Serum mineral reference values of clinically healthy athlete Brazilian four-beat gaited horses

Valores de referências de minerais séricos de cavalos marchadores brasileiros clinicamente saudáveis

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Abstract

Little is known about the serum mineral profiles of Brazilian four-beat gaited horses, and this information could be used to improve their nutritional and exercise evaluation programs. This study aimed to determine the mineral values of athlete Campolina and Mangalarga Marchador horses performing two different gait types. One hundred adult horses (7 ± 3 yr and 432 ± 59 kg; mean ± SD) were grouped as follows: 25 *batida* gait Campolinas (CAMPb), 25 *picada* gait Campolinas (CAMPp), 25 *batida* gait Mangalarga Marchadors (MMb), and 25 *picada* gait Mangalarga Marchadors (MMp). They were fed fresh elephant grass (15 - 20 kg as-fed/day) and commercial concentrate (5 - 7 kg as-fed/day) as well as commercial mineral salt and water *ad libitum*. Horses were exercised three times a week (~40 – 50 min/day), and ~60% of the exercise comprised a speed session ranging from 3.0 to 4.0 m/s. Blood samples were collected after 12 h feed fasting and at least 24 h after exercise. Mineral concentrations were analyzed using ANOVA and Tukey's test ($p \leq 0.05$). No breed or gait effects were observed ($p > 0.05$) on serum levels of chloride, potassium, sodium, magnesium, iron, and selenium. There was a breed by gait effect ($p < 0.05$) on calcium and phosphorus levels. Lower calcium levels were found in MMb (2.13 ± 0.02 mmol/L, $p < 0.0032$), while lower phosphorus levels were found in MMp (1.43 ± 0.02 mmol/L, $p < 0.0005$). The mineral values provided by the current study serve as reference values and are useful in the improvement nutritional and exercise evaluation programs of athlete four-beat gaited horses.

Keywords: Electrolytes. Selenium. Sport horses. Gaited horses.

Resumo

Pouco se sabe sobre o perfil de minerais de cavalos marchadores brasileiros, e tal informação colabora para elaboração de programas mais adequados de nutrição e de avaliação de exercício. O presente estudo objetivou determinar os valores minerais de cavalos Campolina e Mangalarga Marchador atletas de dois diferentes tipos de marcha. Cem cavalos adultos (7 ± 3 anos e 432 ± 59 kg; média ± DP) foram agrupados em: 25 Campolinas de marcha batida (CAMPb), 25 Campolinas de marcha picada (CAMPp), 25 Mangalarga Marchadores de marcha batida (MMb) e 25 Mangalarga Marchadores de marcha picada (MMp). Os equinos foram alimentados com capim elefante (15 – 20 kg de massa fresca/dia) e concentrado comercial (5 – 7 kg de massa fresca/dia), bem como suplemento mineral e água ad libitum. *Os cavalos foram exercitados três vezes por semana (~40 – 50 min/dia) com cerca de 60% do exercício sendo realizado com sessão de velocidade que variou de 3.0 a 4.0 m/s. Amostras de sangue foram coletadas após 12 h de jejum sólido e, pelo menos, 24 h pós-exercício. As concentrações de minerais foram submetidas à ANOVA e comparadas usando o teste de Tukey ($p \leq 0.05$). Não foram observados efeitos de raça ou tipo de marcha ($p > 0.05$) sobre as concentrações séricas de cloreto, potássio, magnésio, ferro e selênio. Houve um efeito da interação raça x tipo de marcha ($p < 0.05$) sobre os níveis de cálcio e fósforo. Níveis de cálcio inferiores foram observados em cavalos MMb (2.13 ± 0.02 mmol/L, $p < 0.0032$), enquanto menores níveis de fósforo foram observados em cavalos MMp (1.43 ± 0.02 mmol/L, $p < 0.0005$). Os valores de minerais determinados no presente estudo servem como valores de referência e são úteis para promover programas de nutrição e de avaliação de exercício mais adequados para cavalos marchadores brasileiros atletas.*

Palavras-chave: *Eletrólitos. Selênio. Cavalos atletas. Cavalos marchadores.*

Introduction

Four-beat gaited Brazilian horses, such as Mangalarga Marchador and Campolina, exhibit distinct types of gaits known as "marcha." There are two types of *marcha*, one with a more lateral association, known as "marcha picada," and another with a more diagonal association, known as "marcha batida," with movements of triple support and no suspension, that makes horses smooth and comfortable for the rider (Wanderley et al., 2010). These horses are usually submitted to marcha gait challenges, which are characterized as a submaximal exercise. Additionally, their natural gait variations involve metabolic challenges that differ from the challenges experienced by trotting or galloping breeds (Ferreira et al., 2017). Therefore, reference values that can be used as biomarkers for horses submitted to *marcha* challenges are warranted.

According to Lumsden et al. (1980), reference values are observations performed on a group of individuals in a defined health condition and used to diagnose diseases and physiological alterations. Serum mineral levels are amongst the main biochemical reference values in equine sciences. Blood minerals are also called electrolytes due to their ionic charges and are essential for cellular metabolism, fluid balance, and the formation of bones, teeth, and blood cells, among several other roles (Santos et al., 2001). Alterations in the blood electrolyte levels of horses can lead to inadequate athletic performance and hence decreased athletic performance (Lewis, 1995).

Most of the electrolyte reference values for horses are generalized for several breeds and exercises, and the class of horse and basal diet are rarely specified. Therefore, basal or pre-exercise blood levels provided by some studies may be used as reference values. Nonetheless, basal mineral levels are well established for horses performing primarily anaerobic-metabolism exercises, such as thoroughbred (Inoue et al., 2002; Crocomo et al., 2009) and Quarter horse (Gordon et al., 2013; Santiago et al., 2014), and jumping horses, such as Italian Saddle horse (Piccione et al., 2007). Few studies have evaluated horses under primarily aerobic-metabolism exercises, such as endurance horses (Hambleton et al., 1980; Schott et al., 2006; Adamu et al., 2014).

Studies published to date providing pre-exercise or basal levels for minerals in four-beat gaited horses were not sufficient to serve as reference value guidelines, as no more than four minerals were evaluated (Folador et al., 2014; Martins et al., 2017). Therefore, the basal mineral reference values of four-beat gaited horses warrant investigation. This study aimed to provide reference values for the most critical

minerals in the serum of athlete Brazilian four-beat gaited horses raised in tropical climate conditions. We hypothesized that there would be no breed or gait variation effects on serum macro and trace mineral levels of athlete Brazilian four-beat gaited horses. However, knowledge of these minerals could contribute to the understanding of the metabolism of these biomarkers. Additionally, it can be used to improve nutritional and exercise evaluation programs and health status of athlete Brazilian four-beat gaited horses.

Material and methods

This study was approved by the Committee of Ethics in Animal Utilization (CEUA) of the Universidade Federal Rural de Pernambuco (UFRPE; protocol #140/2015).

Animals and management

In addition to breed, gait, and characterization within the adult working horse class, selection criteria were established based on physical examination and similarity of nutritional and training programs among operations. To ensure consistency, physical examinations were performed by a single veterinarian, who measured heart and respiratory rates, rectal temperature, skin tent duration, capillary refill time, mucous membrane color, abdominal auscultation, peripheral pulses, and general attitude. Horses younger than 3 years, older than 15 years, and with sign of disease (locomotion injuries, and chronic or acute disorders) were excluded from this study.

One hundred mature horses (50 males and 50 females) of the Brazilian four-beat gait breeds Mangalarga Marchador (MM) and Campolina (CAMP) from 13 different horse operations located in Pernambuco state were used. Horses were between 3 and 15 years (7 ± 3 years; mean ± SD) of age and weighed between 310 and 550 kg (432 ± 59 kg; mean ± SD). The age range of the horses used in this study is representative of the horses used in marcha challenge according to the Brazilian Association of Mangalarga Marchador Horse Breeders´ regulations (ABCCMM, 2018).

Horses were divided into four groups based on breed and gait types, as 25 *batida* gait Campolinas (CAMPb; 6 ± 2 years; 482 ± 23 kg), 25 *picada* gait Campolinas (CAMPp; 7 ± 3 years; 482 ± 28 kg), 25 *batida* gait Mangalarga Marchadores (MMb; 7 ± 4 years; 377 ± 37 kg), and 25 *picada* gait Mangalarga Marchadores (MMp; 8 ± 3 years; 388 ± 37 kg). Although MMp horses had a higher average age than the other groups, the age range of the groups overlaps when the standard deviations are considered, therefore, the authors believe the groups are age-wise distributed similarly for a fair comparison.

Nutrition and training programs

All horses were kept under similar feeding and management conditions and were raised in a similar ecological area in Pernambuco. They were fed chopped fresh elephant grass (*Pennisetum purpureum* Schum) four times a day (around 15 - 20 kg as-fed/day) and commercial concentrate (total 5 - 6 kg/day, with 16% crude protein, 4% ether extract, and 2.3 Mcal of digestible energy/kg) provided in three meals per day. Diets were fed to meet the energy requirements of horses performing exercise of moderate duration and intensity according to the NRC (2007). Mineral supplement and water were provided *ad libitum*. Concentrate feed were similar across farm operations; however, mineral supplements differed substantially, and for that reason, the chemical composition will not be presented.

Horses were trained three times a week at the *batida* or *picada* gait for 40 - 50 minutes, with an initial 5 - 10 min warm-up walk at a speed of ~2 m/s, followed by 30 min of marcha at ~3.5 m/s, and a 5 - 10 min cool-down walk at ~2 m/s. Horses participated in regular gait competitions in Pernambuco. All horses had four days off exercise, performing free exercise in dry-lot 3 - 4 hours per day.

Sample collection and analysis

Blood samples were collected by jugular venipuncture using 9 ml evacuated glass tubes with no additive to obtain serum samples. Horses were feed fasted for 12 hours and rested for at least 24 hours after exercise to avoid the effects of diet and exercise on the concentration of the quantified minerals (Franco et al., 2004; Martins et al., 2005). Tubes were transported to the Laboratory of Molecular Biology Applied to

Animal Production (BIOPA), at the UFRPE, within 2 hours after collection. Samples were allowed to clot at room temperature, and the serum was transferred to 2 ml microtubes. Samples were stored at - 20 ˚C for subsequent analysis of the macrominerals calcium (Ca), phosphorus (P), magnesium (Mg), chloride (Cl), sodium (Na), potassium (K) and the trace minerals iron (Fe) and selenium (Se).

Biochemical analyses were performed in duplicate at BIOPA using commercial kits and a semi-automatic biochemical analyzer (D-250, Doles, Goiânia, GO, Brazil) for quantification of Ca (total calcium), P, Mg, Cl, and Fe. Quantification of Na and K was performed in duplicate at the Laboratory of Clinical Pathology at the UFRPE Veterinary Hospital using commercial kits and an automatic biochemical analyzer (Labtest, Labtest Diagnóstica, Lagoa Santa, MG, Brazil).

Analysis of selenium was performed at the Laboratory of Analytical Chemistry at the Research Support Center (CENAPESQ)/UFRPE. Samples were submitted to microwave digestion using digestion tubes with 1 ml of HNO_3 (MARSXpress, CEM Corporation, Matthews, NC, USA) for 50 minutes. Following digestion, 4.5 ml of Milli-Q high-purity deionized water were added to each tube. Blanks were prepared similarly, excluding the addition of the serum sample adapted from Nomura et al. (2005). Lastly, quantification of Se was performed by inductively coupled plasma mass spectrometry and atomic absorption spectrometry using the spectrometer (Varian AA240FS, Varian Corporation, Palo Alto, Ca, USA).

Statistical analysis

Data were collected as a cross-sectional observational study, which is a study design that provides a "snapshot" of the outcome and the exposure to the factors or causes that can influence the outcome, with no influence (treatments application) of the researchers on the sampling individuals, and data were recorded based on observations only (Levin, 2006). Mineral levels were considered the outcome, whereas the recorded factors that could influence the outcome were breed, gait variation, age, body weight, sex, and farm operation. Horses were arranged in groups according to breed and gait variation.

Data were submitted to analysis of variance using the MIXED procedure in SAS version 9.4 (SAS Inst. Inc., Cary, NC), with both breed and gait variation included as fixed effects in the model. Age, body weight, sex, and farm operation were included as random effects. Data are described statistically as mean ± standard error of the mean. Multiple comparisons of the mean mineral concentrations between groups were performed using the Tukey-Kramer test with a significance level of 5%. Additionally, we investigated the association between P level and age of the horse using Pearson's correlation test through the CORR procedure in SAS. Overall means of serum mineral concentrations, age, and body weight were obtained using the MEANS procedure in SAS to obtain the reference values for each mineral within each group.

Results and discussion

Means of mineral concentrations of the four-beat gaited horses by breed and gait interaction are shown in Table 1. There was no breed or gait variation effect ($p > 0.05$) on concentrations of Mg, Na, K, Cl, Se, and Fe (Table 1). A breed by gait variation effect was observed on concentrations of Ca ($p = 0.0032$) and P ($p = 0.0005$). *Batida* gait Mangalarga Marchador horses had significantly lower Ca serum levels (2.13 ± 0.02 mmol/L), while *picada* gait Mangalarga Marchador horses had significantly lower P serum levels (1.43 ± 0.02 mmol/L) than did the other groups of horses.

According to Lumsden et al. (1980), it is essential to emphasize that for interpreting serum concentrations, individual history, clinical findings, and disorders must be taken into consideration. All horses used in this study were raised in the same ecological area in Pernambuco, where the rainy season occurs between April and August, known as the Atlantic forest. Additionally, variations in the concentrations of biomarkers of mineral metabolism may be a result of different factors, such as the type, intensity, and duration of exercise (Machado et al., 2010).

Serum phosphorus is influenced by age and decreases as the individual ages (Long et al., 1965; Muñoz et al., 2012). Young horses can present alkaline phosphatase concentrations three- to five-fold greater than mature horses due to more remarkable bone growth and remodeling (Muñoz et

al., 2012). This enzyme is responsible for removing phosphate from several molecules and making it available for energetic metabolism, increasing its serum concentration (Oliveira et al., 2016). In the current study, the serum phosphorus level and age of the horses were negatively correlated (r = - 0.2136; p = 0.0328). This may explain the lower concentration of serum P in MMp horses, which had a higher mean age (8 ± 3 years) numerically greater than did the other groups of horses. Another explanation for lower phosphorus concentration in MMp horses could be explained by the higher energetic cost compared with *batida* horses moving at the same speed (Wanderley et al., 2010). However, this might not be the case, as p concentrations in CAMPp did not differ from those in CAMb or MMb horses.

Table 1 - Serum mineral concentrations of athlete Brazilian four-beat gaited horses

	Breed					
	Campolina		Mangalarga Marchador			
Mineral	CAMPb[1]	CAMPp	MMb	MMp	SEM[2]	p-value
Macrominerals (mmol/L[3])						
Calcium (Ca)	2.28a	2.27a	2.13[b]	2.25a	0.02	0.0032
Chloride (Cl)	86.94	85.40	87.26	83.77	1.85	0.5954
Magnesium (Mg)	0.49	0.50	0.49	0.43	0.02	0.0756
Phosphorus (P)	1.50[a]	1.51a	1.54[a]	1.43[b]	0.02	0.0005
Potassium (K)	3.75	3.86	3.93	3.75	0.14	0.3107
Sodium (Na)	136.14	137.83	137.48	140.06	1.08	0.6833
Trace minerals (µmol/L[4])						
Iron (Fe)	32.88	30.80	34.80	31.41	1.63	0.6867
Selenium (Se)	0.53	0.62	0.69	0.69	0.03	0.1373

Note: [1] Breed by gait variation grouping: CAMPb = *batida* gait Campolina; CAMPp = *picada* gait Campolina; MMb = *batida* gait Mangalarga Marchador; and MMp = *picada* gait Mangalarga Marchador. [2] SEM = standard error of the mean. [3] mmol/L = millimole per litter. [4] µmol/L = micromole per litter. [a,b] Different superscripts within a row differ significantly at $p < 0.05$.

The blood calcium level is known to be relatively constant, yielding a poor indicator of calcium status (De Behr et al., 2003). It is maintained within a narrow range (2.69 - 3.36 mmol/L) due to the regulation of calcium homeostasis performed by the endocrine system that regulates calcium absorption and excretion via the recognition of calcium levels (Schryver et al.,1970; Garcia-Lopez et al., 2001; Toribio et al., 2003). Although the difference in calcium levels in the current study was not expected, the authors believe that differences in training adaptation might play a role in that difference. We hypothesized that, because some groups of horses were housed in the same operations, it is possible that MMp horses were in the training adaptation phase, where there is a switch from bone resorption to bone formation, which decreases serum calcium levels (Nielsen et al., 1998; Pipkin et al., 2001).

Reference values for serum minerals

Reference values for serum mineral levels in athlete Brazilian four-beat gaited horses and other horse breed populations are shown in Table 2. The reference Ca level found in the current study (2.24 ± 0.12 mmol/L; mean ± SD) was similar to the basal Ca concentrations of 2.25 mmol/L reported for thoroughbreds (Crocomo et al., 2009) and of 2.46 mmol/L for Arabian horses (Franco et al., 2004). Greater basal Ca concentrations were reported for the same horse breeds (2.66 mmol/L; Silva et al., 2014), thoroughbreds (2.81 mmol/L; Inoue et al., 2002), and Arabian horses (3.03 mmol/L; Robert et al., 2010); and lower concentrations were reported for endurance horses (1.56 mmol/L; Hess et al., 2008).

The mean serum phosphorus concentration found in this study (1.15 ± 0.09 mmol/L) was similar to the 1.36 ± 0.38 mmol/L reported for Campolina and Mangalarga Marchador (Silva et al., 2014), and greater than the 1.27 ± 0.30 mmol/L reported for thoroughbred horses (Inoue et al., 2002) and the 1.02 ± 0.02 mmol/L reported for Shetland pony mares (Gromadzka-Ostrowska et al., 1985). Lower values were reported by Crocomo et al. (2009), of 1.87 ± 0.47 mmol/L for thoroughbred horses, and of 2.11 ± 0.15 mmol/L for endurance horses (Hess et al., 2008).

The mean concentration of Mg (0.48 ± 0.11 mmol/L) reported in the current study was lower than those means (0.70 ± 0.04 mmol/L) obtained for the same horse breeds (Silva et al., 2014), as well as the 0.68 - 0.81 mmol/L reported for thoroughbred horses (Inoue et al., 2002; Crocomo et al., 2009; Sales et al., 2013), and greater than the 0.26 ± 0.02 mmol/L reported for endurance horses (Hess et al., 2008).

Table 2 - Basal serum mineral concentrations (mean ± SD) of athlete Brazilian four-beat gaited horses and other horse breeds populations

Mineral	Brazilian four-beat gaited horses	Silva et al. (2014)[A]	Crocomo et al. (2009)[B]	Inoue et al. (2002)[B]	Montgomery et al. (2011)[C]
Macrominerals (mmol/L[1])					
Calcium (Ca)	2.24 ± 0.12	2.65 ± 0.37	2.25 ± 0.73	2.81 ± 0.51	-
Chloride (Cl)	85.87 ± 9.05	95.44 ± 0.81	101.47 ± 6.59	-	-
Magnesium (Mg)	0.48 ± 0.11	0.70 ± 0.04	0.77 ± 0.14	0.68 ± 0.03	-
Phosphorus (P)	1.50 ± 0.09	1.36 ± 0.38	1.87 ± 0.47	1.27 ± 0.30	-
Potassium (K)	3.82 ± 0.69	3.22 ± 0.16	4.16 ± 0.34	-	-
Sodium (Na)	137.85 ± 5.52	111.94 ± 2.95	134.05 ± 1.95	-	-
Trace minerals (µmol/L[2])					
Iron (Fe)	32.47 ± 8.16	-	-	30.8 ± 2.51	-
Selenium (Se)	0.63 ± 0.15	-	-	-	0.79*

Note: [A] Brazilian four-beat gaited horses; [B] Thoroughbred horses; and [C] Standardbred horses. [1] mmol/L = millimole per litter. [2] µmol/L = micromole per litter. * No standard error or standard deviation was provided. - Not reported.

Silva et al. (2014) and Martins et al. (2017) reported sodium concentrations for gaited horses ranging between 136 and 146 mmol/L, regardless of the mineral amount in the diet. The mean concentration of serum Na found in the current study (137.85 ± 5.52 mmol/L) was within the range reported. Lower values (134.05 ± 1.95 mmol/L) were published for thoroughbred horses (Crocomo et al., 2009), whereas greater values (141.5 ± 0.27 mmol/L) were reported for endurance horses (Hess et al., 2008). According to Silva et al. (2014), a Na concentration greater than 145 mmol/L suggests water deficiency relative to the solute concentration in body fluids.

The average serum concentration of potassium reported in this study (3.83 ± 0.12 mmol/L) was greater than the basal potassium concentrations of 3.22 - 3.53 mmol/L for Mangalarga Paulista horses, another Brazilian gaited breed (Fernandes and Larsson, 2000; Silva et al., 2014) and of 3.61 ± 0.05 mmol/L for endurance horses (Hess et al., 2008); and lower than K concentrations (4.16 ± 0.34 mmol/L) reported for thoroughbred horses (Crocomo et al., 2009). The mean Cl value found in the current study (85.87 ± 9.05 mmol/L) was lower than those reported in the literature, ranging from 95.44 to 102.0 mmol/L (Crocomo et al., 2009; Robert et al., 2010; Silva et al., 2014).

Inoue et al. (2002), evaluating racing horses in Japan, reported a basal iron concentration of 172 µmol/L. Normal ranging suggested in the literature ranges from 21.48 to 37.59 µmol/L (Lewis, 2000). Seckington et al. (1967) found Fe serum levels to be lower in mares kept in stalls fed a high-grain diet compared to grass-fed mares. The greater iron level in the current study compared with the one

conducted in Japan with racing horses might be a function of higher proportions of grains fed to racing horses when compared with horses performing predominantly aerobic exercise.

The reference value for selenium determined in the present study for Brazilian four-beat gated horses (0.63 ± 0.15 µmol/L) suggests that the horses in the present study could be selenium deficient, as their Se values were substantially lower than the expected values for equine species of 1.64 - 2.02 µmol/L reported by Stowe and Herdt (1992). However, serum Se in this study was similar to that reported for Standardbred horses (0.79 µmol/L), indicating that Brazilian four-beat gaited horses indeed have lower basal serum Se concentrations. According to Montgomery et al. (2011), selenium-deficient horses are typically fed forage without trace mineral supplementation. Because the use of supplements rich in compounds that supports exercise performance and recovery is widespread across Brazilian four-beat gaited horse facilities, we believe that their basal and healthy serum Se levels are below the reference levels reported by Stowe and Herdt (1992).

Differences in reference range values among studies might be explained by the type of exercise, breed, sex, age, feeding management, and farm location (Seckington et al., 1967; Pritchard et al., 2009; Silva and Macedo, 2011). For instance, during training, the animal undergoes chronic adaptation to exercise that can change basal biochemical concentrations (Silva and Macedo, 2011).

The reference range values obtained for all minerals evaluated in the present study are within the range for equine species under various health conditions reported by Kaneko et al. (2008). No deficit or excess of minerals was observed, except for Se concentrations, which were below the range reported by Kaneko et al. (2008). The lack of serum mineral imbalance suggests that the horses in this study were fed the recommended amount of minerals to meet their mineral requirements.

Because athlete Brazilian four-beat gaited horses are used for the same purpose, the *marcha* gait challenge, the feeding management, and training protocol practices are performed similarly among the horse facilities included in this study (Bem et al., 2013). The similarity in management can explain the similarity in mineral concentrations, except for calcium and phosphorus. Therefore, there is evidence that equine breeds, used for the same purpose, with a similar genetic standard origin, can show some variation in the concentrations of some blood minerals, and that should be taken into consideration when evaluating the health and nutritional management of the horse.

Conclusion

Data evaluating several minerals in the same horse population are scarce and can enhance our understanding of mineral parameters in Brazilian four-beat gaited horses. These reference range values allow horse nutritionists and veterinarians to properly interpret data on serum minerals when evaluating athlete Brazilian four-beat gaited horses. Additionally, these values may be used to monitor health status, improve feeding management, and diagnose diseases in Brazilian four-beat gaited horses.

The mineral values determined in the present study can serve as reference values, contribute to a better understanding of these metabolism biomarkers, and be used to improve nutritional and exercise evaluation programs and, consequently, the health status of athlete four-beat gaited horses.

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