAIR), and $P < 0.05$ was used as a preset value for statistical significance. All data were used to detect differences between collection periods (ECP1 and ECP2) within feeding periods using an ANOVA procedure for repeated measurements. Genstat release 4.2 for PC (Genstat Committee, 2000) was used for all statistical analyses. One pony was excluded from the total data set because of very low feed intake and weight loss. One PTH measurement was excluded because a clot was formed in the serum aliquot subsequent to serum separation.

RESULTS

Body Weight, Water Intake, Fecal Dry Matter Content, and Urine Production

Body weight of the ponies did not differ between treatments (Table 2). During ECP1 there were no diet effects on water intake, but during ECP2 water intake for the Control ration was greater ($P < 0.05$) than that for the HCaHP ration. Urine production on the Control diet was greater ($P < 0.05$) than that on HCaHP during ECP1, but no treatment effects were observed during ECP2. Dry matter intake and apparent DM digestibility did not differ between treatments in either collection period. The apparent DM digestibility values were lower during ECP2 vs. ECP1 ($P < 0.05$), but there was a significant interaction ($P < 0.05$) between time and diet.

Table 2. Analysis of treatment effects of the total ration (concentrate plus hay) on intake, absorption, and excretion variables and time effects (ECP1 vs. P2)[1]

Item	Diet ECP1[3]					Diet ECP2[3]					Time effect
	Control	HCaHP	HP	SD	P-value	Control	HCaHP	HP	SD	P-value	
BW, kg	140	138	136	1.5	0.331	140	140	138	1.2	0.341	NS
Water intake, L	12.30	10.65	10.51	0.97	0.408	14.79^{a}	11.45^{b}	12.64^{a,b}	0.91	0.099	NS
Urine production, L	7.48^{a}	4.87^{b}	5.84^{a,b}	0.75	0.120	10.17	6.84	7.45	1.16	0.178	NS
DM intake, g	2,261.0	2,310.0	2,254.0	20.8	0.196	2,275.0	2,327.0	2,236.0	33.8	0.241	NS
Apparent DM, %	56.45	56.89	57.49	0.53	0.430	52.63	53.05	53.42	1.03	0.864	<0.05
P											
Intake, mg·kg BW^{-1}·d^{-1}	38.5^{a}	123.8^{b}	126.2^{b}	5.0	<0.001	33.2^{a}	117.4^{b}	118.2^{b}	4.42	<0.001	<0.05
Urine, mg·kg BW^{-1}·d^{-1}	1.6^{a}	14.2^{b}	21.4^{b}	2.19	0.002	1.5^{a}	10.6^{a,b}	20.3^{b}	3.0	0.013	NS
Feces, mg·kg BW^{-1}·d^{-1}	35.4^{a}	94.7^{b}	88.8^{b}	3.79	<0.001	28.9^{a}	90.8^{b}	89.0^{b}	5.28	<0.001	NS
Retention, mg·kg BW^{-1}·d^{-1}	1.5^{a}	14.9^{b}	16.0^{b}	2.04	0.004	2.8^{a}	15.9^{b}	8.8^{a,b}	2.18	0.015	NS
Apparent absorption,%	6.1^{a}	23.1^{b}	29.2^{b}	2.12	<0.001	10.3	21.7	24.7	6.3	0.305	NS
$\%P_u$,[2] %	3.32^{a}	11.01^{b}	16.88^{c}	1.41	0.002	4.8^{a}	9.1^{a,b}	16.9^{b}	2.88	0.064	NS
Ca											
Intake, mg·kg BW^{-1}·d^{-1}	57.0^{a}	150.2^{b}	58.2^{a}	4.71	<0.001	50.0^{a}	142.5^{b}	50.4^{a}	4.28	<0.001	<0.05
Urine, mg·kg BW^{-1}·d^{-1}	33.5^{a}	35.1^{a}	23.1^{b}	3.16	0.071	30.7^{ab}	37.5^{a}	23.8^{b}	3.38	0.076	NS
Feces, mg·kg BW^{-1}·d^{-1}	29.4^{a}	100.5^{b}	33.1^{a}	6.95	<0.001	24.2^{a}	90.1^{b}	32.8^{a}	4.15	<0.001	NS
Retention, mg·kg BW^{-1}·d^{-1}	−6.0^{a}	14.6^{b}	2.0^{a}	3.36	0.014	−4.9^{a}	14.9^{b}	−6.2^{a}	5.0	0.042	NS
Apparent absorption, %	47.5^{a}	33.5^{b}	40.6^{a,b}	2.95	0.042	49.5	35.7	34.9	4.26	0.089	NS
$\%Ca_u$,[2] %	61.7^{a}	22.8^{b}	38.5^{c}	3.41	<0.001	62.2^{a}	26.8^{b}	47.1^{c}	3.89	0.002	NS
Mg											
Intake, mg·kg BW^{-1}·d^{-1}	23.44	24.18	23.41	0.51	0.519	20.16^{a,b}	21.07^{b}	19.86^{a}	0.28	0.051	0.05
Urine, mg·kg BW^{-1}·d^{-1}	11.03^{a}	8.64^{b}	9.23^{a,b}	0.61	0.072	10.88^{a}	8.51^{b}	7.76^{b}	0.56	0.018	NS
Feces, mg·kg BW^{-1}·d^{-1}	11.37^{a}	13.66^{b}	13.01^{a,b}	0.65	0.107	9.65^{a}	12.78^{b}	12.74^{b}	0.32	<0.001	NS
Retention, mg·kg BW^{-1}·d^{-1}	1.03	1.88	1.17	0.45	0.417	-0.37	-0.23	-0.64	0.27	0.582	0.05
Apparent absorption, %	50.0^{a}	44.0^{a,b}	42.1^{b}	1.90	0.058	51.1^{a}	38.3^{b}	34.9^{b}	1.82	0.002	NS
$\%Mg_u$,[2] %	47.8^{a}	36.6^{b}	37.9^{b}	2.59	0.042	53.3^{a}	39.5^{b}	37.8^{b}	3.83	0.013	NS
HCO_3^-											
Feces, mg·kg BW^{-1}·d^{-1}	22.1^{a}	96.8^{b}	33.3^{a}	4.91	<0.001	19.6^{a}	88.9^{b}	29.3^{a}	4.90	<0.001	NS
Urine, mg·kg BW^{-1}·d^{-1}	125.8^{a}	72.6^{b}	50.8^{b}	11.06	0.008	108.1^{a}	74.8^{b}	17.8^{c}	7.83	<0.001	NS
pH											
Feces	6.71^{a}	6.35^{b}	6.18^{b}	0.11	0.007	6.86^{a}	6.42^{b}	6.27^{b}	0.07	<0.001	NS
Urine	8.49^{a}	8.46^{a}	7.63^{b}	0.27	0.033	8.35^{a}	8.33^{a}	6.39^{b}	0.54	0.017	NS

$^{a–c}$Values with different superscripts indicate differences between treatments within a collection period; t probabilities of pairwise differences ($P < 0.05$).

[1]Values are the mean for 5 ponies. The data were analyzed with ANOVA, and the significance level was preset at $P < 0.05$. Observed time effects (ECP1 vs. ECP2) >0.05 are indicated as nonsignificant (NS).

[2]$\%P_u$, $\%Ca_u$, and $\%Mg_u$: urinary mineral excretion expressed as percentage of mineral intake.

[3] ECP1 = excreta collection period 1; Control = ration with Ca and P fed to requirement; HCaHP = high-Ca, high-P ration; HP = ration containing adequate Ca but a high P level; ECP2 = excreta collection period 2.

P Balance

Phosphorus intake was in accordance with diet formulation (Table 2). Urinary P excretion was increased ($P < 0.05$) when high levels of P were fed, but the greatest levels were observed for the HP diet. Fecal P excretion was greater for the HCaHP and HP diets compared with the Control diet ($P < 0.05$). The apparent P absorption increased in ECP1 ($P < 0.05$) when larger amounts of P were fed, but statistical significance was not reached during ECP2. The ponies showed positive P retention on the HCaHP and HP diets and approached zero balance on the Control diet, but P retention on the HP diet during ECP2 did not reach statistical significance. The average P intake and P retention (expressed in mg·kg BW^{-1}·d^{-1}) of ponies fed diets with different Ca:P ratios are graphically presented in Fig. 2.

Ca Balance

Calcium intake was more than 2-fold higher on the HCaHP diet than on the other diets (Table 2). Because of differences in hay composition, Ca intake was lower during ECP2 ($P < 0.05$) than during ECP1 (Table 2). Urinary Ca excretion on the Control diet was similar to that on the HCaHP diet but was lower ($P < 0.05$) than that on the HP diet. The $\%Ca_u$ was greatest ($P < 0.05$) for the Control diet

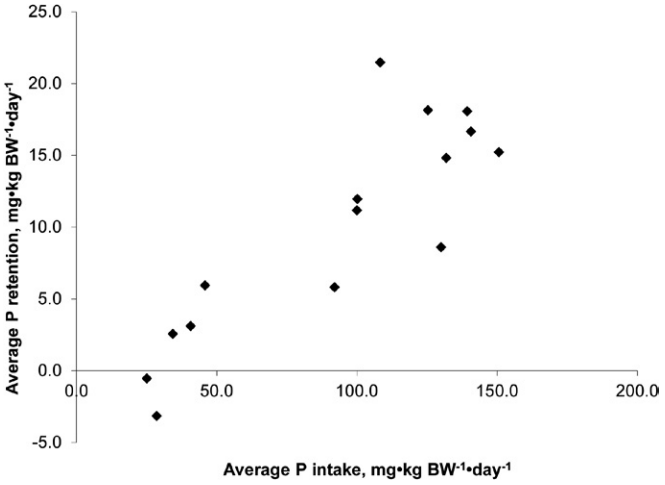

Figure 2. The average P intake and P retention (expressed in mg·kg $BW^{-1}·d^{-1}$) of ponies fed with different Ca:P ratios. The average mineral intake or output was calculated by averaging the intake and retention values from excreta collection periods 1 and 2. The correlation (*R*) between P intake and P retention was 0.866.

and lowest ($P < 0.05$) for the HCaHP diet. The apparent Ca was greater ($P < 0.05$) for the Control diet than for the HCaHP diet in ECP1. The Ca balances on the Control diet did not differ from those on the HP diet. The ponies showed positive Ca retention on the HCaHP diet ($P < 0.05$).

Mg Balance

Magnesium intake was somewhat greater ($P < 0.05$) for the HCaHP diet, although intakes were lower ($P < 0.05$) during ECP2 (Table 2). Urinary Mg excretion and $\%Mg_u$ were greater ($P < 0.05$) for the Control diet than for the other diets. Fecal Mg excretion was higher ($P < 0.05$) for the HP diet in ECP2 and the HCaHP diet (ECP1 and ECP2) than for the Control diet. This difference reflected the greater apparent Mg absorption for the Control diet as a lower ($P < 0.05$) absorption was observed for the HCaHP diet (ECP2) and HP diet (ECP1 and ECP2). During ECP1, group mean balances were positive, and during ECP2 they were negative, but they did not differ significantly from zero.

Fecal and Urinary HCO_3^- Excretion and pH Values

Compared with the other diets, the HCaHP diet produced greater ($P < 0.05$) fecal HCO_3^- excretion. Urinary $HCO3^-$ was greater ($P < 0.05$) for the Control diet and lowest ($P < 0.05$) for the HP diet in ECP2. Urinary pH was lowest ($P < 0.05$) for the HP diet, and fecal pH was greatest ($P < 0.05$) for the Control diet.

Plasma Values

Plasma pre- and postfeeding P concentrations were lower ($P < 0.05$) for the Control diet compared with those for the diets with a high P level (Table 3) in ECP1, but levels for the HP diet did not reach significance in ECP2. Plasma prefeeding Ca concentration did not differ between treatments. Postfeeding Ca concentration was greater for the Control diet than for the other diets, but the difference was not statistically significant during ECP2. Plasma Mg concentrations were not systematically influenced by the type of ration.

Markers of Bone Metabolism

There were no significant diet effects on serum concentrations of osteocalcin and serum bone-specific alkaline phosphatase (Table 3). Serum bone-specific alkaline phosphatase tended ($P = 0.088$) to be greater for the Control diet than for the HP diet during ECP1.

There were no differences in prefeeding I-PTH between diet treatments. Postfeeding values were lowest ($P < 0.05$) for the Control diet, but the difference was not significant during ECP2. Low prefeeding W-PTH values ($P < 0.05$) were observed for the HCaHP diet during ECP2. Postfeeding values were greatest for the HP diet, but the difference vs. the Control diet reached significance only during ECP1. There was no significant time effect on the bone markers.

DISCUSSION

The Control diet contained a P level comparable to the requirement set by the NRC and resulted in a P balance not significantly different from zero, a low urinary P excretion, and normal plasma P concentrations. When P intake was high (about 126 mg·kg $BW^{-1}·d^{-1}$), the ponies had increased P absorption, retained 8.8 to 16 mg·kg $BW^{-1}·d^{-1}$, and had high urinary excretion of P, especially when the HP diet was fed. As a result of P loading, plasma P values increased. No time effect was observed for the P balance and plasma P. The fact that urinary excretion of P was higher for the HP diet than for the HCaHP diet may be explained by the formation of insoluble Ca phosphate complexes (Herrera et al., 2010; Jongbloed, 1987). In contrast to the study by Toribio et al. (2003), who measured serum ionized Ca, this study found no evidence for a response of PTH. This could be explained by the lack of a hypercalcemic effect for the HCaHP diet. Moreover, the bone markers measured in our study did not point to alterations in bone metabolism. Possibly, the addition of Ca to the diet results in insoluble Ca phosphate complexes (Jongbloed, 1987). However, we cannot exclude the possibility that the nonresponse of the bone markers on the HCaHP diet measured in this study rules out the role of bone as an explanation for the positive mineral balances.

Table 3. Treatment and time effects (ECP1 vs. ECP2) for plasma minerals, biochemical bone markers, and parathyroid hormone (PTH) values of ponies receiving various amounts of P and Ca[1]

	Diet ECP1[3]					Diet ECP2[3]					Time
Item[2]	Control	HCaHP	HP	SD	*P*-value	Control	HCaHP	HP	SD	*P*-value	effect
Plasma P											
Prefeeding, mmol/L	1.39[a]	1.61[a,b]	1.70[b]	0.1173	0.089	1.25[a]	1.54[b]	1.51[a,b]	0.1192	0.094	NS
Postfeeding, mmol/L	1.14[a]	1.40[b]	1.60[b]	0.1034	0.012	1.03[a]	1.50[b]	1.45[a,b]	0.1756	0.068	NS
Plasma Ca											
Prefeeding, mmol/L	3.35	3.41	3.38	0.0609	0.676	3.28	3.27	3.47	0.1207	0.262	NS
Postfeeding, mmol/L	3.62[a]	3.38[b]	3.42[b]	0.0713	0.031	3.63	3.56	3.50	0.0817	0.310	NS
Plasma Mg											
Prefeeding, mmol/L	0.61	0.60	0.63	0.0215	0.422	0.63[ab]	0.59[b]	0.69[a]	0.0417	0.115	NS
Postfeeding, mmol/L	0.84	0.76	0.82	0.0545	0.418	0.88	0.84	0.84	0.0350	0.466	NS
Serum osteocalcin											
Postfeeding, ng/mL	38.5	38.3	44.2	10.11	0.808	38.2	44.2	38.8	9.67	0.801	NS
Serum b-ALP											
Postfeeding, U/L	49.2	47.8	40.9	4.03	0.167	45.9	59.4	44.4	9.32	0.283	NS
I-PTH											
Prefeeding, pg/ml	10.2	10.0	10.3	1.150	0.970	23.0	12.3	12.3	8.8	0.438	NS
Postfeeding, pg/mL	5.4[a]	10.3[b]	10.5[b]	1.765	0.047	7.5	9.2	10.4	2.2	0.455	NS
W-PTH											
Prefeeding, pg/mL	67.7	90.5	58.8	18.68	0.290	100.4[a]	35.0[b]	70.3[a,b]	24.10	0.090	NS
Postfeeding, pg/mL	26.4[a]	56.0[a,b]	69.7[b]	15.64	0.079	32.8	24.6	62.1	19.91	0.221	NS

[a,b]Values with different superscripts indicate differences between treatments within a collection period; t probabilities of pairwise differences ($P < 0.05$).

[1]Values are the mean for 5 ponies. The data were analyzed with ANOVA for repeated measurements, and the significance level was preset at $P < 0.05$ and observed time effect (ECP1 vs. ECP2) >0.05 are indicated as nonsignificant (NS). Blood samples were taken before (0900 h) and after (1200 h) the supply of concentrate in the morning (0900 h).

[2]b-ALP = bone-specific alkaline phosphatase; I-PTH, intact PTH; W-PTH, whole PTH.

[3]Control = ration with Ca and P fed to requirement; HCaHP = high-Ca, high-P ration; HP = ration containing adequate Ca but a high P level; ECP1 = excreta collection period 1; ECP2 = excreta collection period 2.

The Control and HP diets contained a Ca level comparable to the requirement set by NRC (2007) and resulted in a Ca balance not significantly different from zero and a considerable excretion of Ca with the urine. Plasma Ca was within the normal range and was higher after feeding than before feeding. This result was also observed by Meyer et al. (1992). When Ca intake increased to about 150 mg·kg BW-1·d-1, absolute urinary Ca excretion did not increase significantly compared with that for the control treatment. There was an increase in fecal Ca excretion on the HCaHP diet, but it did not prohibit Ca retention. This result was also observed previously (Van Doorn et al., 2004b).

During the entire experiment, Mg balances were not different from zero. The plasma Mg concentration was low (Meyer et al., 1992), but a considerable fraction of ingested Mg was excreted with the urine. The lowest absorption of Mg was observed for the HP diet, and the highest was observed for the Control diet, indicating that dietary P reduced Mg absorption as observed by Hintz and Schryver, (1972b).

The retention of Ca, P, and Mg on the Control diet did not differ from zero. Feeding the HCaHP diet resulted in positive balances for Ca and P. The ratio of retained Ca to retained P was about 1:1. With bone formation a ratio of about 2.15:1 would be expected as the crystal structure of bone salt resembles hydroxyapatite, which contains Ca and P in the proportion 2.15:1 g/g. (Russell et al., 1986). Therefore, storage of Ca and P in bone seems unlikely in the current study.

As mentioned above, it is not likely that the P retention seen for the HCaHP ration reflects P accumulation in bone. Thus, it would be expected that the bone markers would not point to enhanced bone metabolism associated with P retention. There was, indeed, no treatment effect on the activity of the serum bone formation markers osteocalcin and bone-specific alkaline phosphatase in the present study. Phosphorus balances were systematically positive when the ponies were fed the HP diet. These observations may suggest that bone turnover is not altered during P loading and consequent P retention.

If the process of bone formation does not serve as a storage pool for P, the physiological phenomenon that explains the observed P retention remains undetermined. It has been suggested (Bigi et al., 1997) that carbonate ions may be substituted for phosphate in hydroxyapatites. However, the results of this study did not point to a higher urinary bicarbonate excretion when the ponies

were fed the HCaHP diet. It should be noted that the dietary carbonate intake in the present study was different for the HCaHP diet, and the observed differences are difficult to interpret. Phosphorus storage in soft tissue also seems unlikely, as indicated by Schryver et al. (1974).

It is possible that P retention is related to the relative inactivity of the ponies. Buchholz-Bryant et al. (2001) studied the effect of Ca and P supplementation in young (2 to 3 yr old), mature (7 to 11 yr old), and aged (15 to 21 yr old) horses with different exercise regimens. The authors reported higher retentions for all age groups when the horses were at rest than when they were intensively exercised. This suggests that the P balance is influenced by physical activity. The same conclusion can be derived from the work of Lepage et al. (2001) and Mansell et al. (2001). On the other hand, Nielsen et al. (1998) did not observe differences in P retention in young horses subjected to different exercise regimens. In contrast, studies exist that describe a higher bone mineral retention in exercised horses compared with stall confined and deconditioned horses (Porr et al., 1998). Further, Vervuert et al. (2005) observed that the intake of different Ca and P levels and the type of exercise influence Ca, P, and Mg homeostasis and intact PTH response. However, P balance was not measured in those studies. The metabolic basis for the relation between activity and P balance, if any, is unknown.

The balances in this study were systematically positive, which would exclude analytical error as the cause. Thus, we conclude that somewhere in the body P is retained when high P levels are fed. One explanation for P retention in this study is the formation of insoluble calcium phosphate complexes that are not absorbed and may accumulate in the (large) intestine. Clearly, the accumulation, if any, can only proceed for a limited period of time before intestinal disorders develop. Bray (1995) indicated that excess P and Mg combined with high levels of Ca may favor the formation of enteroliths. These enteroliths consist of struvite and Mg vivianite, but the presence of Ca has also been reported (Hassel et al., 2001). Assuming a P content of 22% of an enterolith (Hassel et al., 2001), a mean P retention on the HP diet equals about 2 g/d, which should theoretically result in 9 g of enterolith. A 42-d period would then result in a 378-g increase of the enterolith. This seems possible as stones between 200 g and 9 kg have been reported (Pierce, 2009).

The use of biochemical bone markers has been validated for horses, but the values for these markers may vary considerable because of age and breed of the pony, season, diurnal pattern, and the type of assays used (Lepage et al., 2001). Lepage et al. (1998) observed that biochemical bone markers may vary between draft and warmblood horses. Serum osteocalcin concentration in the present study was much greater than that observed by Lepage et al. (1990, 1991, 1998) and Chiappe et al. (1999) but similar to values observed by Black et al. (1999) and Frisbie et al. (2008). The values for bone-specific alkaline phosphatase were similar to those reported by Hank et al. (1993) and somewhat higher than those reported by Weisrock et al. (2011), who observed bone-specific alkaline phosphatase values ranging from 35 to 41 ng/mL.

Estepa et al. (2003) studied the responses of I-PTH and W-PTH in 5 healthy horses with hypocalcaemia induced by infusion of EDTA and with hypercalcaemia induced by infusion of $CaCl_2$. Parathyroid hormone decreased in response to an increase in plasma Ca concentration, and PTH increased when plasma Ca decreased. The changes in W-PTH and I-PTH showed the same pattern, but the W-PTH concentration was always greater than the I-PTH concentration. This finding is consistent with the results of the present study. Estepa et al. (2003) reported a mean W-PTH concentration of 55.7 ± 8.1 pg/mL and a mean I-PTH concentration of 20.8 ± 3.3 in normal horses. The I-PTH values observed in that study seem to be greater than those observed in the present experiment using ponies. Compared to the results of Estepa et al. (2003), this study shows 2 ponies with high W-PTH values (144 and 177 pg/mL) on the HP diet. This could suggest a very mild form of secondary hyperparathyroidism with a nutritional origin.

In summary, high P and Ca induced positive retention of P and Ca. It seems unlikely that this represents bone formation because bone markers did not respond. The formation of enteroliths can probably be suggested because Mg also seems to be affected by high P levels (Hassel et al., 2004).

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