Influence of covering and season on vitamin D synthesis, and its relation to parathyroid hormone, calcium, phosphorus and magnesium homeostasis in horses in New Zealand

Introduction

Vitamin D plays an important role in calcium and phosphorus absorption, bone metabolism, and maintenance of a healthy skeleton (Holick 2006b). Vitamin D deficiency leads to metabolic bone diseases such as rickets in the young, and osteomalacia in adults. In addition, deficiency of vitamin D may also be a risk factor for the development of osteoporosis and subsequently increased fracture risk in the elderly (Bouillon *et al.* 2008, Holick 2007, Holick 2006a).

Humans obtain most of their vitamin D from casual exposure to sunlight (Chen *et al.* 2007). Solar ultraviolet B radiation of wavelength 290-315 nm, results in synthesis of vitamin D in skin (Maclaughlin *et al.* 1982, Holick *et al.* 1980). Vitamin D may also be obtained through the diet (i.e. fatty fish, cod liver oil, and egg yolk), and/or dietary supplements (Holick 2007, DeLuca 2004).

The production of vitamin D in the skin starts with the photolytic conversion of 7-dehydrocholesterol (7-DHC) to previtamin D_3 under the influence of ultraviolet B photons from sun, followed by thermal isomerisation to vitamin D_3 (Holick 2007). The amount and number of ultraviolet photons that reach the skin can dramatically influence the efficiency of vitamin D_3 photosynthesis.

Vitamin D_3 produced in the skin and dietary vitamin D (D_2 and/or D_3) are transported to the liver by vitamin D-binding protein (DBP), whereby vitamin D is hydroxylated by 25-hydroxylases (CYP27A1 and CYP2R1) forming 25-hydroxyvitamin D (25OHD) (Deluca 2004). This form of vitamin D, 25OHD, is stable and the main form of vitamin D in the circulation, thus its concentration is commonly used to determine an individual's vitamin D status (Radlovic *et al.* 2012, Dusso *et al.* 2005). When it is needed, 25OHD is transported to the kidneys and undergoes 1α-hydroxylation by renal 25-hydroxyvitamin-D-1α-hydroxylase (CYP27B1), resulting into the production of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the

biologically active form of vitamin D (Morris & Anderson 2010, Hewison *et al.* 2007, Holick 2004).

Vitamin D is considered one of the calcitropic hormones, a group of hormones that through their actions on bone, kidney and the gastrointestinal tract (GIT), maintain serum calcium concentrations within the normal range required for calcium-requiring physiological functions in body. The functions of vitamin D include increased absorption of dietary calcium function, increased mobilization of calcium from bone, and increased reabsorption of calcium from the kidney (Lips 2012, Bouillon et al. 2008).

The other major calcitropic hormone is parathyroid hormone (PTH), and PTH is regulated by plasma calcium and vitamin D metabolite concentrations. Decreased plasma calcium concentration stimulates the parathyroid glands, leading to the secretion of PTH (Lips 2012). PTH stimulates the production of $1,25(OH)_2D$ in the kidney which increases calcium mobilization from bone (Lips 2012, Bouillon *et al.,* 2008). PTH results in increased absorption of calcium and decreased absorption of phosphorus in kidney (Kumar *et al.*, 2012). Active vitamin D has a negative feedback on PTH secretion (Kumar & Thompson, 2011).

When compared to other domestic animals, horses have several features with regards to calcium balance in the body, including high serum total and ionised calcium concentration (Toribio 2004), poorly regulated intestinal absorption of calcium (Schryver et al. 1970), decreased renal reabsorption and high urinary excretion of calcium (Toribio *et al.* 2001), low serum concentrations of vitamin D metabolites (Breidenbach *et al.* 1998; Maenpaa *et al.* 1988), and decreased parathyroid gland sensitivity to calcium (Toribio *et al.* 2003).

Many factors have an influence on the ability of skin to synthesise vitamin D_3. Skin melanin pigmentation acts as a natural sunscreen which efficiently absorbs ultraviolet B radiation (Clemens *et al.* 1982),and it has been shown that the darker skin is, the lower the percentage of ultraviolet radiation that is transmitted through it (Loomis 1967). Sunscreen usage with a high sun protection factor (SPF) has the same effect, and prevents the penetration of ultraviolet B photons (Matsuoka 1987). Other factors that influence vitamin D_3 production in the skin include season, latitude (Ladizesky *et al.* 1995, Webb *et al.* 1988),

type of clothing (Salih 2004, Matsuoka *et al.* 1992), aging (Holick *et al.* 1989, Maclaughlin & Holick 1985), and the timing of sunlight exposure (Holick 1987).

The importance of clothing's impact on vitamin D_3 synthesis in the skin is well documented. The effect of ultraviolet B radiation on the photosynthesis of vitamin D_3 using different fabrics (cotton, wool, and polyester) with different skin types (racial pigmentation) found that clothing significantly prevents the formation of vitamin D_3 in the skin (Matsuoka *et al.* 1992). Similarly, 15 different types of fabrics were tested for their effect on the efficiency of solar conversion of 7-DHC to vitamin D_3; the results of which showed that the fabric had a direct effect on sunlight attenuation and therefore amount of vitamin D_3 production in skin (Salih 2004). A recent study on Danish Holstein dairy cows showed that covered cows had lower plasma concentration of 25(OH)D compared to non-covered ones, and this change occurred within 28 days (Hymoller and Jensen 2010).

The majority of horses in New Zealand have dark skin and spend a large proportion of their time outside in paddocks wearing a cover, and this may adversely affect the ability of horses to synthesise vitamin D_3 in their skin. In order to assess this hypothesis, a thirteen-month trial was designed to study whether horses that are covered for substantial periods of time will have lower serum vitamin D concentrations than horses that are not covered. In addition we aimed to study the effect of season and ultraviolet exposure level on vitamin D concentration, assess the annual rhythm of calciotropic hormones (PTH and vitamin D metabolites), calcium, phosphorus, and magnesium, and determine a normal baseline concentration of calciotropic hormones (PTH and vitamin D metabolites), calcium, phosphorus, and magnesium in New Zealand horses at pasture.

Material and methods

The animal procedures were approved by the Massey University Animal Ethics Committee (approval no. 12/93).

Animal used

Twenty one healthy adult horses (*Equus caballus*) were included in the study, with a mean age of 12.19 ± 1.03 years (Table 1). The horses were housed in paddocks at the Veterinary Large Animal Teaching Unit (VLATU), Massey University, Palmerston North, New

Zealand. Five of the horses (Table 1) were covered with standard horse rugs, including a neck rug, for one year (Figure 1). The covers were of the appropriate weight for the time of year. Covers were removed at least weekly while the horses were indoors, and the horses were groomed. All horses were fed *ad libitum* grass pasture, and hay when appropriate, and had free access to water.

Table 1- List of horses in the study

ID	Breed	Sex	Age	Colour	Cover
1	Thoroughbred	Mare	18	Bay	Covered
2	Standardbred	Mare	25	Bay	Covered
3	Standardbred	Mare	8	Black	Covered
4	Standardbred	Mare	12	Bay	Covered
5	Thoroughbred	Mare	11	Bay	Covered
6	Standardbred	Mare	16	Bay	Non-covered
7	Standardbred	Mare	7	Bay	Non-covered
8	Standardbred	Mare	11	Bay	Non-covered
9	Thoroughbred	Mare	9	Bay	Non-covered
10	Standardbred	Gelding	9	Bay	Non-covered
11	Thoroughbred	Mare	14	Bay	Non-covered
12	Standardbred	Mare	7	Bay	Non-covered
13	Standardbred	Gelding	9	Bay	Non-covered
14	Standardbred	Gelding	8	Dark brown	Non-covered
15	Standardbred	Mare	13	Bay	Non-covered
16	Standardbred	Mare	6	Bay	Non-covered
17	Standardbred	Gelding	14	Bay	Non-covered
18	Thoroughbred	Mare	19	Chestnut	Non-covered
19	Thoroughbred	Mare	18	Chestnut	Non-covered
20	Thoroughbred	Mare	13	Bay	Non-covered
21	Standardbred	Mare	9	Bay	Non-covered

Sample Collection and Analysis

Samples were collected monthly at the same time of day for 13 months (January 2013- January 2014).

One serum separator tube (SST) and two plain tubes were collected (Vacutainer, Becton–Dickinson) from each horse by jugular venipuncture. All tubes were kept chilled and sealed, pending processing within 4 hours.

The serum was separated by centrifugation at 3500 × g for 15 min. The serum from the SST tubes were removed, and transferred to 10 mL plain tubes in an anaerobic manner, followed by ionised calcium estimation within 4 hours of collection, FLEX Analyse. Serum from plain tubes was transferred to 1.5 mL micro centrifuge tubes and stored at -80˚C until further analysis.

Analysis of Serum Samples

The serum parathyroid hormone (PTH) concentration was measured using the ARCHITECT Intact PTH assay (Abbott Architect Ci8200, ABBOTT Diagnostics Division, Germany) at Endolab, Canterbury Health Laboratories, Christchurch, New Zealand. The PTH assey was performed in duplicate, and had an intraassay CV of < 6.2%, and interassay CV of < 7.3%.

Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry (LC/MS) was used to measure 25-hydroxyvitamin D_3 ($25OHD_3$) and 25-hydroxyvitamin D_2 ($25OHD_2$) concentrations, also at Endolab.

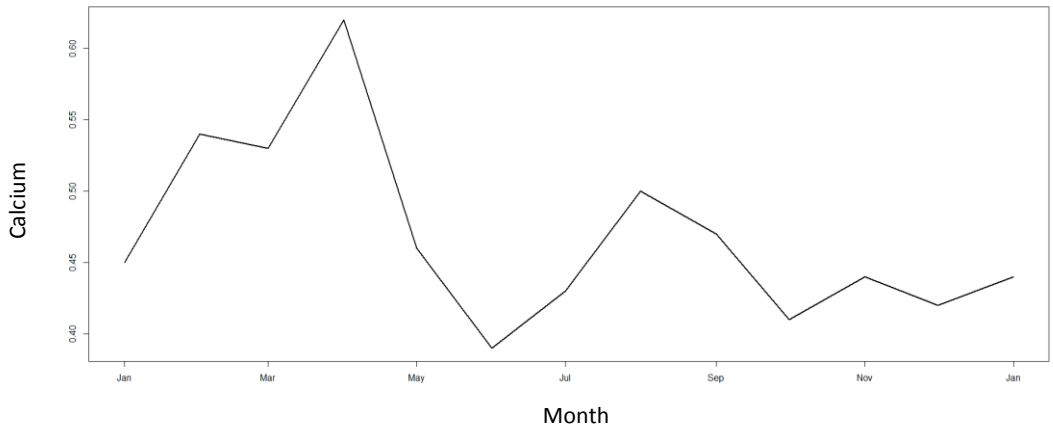
Serum total Calcium (tCa^{2+}), Phosphorus (PO_4^{3-}) and total Magnesium (tMg^{2+}) concentrations were measured using a Roche Hitachi 911 Chemistry Analyser (Roche Diagnostics, USA) at a commercial veterinary diagnostic laboratory (New Zealand Veterinary Pathology, Palmerston North, New Zealand). Ionised Calcium (iCa^{2+}) was measured using Radiometer ABL800 FLEX analyzer (Radiometer Medical ApS, Denmark) at MedLab Central, Palmerston North Hospital, New Zealand.

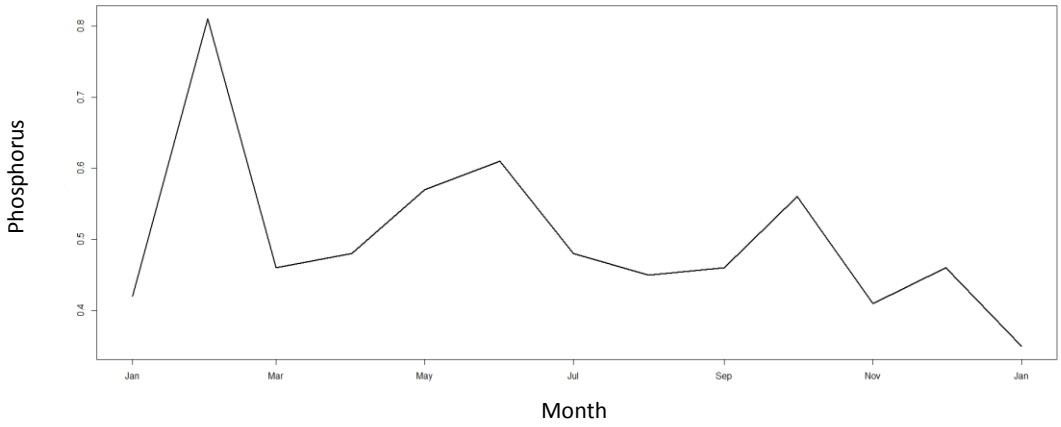
Serum 1,25-dihydroxyvitamin D (1,25(OH)D) concentration was measured using the DiaSorin 1,25-dihydroxyvitamin D RIA kit (Stillwater, Minnesota, USA) as per manufactures' instruction. Samples were analysed in duplicate intraassay and interassay CV were always < 10%.

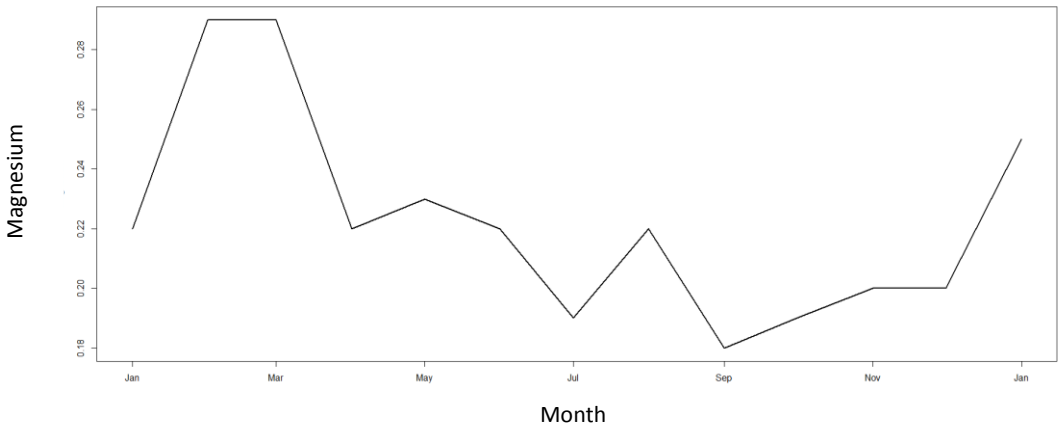
Pasture Collection and feed analysis

The horses were housed in paddocks at VLATU, Massey University, and pasture samples were collected monthly the day prior to blood collection, from paddocks the horses were kept in during the month prior to sample collection, in order to determine the calcium, phosphorus, magnesium and vitamin D content of the herbage consumed by the horses. Horses are selective grazers, and actively avoid areas where they have defecated, so pasture samples were collected from areas where horses were actively grazing (i.e. short grass) and not from areas they were avoiding (i.e. long grass). Each starting transect point was chosen in areas where the horses preferred to graze. If pasture cover was low, most of the sward was collected, if there was higher pasture cover the top two-thirds of the sward was collected. Photos from each paddock were taken to record the quality of pasture. Pasture samples were collected before 12 noon, and a straight lines transect method was used for sampling. Samples were taken at around 10-15 m intervals along the transect line, using scissors, resulting in a handful of pasture (approximately 10-20 g per hand scissor clip). At least 50 clips per paddock were taken, resulting in approximately 0.5 kg fresh weight of pasture from each paddock. All grass was wrapped in a dark plastic cover to protect it from sunlight, weighed and transported to the Nutrition Lab, Institute of Food, Nutrition and Human Health, Massey University, Plamerston North, New Zealand.

Samples from different paddocks were mixed, freeze-dried and ground to pass through a 1 mm screen. The ground sample was analysed for calcium, phosphorus and magnesium using Plasma Emission Spectrometry subcontracted method, and for vitamin D_2 and vitamin D_3 using HPLC NMKL 167, 2000 method.







Climate Data

The daily sunshine hours and amount of UV radiation (January 2013 – January 2014) was obtained from the Palmerston North climate station at the Palmerston North airport run by The National Institute of Water and Atmospheric Research (NIWA)/Taihoro Nukurangi, New Zealand.

The mean (± SE) monthly sunshine (Hrs) and UV radiation (MJ/m^2) was recorded at the Palmerston North region during the whole experiment (Table 4).

Table 2- Mean (± SE) serum analyte concentration from January 2013 to January 2014 in covered and non-covered horses

		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan
iCa^{2+} (mmol/L)	C	1.45 (± 0.01)	1.53 (± 0.02)	1.53 (± 0.01)	1.52 (± 0.02)	1.53 (± 0.01)	1.42 (± 0.02)	1.5 (± 0.01)	1.53 (± 0.01)	1.42 (± 0.01)	1.49 (± 0.01)	1.49 (± 0.01)	1.52 (± 0.01)	1.52 (± 0.01)
	NC	1.47 (± 0.01)	1.54 (± 0.009)	1.52 (± 0.01)	1.45 (± 0.01)	1.48 (± 0.01)	1.53 (± 0.02)	1.51 (± 0.01)	1.49 (± 0.01)	1.43 (± 0.01)	1.5 (± 0.01)	1.46 (± 0.007)	1.53 (± 0.01)	1.53 (± 0.01)
tCa^{2+} (mmol/L)	C	2.98 (± 0.04)	2.61 (± 0.11)	3.02 (± 0.04)	3.1 (± 0.04)	3.12 (± 0.06)	2.85 (± 0.06)	2.68 (± 0.19)	2.58 (± 0.11)	2.89 (± 0.05)	3.42 (± 0.22)	3.26 (± 0.14)	2.68 (± 0.2)	3.08 (± 0.12)
	NC	2.91 (± 0.03)	3 (± 0.03)	3.01 (± 0.02)	2.94 (± 0.05)	2.98 (± 0.04)	3.05 (± 0.03)	2.72 (± 0.02)	2.53 (± 0.04)	3 (± 0.02)	3.76 (± 0.14)	2.91 (± 0.11)	3.12 (± 0.11)	2.42 (± 0.06)
PO_4^{3-} (mmol/L)	C	1.18 (± 0.06)	1.03 (± 0.08)	1.1 (± 0.03)	0.9 (± 0.04)	1.07 (± 0.05)	1.02 (± 0.03)	0.75 (± 0.03)	0.66 (± 0.03)	1.18 (± 0.03)	1.27 (± 0.07)	1.13 (± 0.03)	0.91 (± 0.06)	0.88 (± 0.08)
	NC	1 (± 0.04)	1.05 (± 0.03)	1.13 (± 0.04)	1.04 (± 0.03)	1.05 (± 0.04)	0.87 (± 0.04)	0.85 (± 0.02)	0.88 (± 0.03)	1.18 (± 0.04)	1.2 (± 0.06)	1.04 (± 0.07)	1.01 (± 0.09)	0.75 (± 0.03)
tMg^{2+} (mmol/L)	C	0.74 (± 0.007)	0.72 (± 0.04)	0.82 (± 0.01)	0.76 (± 0.02)	0.75 (± 0.01)	0.68 (± 0.005)	0.56 (± 0.02)	0.61 (± 0.02)	0.65 (± 0.01)	0.81 (± 0.06)	0.81 (± 0.04)	0.69 (± 0.04)	0.74 (± 0.02)
	NC	0.72 (± 0.01)	0.81 (± 0.02)	0.79 (± 0.01)	0.72 (± 0.01)	0.66 (± 0.01)	0.71 (± 0.01)	0.61 (± 0.009)	0.56 (± 0.01)	0.68 (± 0.01)	0.89 (± 0.03)	0.71 (± 0.03)	0.76 (± 0.03)	0.61 (± 0.01)
$25OHD_2$ (nmol/L)	C	8.8 (± 0.43)	8.6 (± 0.21)	11.2 (± 0.33)	11.8 (± 0.76)	9 (± 0.48)	6.2 (± 0.65)	5.6 (± 0.72)	6.2 (± 0.33)	7.8 (± 0.43)	10.2 (± 0.59)	10.8 (± 0.52)	15.2 (± 1.03)	11.6 (± 1.11)
	NC	8.43 (± 0.6)	10.31 (± 0.71)	11.62 (± 0.55)	9.87 (± 0.53)	6.37 (± 0.3)	6 (± 0.39)	6.37 (± 0.44)	6.12 (± 0.37)	7 (± 0.63)	8.18 (±0.54)	9.25 (± 0.63)	11.75 (± 0.97)	10.12 (± 0.48)
$25OHD_3$ (nmol/L)	C	0	0.2 (± 0.17)	0.6 (± 0.21)	0.2 (± 0.17)	0.2 (± 0.17)	0.2 (± 0.17)	0.2 (± 0.17)	0	0	0.4 (± 0.21)	0	0	0.4 (± 0.21)
	NC	0.87 (± 0.08)	0.81 (± 0.09)	0.56 (± 0.12)	0.31 (± 0.11)	0.06 (± 0.06)	0.06 (± 0.06)	0.12 (± 0.08)	0.06 (± 0.06)	0.06 (± 0.06)	0	0	0.06 (± 0.06)	0.12 (± 0.08)
$1,25(OH)_2D$ (pmol/L)	C	29.85 (± 3.91)	36.43 (± 1.68)	36.38 (± 7.17)	47.42 (± 8.84)	67.82 (± 21.05)	20.54 (± 6.35)	29.23 (± 1.69)	25.82 (± 2.56)	26.35 (± 1.27)	23.23 (± 2.23)	26.88 (± 2.67)	26.25 (± 4.44)	34.89 (± 4.1)
	NC	55.68 (± 6.8)	49.69 (± 9.46)	45.97 (± 5.1)	38.56 (± 3.67)	37.15 (± 3.73)	24.59 (± 2.97)	31.93 (± 2.69)	29.94 (± 2.15)	33.72 (± 3.25)	31.99 (± 2.75)	32.8 (± 2.53)	39.48 (± 3.75)	41.85 (± 4.15)
PTH (pg/ml)	C	6.96 (± 1.63)	7.05 (± 2.47)	4.73 (± 0.46)	6.28 (± 2.15)	7.02 (± 0.97)	24.12 (± 5.67)	10.84 (± 3.55)	12.84 (± 7.19)	15.84 (± 5.35)	9 (± 2.23)	9.9 (± 2.3)	6.87 (± 0.7)	4.78 (± 0.8)
	NC	8.77 (± 2.72)	7.78 (± 1.58)	9.33 (± 0.94)	15.6 (± 1.8)	16.79 (± 3.11)	15.12 (± 3.83)	9.74 (± 3.52)	12.78 (± 2.6)	14.34 (± 3.25)	10.18 (± 2.19)	12.26 (± 4.23)	9.22 (± 1.7)	7.8 (± 1.86)

C: covered horses, NC: non-covered horses, iCa^{2+}: ionised calcium, tCa^{2+}: total calcium, PO_4^{3-}: Phosphorus, tMg^{2+}: total magnesium, $25OHD_2$: 25-hydroxyvitamin D_2, $25OHD_3$: 25-hydroxyvitamin D_3, 1,25(OH)2D: 1,25-dihydroxyvitamin D and, PTH: parathyroid hormone

Table 3- Concentration of calcium (%), phosphorus (%), magnesium (%) and vitamin D (μg/g) in the pasture consumed by horses from January 2013 to January 2014

		January	February	March	April	May	June	July	August	September	October	November	December	January
Calcium	Pasture	0.45	0.54	0.53	0.46	0.46	0.39	0.43	0.42	0.46	0.41	0.44	0.42	0.44
	Hay	-	-	-	0.78	-	-	-	0.58	0.48	-	-	-	-
Phosphorus	Pasture	0.42	0.81	0.46	0.64	0.57	0.61	0.48	0.54	0.64	0.56	0.41	0.46	0.35
	Hay	-	-	-	0.32	-	-	-	0.27	0.28	-	-	-	-
Magnesium	Pasture	0.22	0.29	0.29	0.24	0.23	0.22	0.19	0.22	0.18	0.19	0.20	0.20	0.25
	Hay	-	-	-	0.19	-	-	-	0.17	0.18	-	-	-	-
Vitamin D	Pasture	5.22	5.44	5.41	3.05	2.54	3.31	3.48	1.07	0.52	0.49	0.57	2.35	1.24
	Hay	-	-	-	15.09	-	-	-	7.99	3.67	-	-	-	-

Table 4- Climate variables of mean (± SE) monthly sunshine (Hrs) and UV radiation (MJ/m^2) obtained from the Palmerston North climate station at Palmerston North airport (NIWA) from January 2013 – January 2014

	January	February	March	April	May	June	July	August	September	October	November	December	January
Sunshine	7.98 (± 0.76)	8.98 (± 0.73)	7.79 (± 0.65)	3.75 (± 0.57)	4.20 (± 0.57)	2.89 (± 0.52)	2.80 (± 0.46)	4.05 (± 0.46)	4.05 (± 0.53)	3.93 (± 0.51)	5.69 (± 0.82)	5.74 (± 0.73)	6.48 (± 0.69)
Radiation	23.92 (± 1.07)	22.16 (± 1)	17.13 (± 0.88)	9.26 (± 0.64)	7.16 (± 0.44)	4.99 (± 0.39)	5.62 (± 0.39)	8.88 (± 0.45)	11.88 (± 0.64)	14.97 (± 1.02)	19.55 (± 1.2)	21.19 (± 1.17)	20.74 (± 1.17)

Statistical Analysis

Results from the ionised calcium, total calcium, phosphorus, total magnesium, parathyroid hormone, and vitamin D metabolite ($25OHD_2$, $25OHD_3$ and, $1,25(OH)_2D$) analysis are expressed in absolute concentrations. Generalized additive mixed models were used to test the influence of coverage, age, sex, breed, the amount of each analyte in feed and the average monthly UV radiation/sunshine on the concentration of each serum analyte incorporating a smooth trend for them, with a random effect for horses. In order to find any differences between covered and non-covered horses, smooth trends through the year were calculated by fitting a generalized additive model for each analyte using the mgcv package (Wood 2011) in R (R Core Team 2014). Spearman's pairwise correlation and scatterplots were used for the serum analytes to study the correlation between different analytes. For all statistical comparisons a value of $P < 0.05$ was considered significant.

Results

Effects of covering, sunlight/UV radiation and diet on serum analytes

Climate data, as it was expected, showed in contrast the amount of UV radiation was higher during spring (September-November) and summer (December-February) time to autumn (March-May) and winter (June-August) (Table 4).

The data obtained for each analyte for covered and non-covered horses is shown in Table 2. No significant differences were found in serum concentration of iCa^{2+}, tCa^{2+}, PO_4^{3-}, tMg^{2+},PTH, $25OHD_3$, $25OHD_2$ and $1,25(OH)_2D$ between covered and non-covered horses. Serum concentrations of $25OHD_3$ were surprisingly low, mostly zero in all horses both covered and non-covered, and statistical analysis was unable to be performed. The data was put into the generalized additive mixed models, and the results are presented in Table 5.

Average monthly sunshine had a significant positive effect on serum $25OHD_2$, $1,25(OH)_2D$ and, iCa^{2+} concentrations and a significant negative effect on serum tMg^{2+} and tCa^{2+}concentrations ($P< 0.05$), after adjusting for different parameters. In contrast, average monthly radiation had a significant positive effect on serum $25OHD_2$, $1,25(OH)_2D$, tCa^{2+} and, tMg^{2+} concentrations ($P< 0.05$) and a significant negative effect on serum iCa^{2+}concentration ($P< 0.05$) (Table 5).

After adjusting for other covariates, serum tCa^{2+} concentration had a direct significant positive effect on PO_4^{3-} and tMg^{2+} ($P< 0.05$). Similarly, serum PO_4^{3-} and tMg^{2+}concentrations had a significant positive effect on serum tCa^{2+} concentration ($P< 0.05$). In contrast, serum iCa^{2+} concentration had a significant negative effect on serum PO_4^{3-} and PTH concentrations ($P< 0.05$). Serum iCa^{2+} and tMg^{2+} concentrations similarly had a positive effect on serum $25OHD_2$ concentration ($P< 0.05$). Serum $1,25(OH)_2D$ concentration had a significant negative effect on serum PTH concentration ($P<0.05$) and serum PTH concentration had a significant negative effect on serum iCa^{2+} concentration ($P< 0.05$) (Table 5).

The amount of magnesium in pasture had a significant positive effect on serum tMg^{2+} concentration ($P< 0.05$), similarly the amount of vitamin D in pasture and hay had a significant positive effect on serum $25OHD_2$ and $1,25(OH)_2D$ concentrations ($P< 0.05$), after adjusting different parameters in the statistical model (Table 5).

Table 5- Statistical analysis of effects of covering, sunlight/UV radiation and diet on serum analytes

	$25OHD_2$	$1,25(OH)_2D$	tCa^{2+}	iCa^{2+}	PO_4^{3-}	tMg^{2+}	PTH
	Estimate (95% C.I.)	Estimate (95% C.I.)	Estimate (95% C.I.)	Estimate (95% C.I.)	Estimate (95% C.I.)	Estimate (95% C.I.)	Estimate (95% C.I.)
Intercept	-1.13 (-8.8, 6.6)	42.11 (-36, 120)	1.27 (0.79, 1.7)	1.56 (-9.1, 12.2)	1.3 (2, 0.5)	-0.21 (-0.4, 0.04)	97.03 (66, 127)
Cover	1.01 (-12, 14)	-3.85 (-11.3, 3.6)	-0.04 (-0.13, 0.05)	-1.04 (-4.7, 2.6)	0.04 (-0.02, 0.09)	0.006 (-0.01, 0.02)	-3.83 (-10.2, 2.5)
Age	5.8 (-6.9, 18.5)	0.003 (-0.72, 0.72)	0.0	8.73 (5.1, 12.3)	-0.008 (0.008)	0.001 (0.002)	0.47 (-0.13, 1.07)
Sex	-2.37 (-17.4, 12.6)	-7.06 (-16.5, 2.4)	-0.001 (-0.11, 0.11)	-5.9 (-10, -1.7)	-0.02 (-0.09, 0.05)	0.006 (-0.01, 0.02)	-2.31 (-9.5, 4.9)
Breed	1.09 (-12, 14.2)	2.09 (-5.5, 9.7)	0.08 (-0.01, 0.17)	1.3 (-2.4, 5)	-0.03 (-0.09, 0.02)	-0.02 (-0.03)	0.97 (-5.5, 7.4)
tCa^{2+}	–	–	–	–	**0.27 (0.2, 0.3)**	**0.21 (0.19, 0.22)**	-3.21 (-7.7, 1.35)
iCa^{2+}	**7.46 (2.7, 12.1)**	-1.32 (-51.2, 48.6)	–	–	**-0.91 (-1.4, -0.4)**	-0.1 (-0.25, 0.05)	**-53.08 (-73.7, -32.5)**
PO_4^{3-}	–	-0.08 (-12.3, 12.1)	**0.29 (0.17, 0.4)**	-3.51 (-5.7, -0.5)	–	0.007 (-0.01, 0.02)	3.15 (-1.47, 7.7)
tMg^{2+}	**3.47 (1.3, 5.6)**	-9.9 (-34.8, 15)	**2.59 (2.3, 2.8)**	**9.88 (4.3, 15.3)**	-0.03 (-0.4, 0.3)	–	7.17 (-8.2, 22.5)
$25OHD_2$	–	-0.2 (-1.4, 1)	–	3.39 (0.9, 5.9)	–	–	-0.13 (-0.56, 0.3)
$1,25(OH)_2D$	3.91 (-8.1, 15.9)	–	–	-2.01 (-4.7, 0.6)	–	–	**-0.06 (-0.09, -0.02)**
PTH	-1.85 (-4.9, 1.2)	0.15 (-0.29, 0.29)	-0.002 (-0.003)	**-1.96 (-8.9, 5)**	0.001 (0.001)	-0.0001 (0.002)	–
Pasture Ca	–	–	-0.14 (-1.4, 1.21)	-3.83 (-6.8, -0.8)	–	–	–
Hay Ca	–	–	-0.14 (-0.2, -0.04)	-1.6 (-4, 0.8)	–	–	–
Pasture PO_4	–	–	–	–	0.001 (0.23)	–	–
Hay PO_4	–	–	–	–	0.11 (-0.12, 0.34)	–	–
Pasture Mg	–	–	–	–	–	**1.73 (1.18, 2.27)**	–
Hay Mg	–	–	–	–	–	-0.11 (-0.2, -0.01)	–
Pasture Vit D	**6.04 (3.9, 8.1)**	**0.63 (-1.6, 2.9)**	–	–	–	–	–
Hay Vit D	**1.79 (-2.7, 6.3)**	**0.26 (-0.28, 0.8)**	–	–	–	–	–
Monthly Sunshine	**0.78 (0.6, 0.9)**	**2.45 (0.49, 4.41)**	**-0.1 (-0.15, -0.04)**	**3.94 (-10, 18.6)**	0.03 (0.06)	**-0.04 (-0.06, -0.02)**	0.05 (-1.5, 1.6)
Monthly Radiation	**3.27 (-9, 15.5)**	**0.63 (0.1, 1.15)**	**0.01 (-0.005, 0.02)**	**-1.04 (-4.4, 2.3)**	0.01 (0.01)	**0.01 (0.01)**	-0.33 (-0.76, 0.1)

	$25OHD_2$	$1,25(OH)_2D$	tCa^{2+}	iCa^{2+}	PO_4^{3-}	tMg^{2+}	PTH
S(Month)	1.794	1	1	1	1.899	1.92	1
R^2	0.49	0.0868	0.693	0.254	0.321	0.724	0.175
P-value	1.34e-08	0.623	0.163	2.8e-06	0.00484	0.000116	0.941

Trend of each analyte through the year

As there were no significant differences between covered and non-covered groups, the full data was analysed to determine trends in the measured analytes across the year (Table 6). The results plotted to visualise the shape and rhythm of each analyte through the year are presented in Figures 1-4. All analytes showed a significant P value in their serum concentration during the year ($P< 0.0001$).

During spring and summer (November-March) the amount of $25OHD_2$ in serum was high compared to autumn and winter (April-October) were it was low (Figure 1). Similarly the lowest amount of $1,25(OH)_2D$ was also measured during winter (July-September) and the concentration of $1,25(OH)_2D$ was higher during spring and summer (November-March) (Figure 1).

Seasonal changes in iCa^{2+} concentration were more obvious than that for the other analytes. The highest concentration of iCa^{2+} was measured in February, July, December and January whereas the lowest concentration was measured during April and September (Figure 2).

Total calcium (tCa^{2+}) did not fluctuate markedly during January-July, but declined during July and August, and then peaked in October. From December-January the trend for it was for it to decline (Figure 2).

PTH showed a very stable rhythm through the year, where the lowest concentration of PTH was measured during January and the highest concentration was measured in July (Figure 3).

Table 6- Mean (± SE) serum analyte concentration from January 2013 to January 2014 in horses in New Zealand

	January	February	March	April	May	June	July	August	September	October	November	December	January
iCa^{2+} (mmol/L)	1.46 (± 0.01)	1.54 (± 0.008)	1.53 (± 0.01)	1.47 (± 0.01)	1.49 (± 0.01)	1.50 (± 0.02)	1.51 (± 0.01)	1.50 (± 0.01)	1.43 (± 0.01)	1.49 (± 0.01)	1.47 (± 0.006)	1.52 (± 0.009)	1.53 (± 0.009)
tCa^{2+} (mmol/L)	2.93 (± 0.02)	2.91 (± 0.04)	3.02 (± 0.02)	2.98 (± 0.04)	3.01 (± 0.03)	3 (± 0.03)	2.71 (± 0.05)	2.54 (± 0.04)	2.97 (± 0.02)	3.68 (± 0.12)	3 (± 0.09)	3.02 (± 0.1)	2.58 (± 0.08)
PO_4^{3-} (mmol/L)	1.04 (± 0.04)	1.05 (± 0.03)	1.12 (± 0.03)	1.01 (± 0.03)	1.06 (± 0.03)	0.9 (± 0.04)	0.83 (± 0.02)	0.83 (± 0.03)	1.18 (± 0.03)	1.22 (± 0.05)	1.06 (± 0.06)	0.98 (± 0.07)	0.78 (± 0.03)
tMg^{2+} (mmol/L)	0.72 (± 0.01)	0.79 (± 0.02)	0.8 (± 0.01)	0.73 (± 0.01)	0.68 (± 0.01)	0.71 (± 0.01)	0.6 (± 0.01)	0.57 (± 0.01)	0.67 (± 0.01)	0.87 (± 0.03)	0.73 (± 0.02)	0.75 (± 0.02)	0.64 (± 0.01)
$25OHD_2$ (nmol/L)	8.52 (± 0.47)	9.9 (± 0.57)	11.52 (± 0.42)	10.33 (± 0.48)	7 (± 0.35)	6.04 (± 0.33)	6.19 (± 0.38)	6.14 (± 0.29)	7.19 (± 0.5)	8.66 (± 0.47)	9.61 (± 0.51)	12.57 (± 0.84)	10.47 (± 0.47)
$25OHD_3$ (nmol/L)	0.66 (± 0.1)	0.66 (± 0.1)	0.57 (± 0.1)	0.28 (± 0.09)	0.09 (± 0.06)	0.09 (± 0.06)	0.14 (± 0.07)	0.04 (± 0.04)	0.04 (± 0.04)	0.09 (± 0.06)	0	0.04 (± 0.04)	0.19 (± 0.08)
$1,25(OH)_2D$ (pmol/L)	49.53 (± 5.79)	46.54 (± 7.32)	43.69 (± 4.34)	40.67 (± 3.6)	44.45 (± 6.43)	23.62 (± 2.74)	31.29 (± 2.1)	28.96 (± 1.79)	31.96 (± 2.58)	29.91 (± 2.31)	31.39 (± 2.1)	36.33 (± 3.2)	40.19 (± 3.37)
PTH (pg/ml)	8.23 (± 1.99)	7.56 (± 1.33)	7.97 (± 0.84)	12.86 (± 1.75)	13.91 (± 2.46)	17.77 (± 3.33)	10.06 (± 2.7)	12.8 (± 2.83)	14.78 (± 2.78)	9.83 (± 1.68)	11.52 (± 3.04)	8.63 (± 1.28)	6.91 (± 1.37)

iCa^{2+}: ionised calcium, tCa^{2+}: total calcium, PO_4^{3-}: Phosphorus, tMg^{2+}: total magnesium, $25OHD_2$: 25-hydroxyvitamin D_2, $25OHD_3$: 25-hydroxyvitamin D_3, $1,25(OH)_2D$: 1,25-dihydroxyvitamin D and, PTH: parathyroid hormone

The concentration of PO_4^{3-} was consistently flat from January-May. In June it started to decrease and the lowest concentration of PO_4^{3-} was measured in July. From August the concentration of PO_4^{3-} was increased and reached its peak in October following it which declined (Figure 4).

There was fluctuation in the trend of tMg^{2+} during the study. The concentration of tMg^{2+} increased during January-March then decreased from April-July and reached its lowest concentration in August. Then the concentration of tMg^{2+} increased and reached its highest peak in October where it declined from there onward (Figure 4).

The trend of tCa^{2+}, tMg^{2+} and to some extent PO_4^{3-} were similar but, in comparison, the trends of PTH and $1,25(OH)_2D$ were opposite to each other throughout the year.

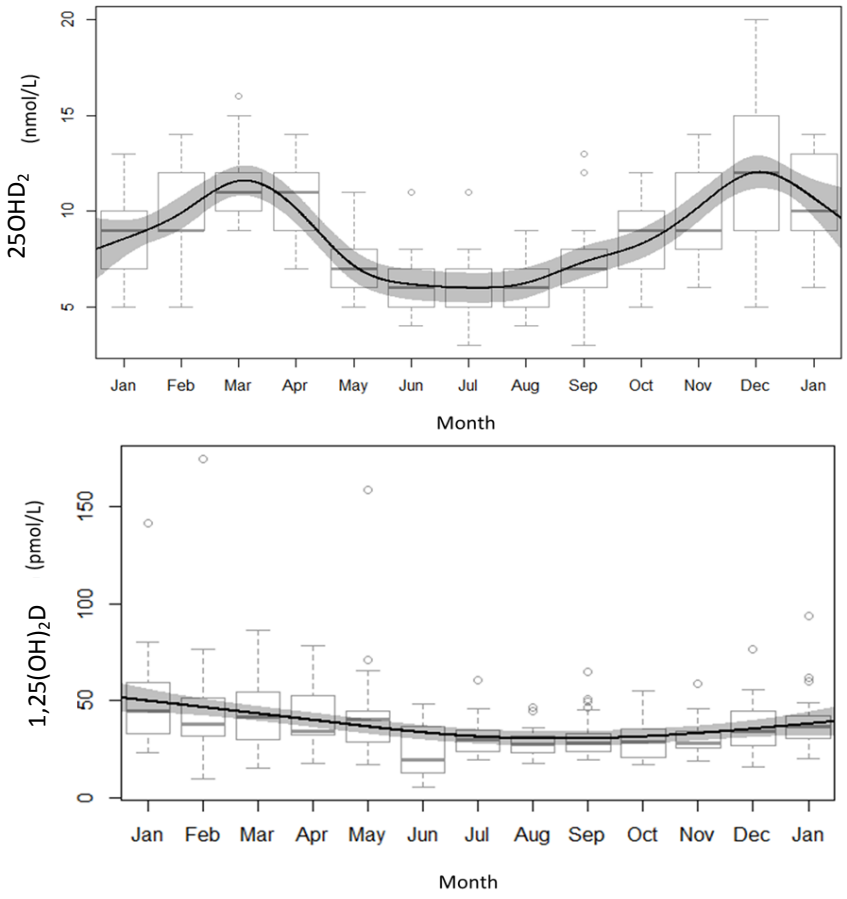


Figure 1- The trend of 25-hydroxyvitamin D_2 ($25OHD_2$) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) through the year-long study in the sample population.

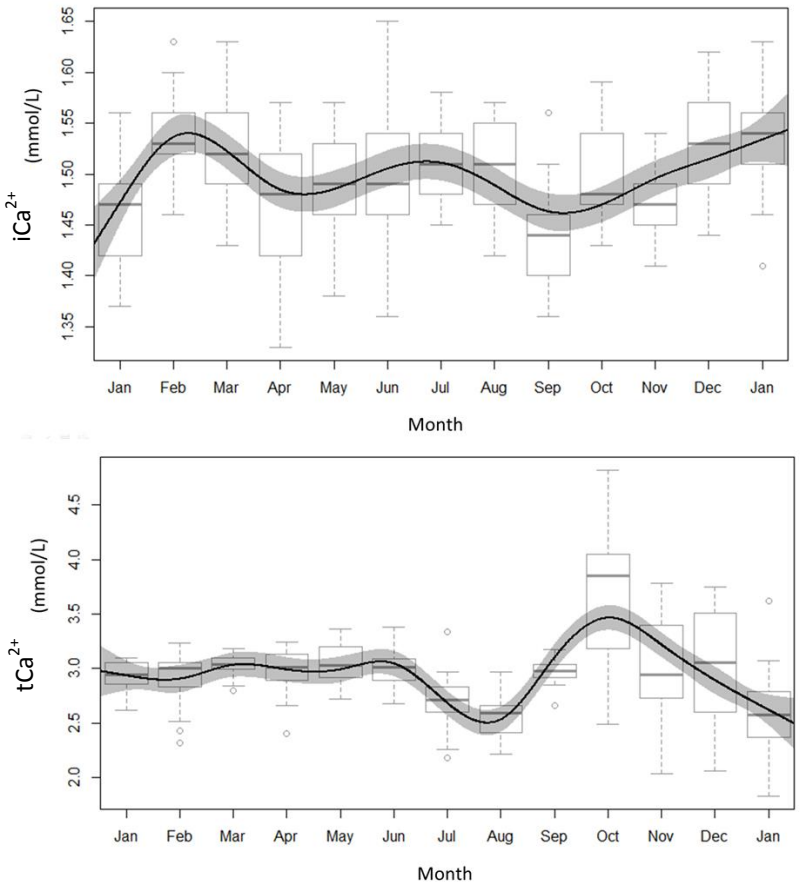


Figure 2- The trend of ionised calcium (iCa^{2+}) and total calcium (tCa^{2+}) through the year-long study in the sample population.

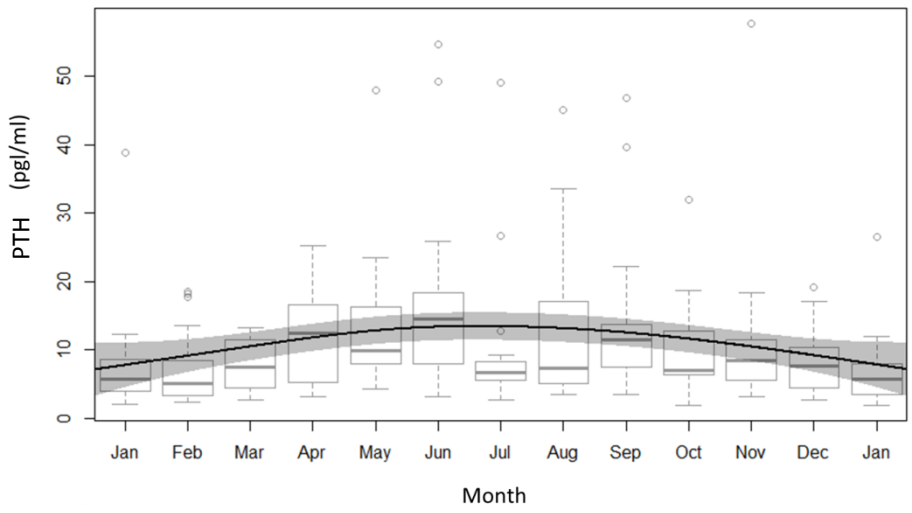


Figure 3- The trend of parathyroid hormone (PTH) through the year-long study in the sample population.

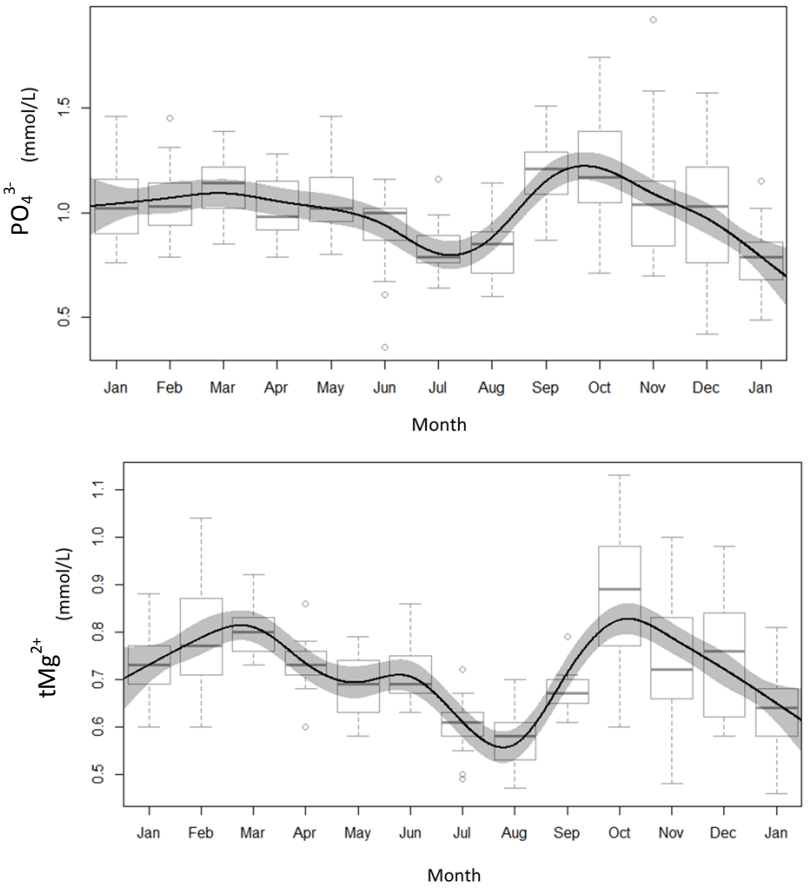


Figure 4- The trend of phosphorus (PO_4^{3-}) and total magnesium (tMg^{2+}) through the year-long study in the sample population.

Pairwise correlation of serum analytes

As there were no significant differences between covered and non-covered groups, the full data was analysed and plotted to determine the pairwise correlation of serum analytes towards each other, and is presented in Figure 5.

Ionised calcium (iCa^{2+}) and parathyroid hormone (PTH) showed the highest negative correlation (R^2= -0.33, 95% CI (-0.44, -0.21)), whereas total calcium (tCa^{2+}) and total magnesium (tMg^{2+}) showed the highest positive correlation (R^2= 0.79, 95% CI (0.74, 0.83)) (Figure 5).

PTH showed a significant negative correlation with 25-hydroxyvitamin D_2 ($25OHD_2$) (R^2= -0.24, 95% CI (-0.36, -0.12)), tMg^{2+} (R^2= -0.17, 95% CI (-0.29, -0.05)) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (R^2= -0.14, 95% CI (-0.26, -0.02)) (Figure 5).

A significant positive correlation of $25OHD_2$ with iCa^{2+} (R^2= 0.28, 95% CI (0.17, 0.39)), phosphorus (PO_4^{3-}) (R^2= 0.25, 95% CI (0.14, 0.36)) and $1,25(OH)_2D$ (R^2= 0.12, 95% CI (0.00, 0.24)) with $25OHD_2$ was seen (Figure 5).

PO_4^{3-} showed a significant positive correlation with tCa^{2+} (R^2= 0.47, 95% CI (0.37, 0.56)) and tMg^{2+} (R^2= 0.42, 95% CI (0.31, 0.51)) and a significant negative correlation with iCa^{2+} (R^2= -0.17, 95% CI (-0.29, -0.06)) (Figure 5).

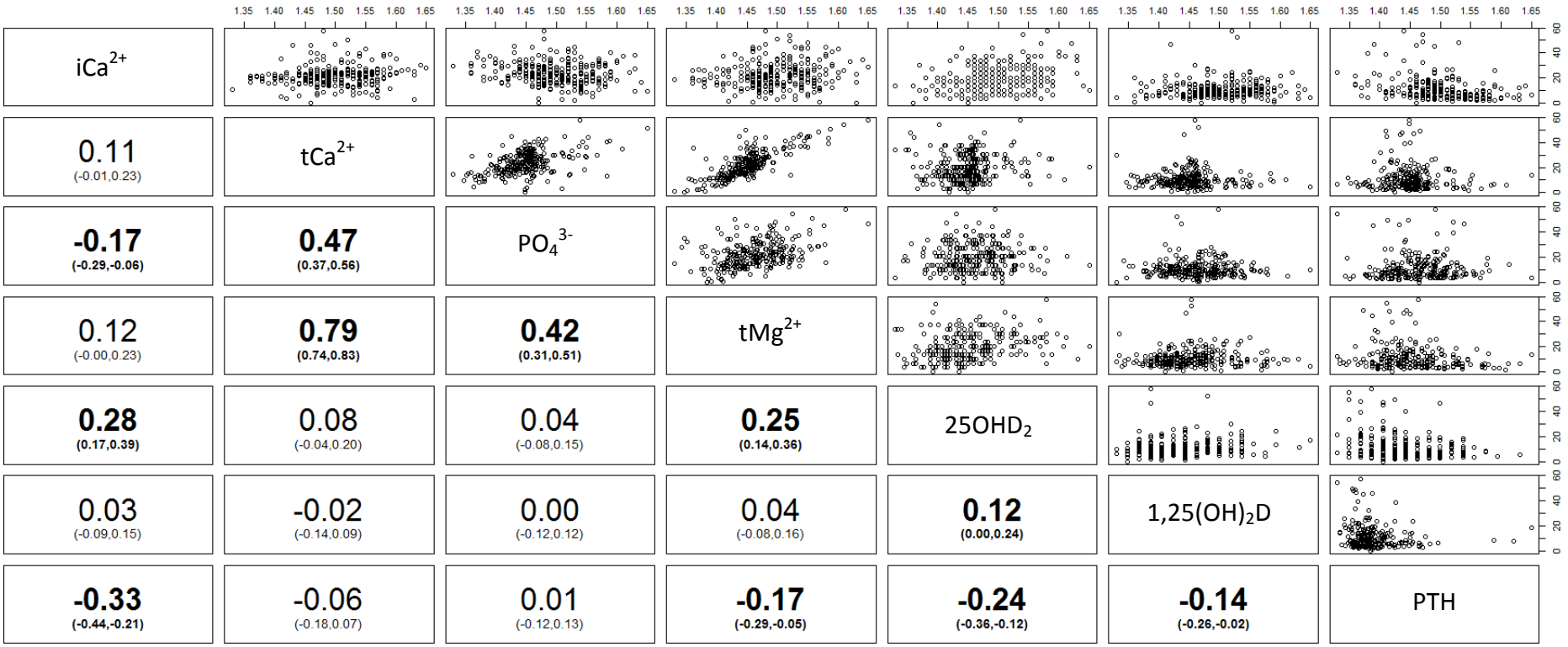


Figure 5- Spearman's correlation (95% Confidence Intervals) between ionised calcium (iCa^{2+}), total calcium (Ca^{2+}), phosphorus (PO_4^{3-}), total magnesium (tMg^{2+}), 25-hydroxyvitamin D_2 ($25OHD_2$) and 1,25-dihydroxyvitamin D ($1,25(OH)_2$) and parathyroid hormone (PTH) in horses. Bold numbers indicate significance (P< 0.05).

Discussion

There are two main forms of vitamin D, vitamin D_3 obtained from irradiation of skin with UVB light (photolysis of 7-DHC to vitamin D_3) and from food (eg. cod liver oil and fatty fish), and vitamin D_2 obtained from irradiated plants.

The major new finding from this study was that, in contrast to other studies, serum $25OHD_3$ was undetectable and $25OHD_2$ was the major form present in the serum.

The principal source of vitamin D in horses was assumed to be through synthesis of vitamin D_3 in their skin when exposed to sunlight/UV light, much like other grazing animals, especially cows and sheep (Hymoller and Jensen 2010, Smith & Wrigh 1984b).

Some previous studies on horses showed that the amount of $25OHD_3$ in horses was much lower than other species but was detectiable, which is in direct contrast to the results of this study (Pozza *et al.* 2014, Piccione *et al.* 2008, Breidenbach *et al.* 1998, Smith & Wrigh 1984a, Maenpaa *et al.* 1988a,b, Maenpaa *et al.* 1987, Elshorafa *et al.* 1979). However, one study mentioned that $25OHD_3$ might not be a good index of vitamin D status in the horse as its concentration was almost undetectable (Smith & Wrigh 1984a). This study clearly showed that the main form of circulating vitamin D metabolite in horse serum was $25OHD_2$. This study, together with our results, suggests that the measured concentration of 25OHD in previous studies included both $25OHD_2$ and $25OHD_3$ due to the lack of speciality of the specific test method.

Skin coverage has a direct impact on the synthesis of $25OHD_3$ in mammalian skin, particularly in humans (Hymoller and Jensen 2010, Salih 2004, Matsuoka *et al.* 1992). Regardless of coverage in the current study, however, no differences between the serum concentrations of total 25OHD in horses were found.

The results of this study suggest that horses are not able to produce much, if any, vitamin D_3 in their skin, whether they spend a large proportion of their time outside in paddocks wearing covers or not. Therefore, horses might be mainly relying on vitamin D_2 that specifically obtained from hay/pasture and their diet to fulfil their need of vitamin D.

There was a seasonal trend of $25OHD_2$ in the serum of horses during the study. During spring and summer (November-March) when the amount of sunshine and UV

radiation was higher, the concentration of $25OHD_2$ in serum was higher compared with autumn and winter (April-October). This supports the direct effect of the amount of sunshine and UV radiation on the synthesis of $25OHD_2$ in the pasture/hay that was consumed by the horses. A seasonal trend was also seen for $1,25(OD)_2D$ in the serum of horses, when the lowest amount of $1,25(OH)_2D$ was measured in winter (July-September), during the time that the amount of sunshine and UV radiation was lower, and the concentration of $1,25(OH)_2D$ in serum was higher during spring and summer (November-March). In contrast, an exact opposite trend for PTH was found when compared to $1,25(OH)_2D$ that supports the negative feedback effect of these hormones on each other in the body.

As there was no significant differences in serum concentrations of vitamin D metabolites ($25OHD_2$ and $25OHD_3$), $1,25(OH)_2D$, iCa^{2+}, tCa^{2+}, PO_4^{3-}, tMg^{2+} and PTH between covered and non-covered groups, it was decided that the full data would be analysed to determine the trend of each analyte during the 13 months of study.

iCa^{2+}showed a rhythm through the study and a seasonal change in this analyte was clearly noticeable. Serum concentration of iCa^{2+} had a direct impact on the concentration of $25OHD_2$ and PTH concentration, where PTH is functioning as the key regulator of plasma calcium homeostasis. The amount of vitamin D in pasture/hay that was consumed by the horses always exceeded the minimum daily recommended intake requirements for horses, even during autumn and winter (NRC, 2007). As PTH showed a very stable trend through the year, the rhythm in $25OHD_2$ and iCa^{2+} could explain the physiological adjustment that the body goes through to keep the concentration of calcium in serum at an appropriate level.

When comparing the fluctuation of ionised Ca through the year to that of total Ca, the marked difference between the two suggests that total calcium might not be a good indicator available calcium and ionised calcium may be a more reliable measure than total calcium.

A very smooth, stable and similar trend of tCa^{2+}, PO_4^{3-} and to some extent tMg^{2+} was identified during the study. This suggests that these ions may have similar pathways and perhaps their concentrations in the body affect each other. The amount of these mineral ions in pasture/hay consumed by the horses exceeded the minimum daily recommended

intake requirements of horses (NRC, 2007) and there stable rhythms in serum suggest that the proper amount of calcium, phosphorus and magnesium in the pasture was consumed by the horses during the year.

The calcitropic hormones (PTH and vitamin D metabolites ($25OHD_2$ and $1,25(OH)_2D$) were significantly correlated thus clearly revealing the combined action of these hormones on one another. A significant negative correlation existed between PTH, $25OHD_2$ and $1,25(OH)_2D$; synthesis of $1,25(OH)_2D$ is stimulated by PTH, and $1,25(OD)_2D$ also has a direct negative feedback effect on PTH production (Lips 2006). In contrast, the correlation between $25OHD_2$ and $1,25(OH)_2D$ was positive; $25OHD_2$ is a precursor component for $1,25(OH)_2D$ in the body, and this was shown by direct relationship between the concentration of these analytes.

The role of calcitropic hormones (vitamin D metabolites ($25OHD_2$ and $1,25(OH)_2D$) and PTH) in regulation and metabolism of mineral ions in body, especially calcium and phosphorus, is well documented (Lips 2012, Lopez *et al.* 2006a,b). In the current study, iCa^{2+} showed a significant negative correlation with PTH which supports the inverse action of these analytes on each other. The concentration of calcium in the blood circulation has a direct impact on the secretion of PTH from the parathyroid glands. Therefore, when the serum calcium concentration is low the secretion of PTH increases, resulting in increased serum calcium by increasing active calcium absorption in the intestine. Increasing the serum calcium concentration then has a suppressing action on the secretion of PTH from the parathyroid glands (Lips 2006).

A positive significant correlation was seen between the serum iCa^{2+} concentration and $25OHD_2$ that supports the interaction between the amount of calcium intake in the body and this vitamin D metabolite (Lips 2012). Similarly, tMg^{2+} showed a significant positive correlation with $25OHD_2$, which suggests that absorption of Mg in the horse may also be stimulated by vitamin D.

A significant positive relationship was seen between the serum mineral ions concentrations (tCa^{2+}, PO_4^{3-} and tMg^{2+}), although the correlation between the serum tCa^{2+} and tMg^{2+} concentrations was almost twice as big as that of serum tCa^{2+} to PO_4^{3-} concentration. Serum PO_4^{3-} concentration showed a similar relationship with both serum

tCa^{2+} and tMg^{2+} concentrations. This finding suggests that $Ca^{2+}$and Mg^{2+} have similar metabolic pathways in the body, and perhaps serum PO_4^{3-} concentration might be similarly regulated by Mg^{2+} as well as Ca^{2+}.

The results of this study show that, regardless of coverage, the serum concentration of $25OHD_3$ in horses is barely detectable, while $25OHD_2$ is the main metabolic precursor form of 25OHD in serum. Therefore, we can conclude that $25OHD_2$ is the main metabolic precursor for $1,25(OH)_2D$ in horses and this vitamin D metabolite should be considered as the best available index of vitamin D status in these species.

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Circadian rhythm of calcitropic hormones, calcium, phosphorus and magnesium during shortest and longest time of the year in horses in New Zealand

Introduction

Life is a cycle which is regulated by different internal chemical processes and biological rhythms such as annual, seasonal, weekly and circadian (24 hr.) rhythms. Chronobiology is the science of studying these biological rhythms.

The term circadian comes from Latin and means around the day. A circadian rhythm is a biological process that is driven by an "internal clock" and has been observed in animals, plants, and even bacteria and fungi (Dunlap, 1999). It is an endogenous self-sustaining rhythm; however, the external environment has a huge impact on it. The circadian clock has a direct link with the perception of light and temperature which provides information about the environment. It allows an association between internal and external time to allow appropriate responses to biochemical, physiological and behavioural activities that are required at certain times of the day (Baker *et al.,* 2012).

All life forms, from the simplest alga through to mammals, depend on sunlight, and use it to regulate their activity in order to optimise survival. Animals adapt and establish their life style in a way that their activities in 24-h cycles are defined by sunrise and sunset. Therefore, most organisms have a biochemical system which is driven by sunlight that is known as the internal, biological and/or, circadian clock (Buijs *et al.,* 2003; Whitmore *et al.,* 2000).

In mammals, the master circadian clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, where it receives light signals from the retina. The SCN works as a circadian pacemaker that coordinates many aspects of mammalian behavioural and physiological rhythms with the daily and seasonal environmental changes, such as physical activity and sleep, hormonal levels, body temperature, and digestive activity (Bernard *et al.,* 2007; Aton & Herzog, 2005).

Sunlight has a direct influence on the synthesis of vitamin D_3 in the skin of most mammals. The amount of ultraviolet B photons that reach the skin has a direct correlation

with the photoconversion of 7-dehydrocholesterol to vitamin D_3 (Chen *et al.,* 2007; Holick, 2003). The axial rotation of the earth causes extensive periodic variations in environmental conditions such as day and night length, and seasons. These are also factors that influence the production of vitamin D_3 in skin and vitamin D status in the body (Holick & Chen, 2008). The most biologically active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), is synthesised in the kidney and this renal synthesis is catalysed by CYP27B1 (1α-hydroxylase) (Morris & Anderson, 2010). Calcitropic hormones (vitamin D and PTH) play important roles in the regulation of calcium homeostasis (Peacock, 2010).

Circadian mechanisms have a specific practical importance in veterinary medicine as they are critical in mediating the timing of seasonal reproduction, annual physiological and behavioural fluctuations, immune function and seasonal prevalence of different diseases. Horses have hugely different patterns in their sleep-wake time compared to other species. Their sleep periods are not limited to just the dark hours, and their average sleeping time is 2.9 hours that obtained in short 15 minute bursts (Dallaire, 1986) and this may affect the circadian and circannual rhythms in horses.

The presence of circadian rhythmicity and photoperiodism and their effect on physiological processes have been well studied in human. Recently in the horse, many diurnal and circadian variations in physiological parameters reported, including locomotor activity, rectal and core body temperature, heart rate, melatonin, glucose, mineral ions and hormones (Piccione *et al.*, 2008; Murphy *et al.*, 2007; Piccione *et al.* 2005).

The aim of this study was to determine the circadian rhythm of calciotropic hormones ($25OHD_3$, $25OHD_2$, $1,25(OH)_2D$ and PTH), calcium, phosphorus and magnesium on the shortest (21st July) and longest (22nd December) days of the year in horses in New Zealand.

Material and methods

The animal procedures were approved by the Massey University Animal Ethics Committee (approval no. 12/93).

Animals

Five healthy adult horses (*Equus caballus*) were included in the study, with a mean age of 13 (± 1.72) years (Table 1). Prior to this study, all horses were housed in paddocks at the Veterinary Large Animal Teaching Unit (VLATU), Massey University for more than 5 months. At the beginning of study all horses were transported from VLATU to Massey University Hospital and housed in individual stalls. They were fed *ad libitum* grass and hay, and had free access to water.

Table 7- List of horses in the study

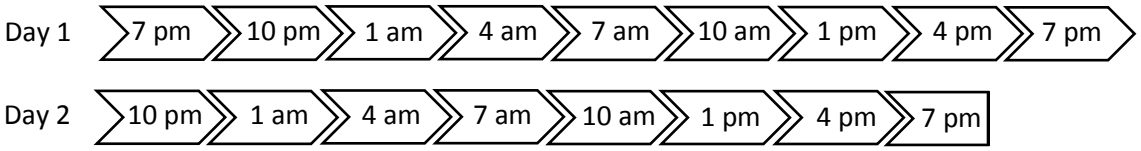
ID	Breed	Sex	Age	Colour
1	Thoroughbred	Mare	18	Chestnut
2	Thoroughbred	Mare	13	Bay
3	Standardbred	Mare	16	Bay
4	Standardbred	Mare	11	Bay
5	Standardbred	Mare	7	Bay

Sample collection

Blood

Samples were collected i: on June (25^{th}-27^{th}), during the shortest days of year (sunrise 7:41 and sunset 16:59) and, ii. on December (16^{th}-18^{th}), during the longest days of the year (sunrise 5:42 and sunset 20:45). One serum separator tube (SST) and two plain tubes were collected (Vacutainer, Becton–Dickinson) from each horse at 3 hr. intervals over a 48 hr. period (starting at 7:00 pm on day 1 and finishing at 7:00 pm on day 2) via an intravenous cannula inserted into the jugular vein (Table 2). The serum was separated by centrifugation at 3500 × g for 15 min. Serum from the SST tubes was removed anaerobically and, transferred to 10 ml plain tubes, followed by ionised calcium analysis. Serum from plain tubes was transferred to 1.5 ml micro centrifuge tubes and stored at -80˚C until further analysis.

Table 8- Blood collection timeframe



Urine

Urine samples were collected by catheterization for urinalysis and urine biochemical analyses. Blood samples for serum biochemical analyses were collected at the same time as urine samples.

Sample analysis

Biochemistry

Ionised calcium concentrations were measured within 4 hours of collection, using the Radiometer ABL800 machine (Medlab Central, Palmerston North).

Parathyroid hormone concentration was measured using the ARCHITECT Intact PTH assay (ABBOTT Diagnostics Division, Germany) at the Endolab (Canterbury Health Laboratories, Christchurch). Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry (LC/MS) was used to measure 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 concentrations also at the Endolab (Canterbury Health Laboratories, Christchurch).

Total calcium, phosphorus and magnesium concentrations were measured using a Roche Hitachi 911 Chemistry Analyser (Roche Diagnostics, USA) at a commercial veterinary diagnostic laboratory (New Zealand Veterinary Pathology, Palmerston North).

The DiaSorin 1,25-dihydroxyvitamin D RIA kit was used to measure total 1,25-dihydroxyvitamin D as per the manufactures instructions (Nutrition laboratory, Massey University (IFNHH), Palmerston North).

Food samples

Samples were taken from the grass and hay the horses were eating in the stalls. Samples from were mixed, freeze-dried and ground to pass through a 1 mm screen. The ground sample was analysed for calcium, phosphorus and, magnesium using Plasma

Emission Spectrometry subcontracted method, and for vitamin D_2 and vitamin D_3 using HPLC NMKL 167, 2000 method (Nutrition laboratory, Massey University).

Urine analysis

Urine was acidified with NHCl to dissolve calcium salts. Urine calcium, phosphorus, magnesium and creatinine concentrations were measured as described for serum. Fractional clearances of calcium, phosphorus and magnesium were calculated, using serum and urine calcium, phosphorus, magnesium and creatinine concentrations.

Data analysis

Results from the ionised calcium (iCa^{2+}), calcium (Ca^{2+}), phosphorus (PO_4^{3-}), magnesium (Mg^{2+}), parathyroid hormone (PTH), and vitamin D metabolite ($25OHD_2$, $25OHD_3$ and, $1,25(OH)_2D$) analysis are expressed in absolute concentrations and all the results express as mean (± SE) (Table 3 and 5). The results were fitting generalized additive models, where it made it possible to have a smooth trend for time and a random effect for each separate horse. In order to find any differences between summer and winter, smooth trends through the 48 hours of sampling were calculated by fitting a generalized additive model for each analyte using the mgcv package (Wood, 2011) in R (R Core Team 2014). For all statistical comparisons a value of $P < 0.05$ was considered significant.

Table 9- Mean (± SE) serum concentration of Ca^{2+} (mmol/L), iCa^{2+} (mmol/L), PO_4^{3-} (mmol/L), Mg^{2+} (mmol/L), $25OHD_2$ (nmol/L), $25OHD_3$ (nmol/L), $1,25(OH)_2D$ (pmol/L) and PTH (pg/ml) during the shortest time of the year (June 25th-27th 2013) in horses in New Zealand

	7 pm	10 pm	1 am	4 am	7 am	10 am	1 pm	4 pm	7 pm	10 pm	1 am	4 am	7 am	10 am	1pm	4 pm	7 pm
Ca^{2+}	3.2 (± 0.03)	3.05 (± 0.1)	3.13 (± 0.04)	3.12 (± 0.04)	3.08 (± 0.03)	3.07 (± 0.05)	2.98 (± 0.05)	2.96 (± 0.09)	2.95 (± 0.06)	2.98 (± 0.08)	2.85 (± 0.06)	2.94 (± 0.09)	2.97 (± 0.13)	2.78 (± 0.1)	2.72 (± 0.04)	2.79 (± 0.11)	2.81 (± 0.)
iCa^{2+}	1.54 (± 0.01)	1.52 (± 0.006)	1.56 (± 0.01)	1.51 (± 0.01)	1.44 (± 0.03)	1.54 (± 0.02)	1.52 (± 0.01)	1.46 (± 0.02)	1.47 (± 0.02)	1.48 (± 0.02)	1.51 (± 0.01)	1.49 (± 0.01)	1.47 (± 0.02)	1.5 (± 0.01)	1.51 (± 0.02)	1.48 (± 0.02)	1.49 (± 0.01)
PO_4^{3-}	0.88 (± 0.04)	0.8 (± 0.02)	0.85 (± 0.02)	0.89 (± 0.03)	0.71 (± 0.02)	0.73 (± 0.1)	0.64 (± 0.02)	0.62 (± 0.03)	0.6 (± 0.03)	0.67 (± 0.03)	0.63 (± 0.00)	0.72 (± 0.04)	0.6 (± 0.04)	0.55 (± 0.04)	0.6 (± 0.02)	0.6 (± 0.04)	0.66 (± 0.02)
Mg^{2+}	0.75 (± 0.01)	0.72 (± 0.01)	0.74 (± 0.01)	0.73 (± 0.01)	0.74 (± 0.01)	0.74 (± 0.02)	0.68 (± 0.02)	0.68 (± 0.03)	0.71 (± 0.02)	0.71 (± 0.02)	0.66 (± 0.01)	0.65 (± 0.01)	0.63 (± 0.03)	0.61 (±0.04)	0.63 (± 0.02)	0.63 (± 0.03)	0.65 (± 0.03)
$25OHD_2$	5.2 (± 0.33)	5.4 (± 0.6)	5.2 (± 0.33)	5.4 (± 0.45)	5.6 (± 0.45)	5.4 (± 0.45)	5.8 (± 0.52)	5.4 (± 0.53)	5.6 (± 0.21)	6.4 (± 0.6)	5.8 (± 0.52)	6 (± 0.4)	6.4 (± 0.45)	6.4 (± 0.35)	7 (± 0.48)	6.4 (± 0.72)	5.6 (± 0.45)
$25OHD_3$	0.4 (± 0.21)	0.2 (± 0.17)	0	0.8 (± 0.52)	0	0	0	0	0	0.5 (± 0.22)	0.2 (± 0.17)	0	0	0.33 (± 0.21)	0	0.2 (± 0.17)	0.2 (± 0.17)
$1,25(OH)_2D$	14.26 (± 4.39)	13.63 (± 2.88)	10.95 (± 2.76)	17.36 (± 3.84)	22.09 (± 4.12)	11.4 (± 3.37)	12.14 (± 3.4)	11.41 (± 2.91)	14.12 (± 4.15)	15.31 (± 3.21)	19.28 (± 3.37)	19.8 (± 0.86)	15.4 (± 3.05)	11.64 (± 3.71)	12.4 (± 2.06)	12.92 (± 1.81)	20.04 (± 4)
PTH	8.48 (± 1.65)	10.14 (± 1.7)	10.98 (± 2.51)	10.16 (± 3.43)	12.72 (± 1.58)	10.7 (± 2.89)	11.56 (± 2.94)	9.22 (± 2.65)	8.12 (± 2.12)	20.78 (± 8.37)	11.2 (± 4.49)	9.96 (± 2.63)	10.06 (± 2.09)	8.7 (± 1.24)	10.82 (± 1.84)	12.3 (± 5.36)	8.92 (± 2.71)

Table 10- Fractional urinary clearance of calcium (FCa), phosphorus (FP) and magnesium (FMg) in horses

	Serum Creatinine	Urine Creatinine	Serum Ca^{2+} (mmol/L)	Urine Ca^{2+} (mmol/L)	FCa %	Serum PO_4^{3-} (mmol/L)	Urine PO_4^{3-} (mmol/L)	FP %	Serum Mg^{2+} (mmol/L)	Urine Mg^{2+} (mmol/L)	FMg %
Horse A	90	20063	3.17	21.85	3.09	0.75	0.27	0.16	0.73	9.82	6.03
Horse B	81	20869	2.81	22.5	3.1	0.52	0.21	0.15	0.61	13.2	8.39
Horse C	101	35053	2.48	21.4	2.17	0.46	0.16	0.1	0.48	8.59	5.15
Horse D	108	13255	3.07	10.91	2.89	0.57	0.13	0.18	0.67	8.14	9.89
Horse E	109	21488	3	10.95	1.85	0.7	0.4	0.28	0.7	5.42	3.92

Table 11-Mean (± SE) serum concentration of Ca^{2+} (mmol/L), iCa^{2+} (mmol/L), PO_4^{3-} (mmol/L), Mg^{2+} (mmol/L), $25OHD_2$ (nmol/L), $25OHD_3$ (nmol/L), $1,25(OH)_2D$ (pmol/L) and PTH (pg/ml) during the longest time of the year (December 16th-18th 2013) in horses in New Zealand

	7 pm	10 pm	1 am	4 am	7 am	10 am	1 pm	4 pm	7 pm	10 pm	1 am	4 am	7 am	10 am	1pm	4 pm	7 pm
Ca^{2+}	2.93 (± 0.12)	3.13 (± 0.09)	2.82 (± 0.17)	2.71 (± 0.06)	2.96 (± 0.07)	3.33 (± 0.14)	2.8 (± 0.11)	3.36 (± 0.28)	3 (± 0.09)	2.97 (± 0.13)	2.92 (± 0.25)	3.12 (± 0.13)	3.06 (± 0.17)	2.92 (± 0.25)	2.75 (± 0.05)	2.94 (± 0.21)	2.75 (± 0.12)
iCa^{2+}	1.55 (± 0.00)	1.58 (± 0.01)	1.58 (± 0.00)	1.55 (± 0.00)	1.5 (± 0.01)	1.5 (± 0.00)	1.48 (± 0.02)	1.53 (± 0.01)	1.53 (± 0.01)	1.53 (± 0.01)	1.51 (± 0.01)	1.51 (± 0.01)	1.48 (± 0.02)	1.52 (± 0.01)	1.51 (± 0.02)	1.51 (± 0.00)	1.53 (± 0.01)
PO_4^{3-}	0.71 (± 0.05)	0.6 (± 0.06)	0.55 (± 0.03)	0.52 (± 0.05)	0.51 (± 0.06)	0.56 (± 0.06)	0.46 (± 0.04)	0.49 (± 0.03)	0.47 (± 0.04)	0.48 (± 0.05)	0.47 (± 0.06)	0.56 (± 0.06)	0.57 (± 0.09)	0.57 (± 0.07)	0.58 (± 0.05)	0.59 (± 0.11)	0.62 (± 0.1)
Mg^{2+}	0.73 (± 0.04)	0.77 (± 0.04)	0.79 (± 0.02)	0.69 (± 0.04)	0.76 (± 0.02)	0.8 (± 0.04)	0.78 (± 0.02)	0.8 (± 0.07)	0.85 (± 0.04)	0.79 (± 0.02)	0.78 (± 0.06)	0.79 (± 0.03)	0.71 (± 0.08)	0.75 (± 0.02)	0.67 (± 0.01)	0.73 (± 0.05)	0.7 (± 0.03)
$25OHD_2$	10 (± 1.01)	10.8 (± 0.33)	10 (± 1.09)	10.8 (± 0.33)	10.2 (± 0.52)	8.8 (± 0.33)	9.8 (± 0.86)	10 (± 0.48)	9.6 (± 0.77)	9.6 (± 0.45)	10 (± 0.84)	9.2 (± 0.33)	9.8 (± 0.71)	10.8 (± 1.45)	9.2 (± 0.95)	10.8 (± 0.76)	9.6 (± 0.77)
$25OHD_3$	0.2 (± 0.17)	0	0	0	0	0	0	0	0	0	0	0	0	0.4 (± 0.35)	0	0.25 (± 0.19)	0.2 (± 0.17)
$1,25(OH)_2D$	21.24 (± 5.45)	17.16 (± 3.16)	16.77 (± 2.39)	21.25 (± 4.14)	19.65 (± 0.55)	18.53 (± 5.87)	19.34 (± 5.13)	25.25 (± 3.31)	24.63 (± 6.77)	21.12 (± 6.04)	25.2 (± 4.14)	24.91 (± 2.35)	22.26 (± 5.85)	22.36 (± 1.8)	26.3 (± 4.65)	27 (± 7.9)	28.39 (± 4.49)
PTH	8.48 (± 1.65)	10.14 (± 1.7)	10.98 (± 2.51)	10.16 (± 3.43)	12.72 (± 1.58)	10.7 (± 2.89)	11.56 (± 2.94)	9.22 (± 2.65)	8.12 (± 2.12)	20.78 (± 8.37)	11.2 (± 4.49)	9.96 (± 2.63)	10.06 (± 2.09)	8.7 (± 1.24)	8.82 (± 1.78)	12.3 (± 5.36)	8.92 (± 2.71)

Table 12- The amount of calcium (%), phosphorus (%), magnesium (%) and vitamin D (µg/g) in the pasture and hay consumed by horses

	Pasture	Hay
Calcium (%)	0.51	1.07
Phosphorus (%)	0.18	0.18
Magnesium (%)	0.25	0.31
Vitamin D (µg/g)	1.03	3.72

Results

The daily mean (± SE) serum concentration of Ca^{2+}, iCa^{2+}, PO_4^{3-}, Mg^{2+}, $25OHD_2$, $1,25(OH)_2D$ and PTH during the shortest day of the year (June 25th-27th 2013) were different to the longest day of the year (December 16th-18th 2013) in horses. Serum concentration of $25OHD_3$ was really low and mostly undetectable during both seasons.

The serum Ca^{2+} concentration was lower during summer compared to winter. There was a slight increase in the concentration of Ca^{2+} at the middle of the sampling time during summer whereas a uniform downward pattern for winter was identified. The smooth trend for this analyte in serum was significantly different between seasons (P= 0.013) (Figure 1).

The serum iCa^{2+} concentration was higher during summer and showed a periodic pattern whereas its concentration in serum of horses was lower during winter with a significantly different rhythm (P= 0.045) (Figure 2).

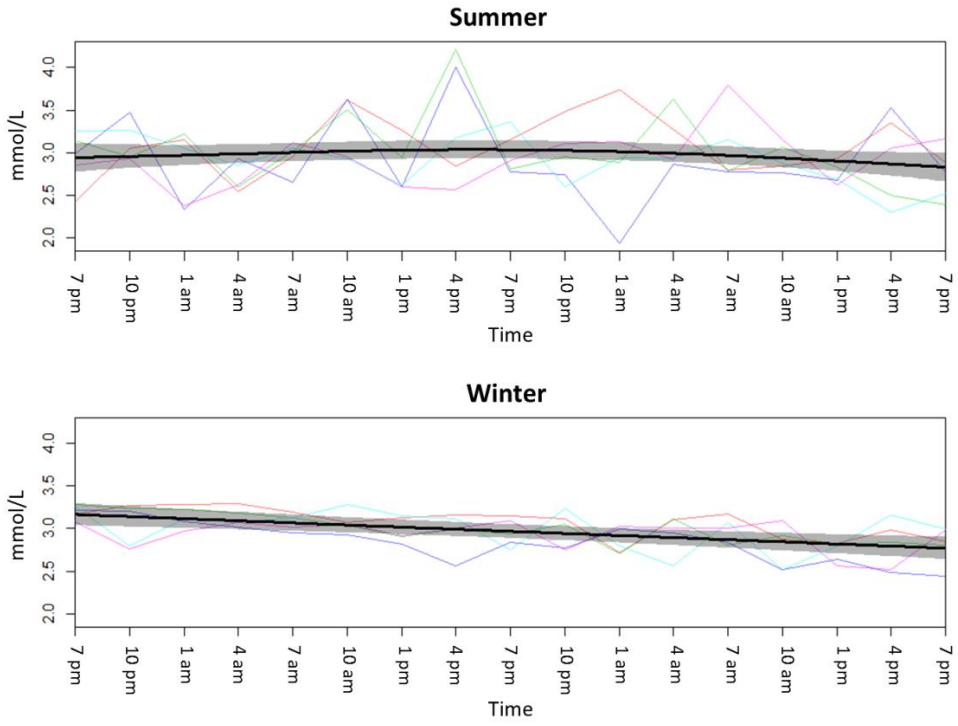


Figure 6-trend of calcium (Ca^{2+}) through summer (December 2013) and winter (June 2013)

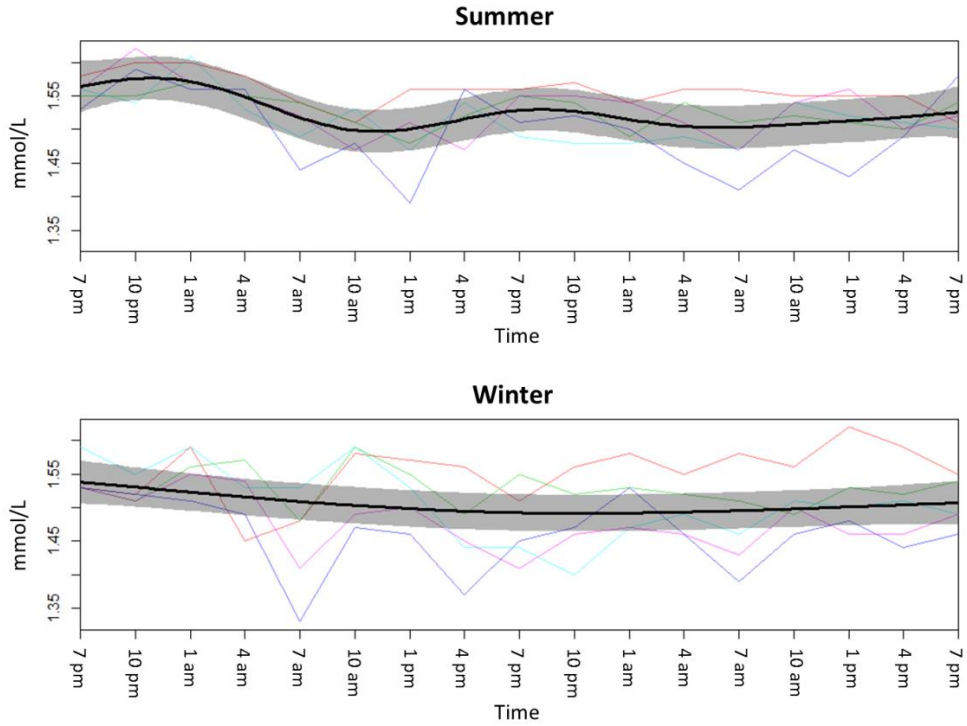


Figure 7- trend of ionised calcium (iCa^{2+}) through summer (December 2013) and winter (June 2013)

Serum PO_4^{3-} concentration was lower during summer with a minimum concentration in the evening, whereas PO_4^{3-} concentration was higher during winter with a downward constant trend through the study. The smooth trend for PO_4^{3-} was significantly different between two seasons (P= 0.0001) (Figure 3).

The concentration of Mg^{2+} in serum was higher during summer with a maximum concentration in the evening compared to its concentration during winter with a constant downward trend. The smooth trend for Mg^{2+} was significantly different between the two seasons (P= 0.003) (Figure 4).

Serum $25OHD_2$ was much higher during summer compared to winter. In both seasons a uniform flat pattern was seen. The smooth trend for this analyte though was also significantly different between seasons (P= 0.003) (Figure 5).

During summer, the serum concentration of $1,25(OH)_2D$ was higher with a periodic upward pattern whereas during winter its concentration was lower with a constant pattern.

The smooth trend for $1,25(OH)_2D$ was significantly different between the two seasons (P= 0.0000001) (Figure 6).

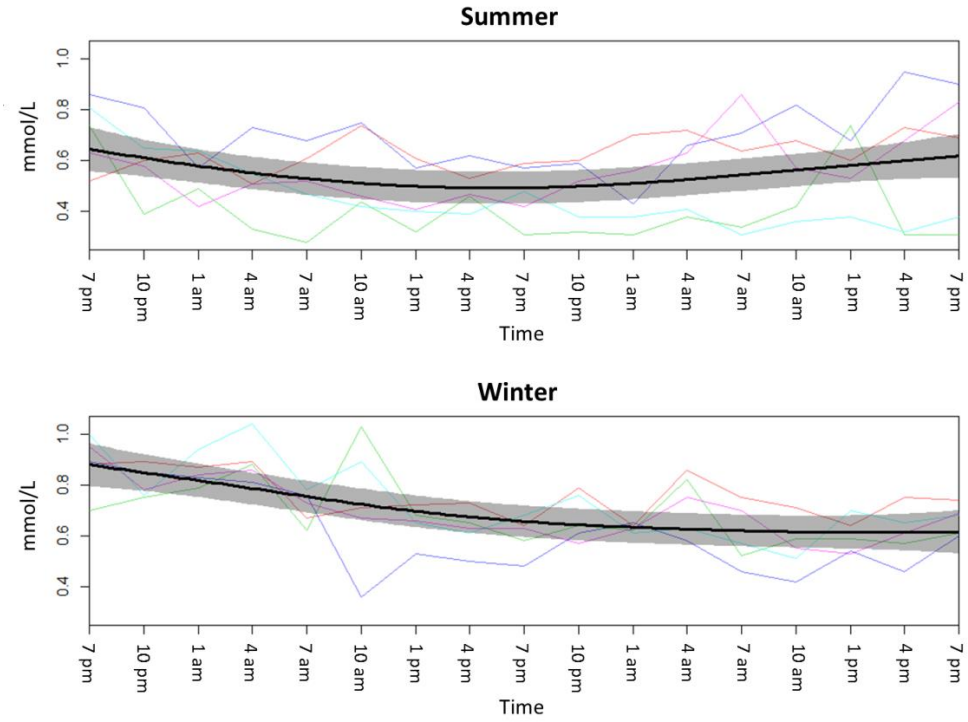


Figure 8- trend of phosphorus (PO_4^{3-}) through summer (December 2013) and winter (June 2013)

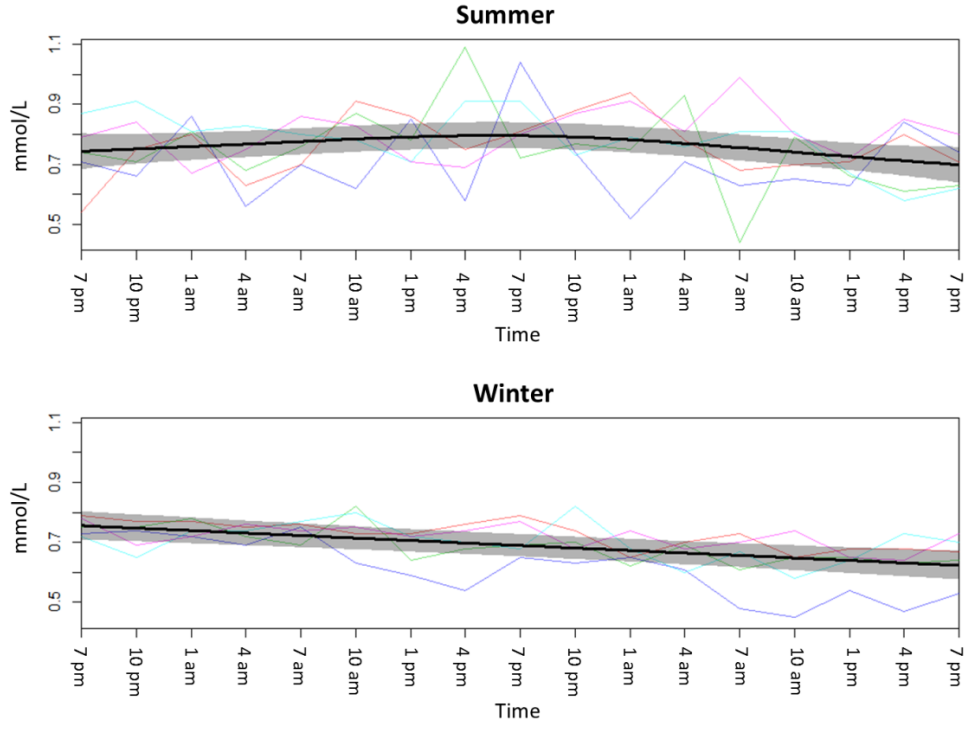


Figure 9- trend of magnesium (Mg^{2+}) through summer (December 2013) and winter (June 2013)

Serum concentration of PTH was lower during summer with a periodic pattern whereas its concentration was much higher during winter with a constant flat pattern. The smooth trend for PTH differed between the two seasons (P= 0.07) (Figure 6).

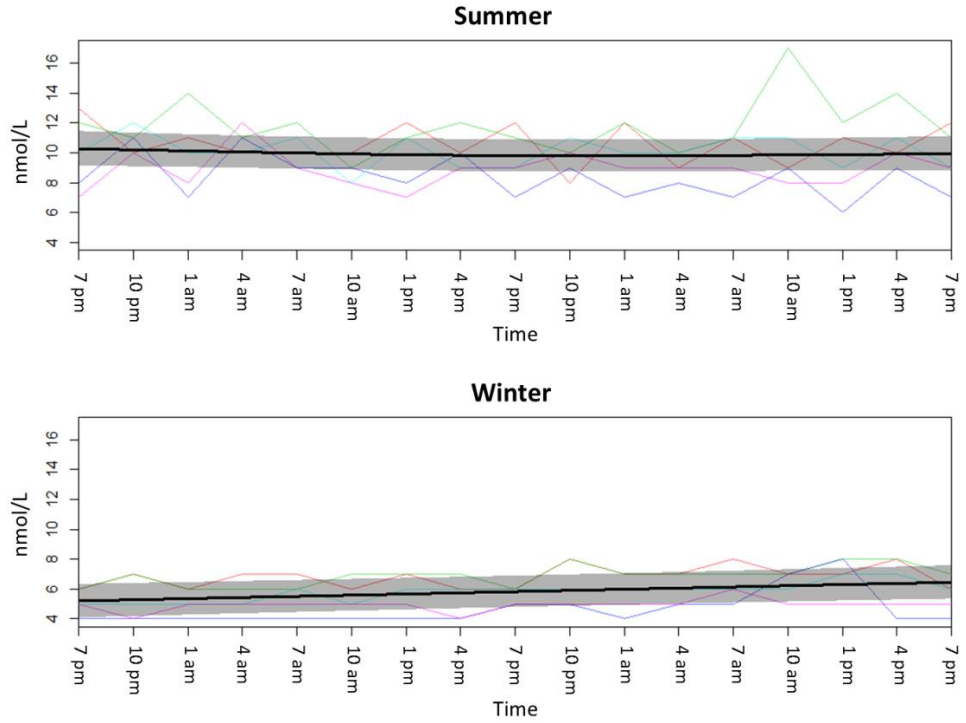


Figure 10- trend of 25-hydroxyvitamin D_2 ($25OHD_2$) through summer (December 2013) and winter (June 2013)

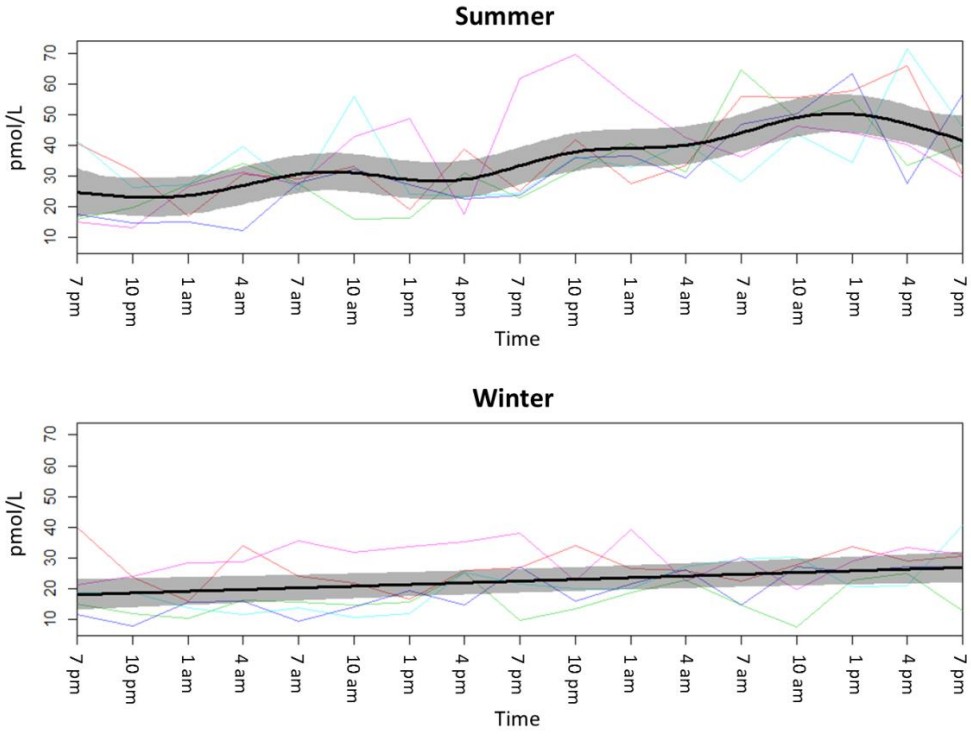


Figure 11- trend of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) through summer (December 2013) and winter (June 2013)

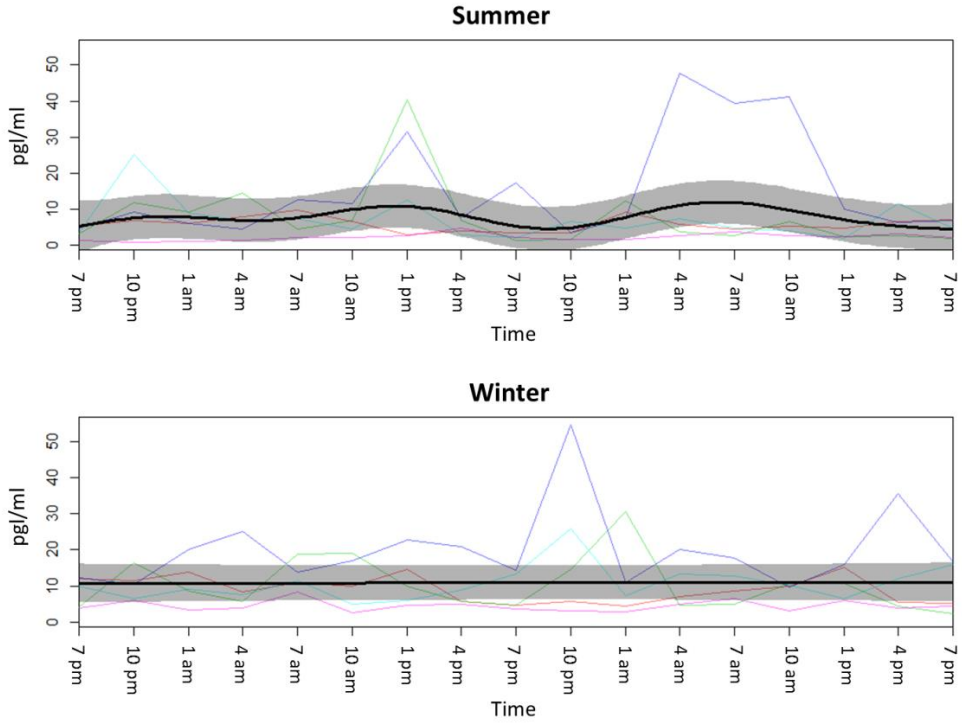


Figure 12- trend of parathyroid hormone (PTH) through summer (December 2013) and winter (June 2013)

Fractional urinary clearance of calcium, phosphorus and magnesium

Urine and blood samples were collected simultaneously from 5 horses that were involved in this study and fractional urinary clearance of calcium (FCa), phosphorus (FP) and magnesium (FMg) were calculated (Table 4).

Discussion

There was a significant difference between the serum concentration of calcitropic hormones ($25OHD_2$, $1,25(OH)_2D$ and PTH), calcium, phosphorus and magnesium between the shortest and longest time of the year in horses, all the analytes however were in the physiological range for horses (Toribio, 2011; Berlin & Aroch, 2009; Estepa *et al,*. 2003). Serum iCa^{2+} and PTH concentrations clearly showed a circadian rhythm during summer and serum tCa^{2+}, PO_4^{3-} and tMg^{2+} concentrations showed a diurnal pattern in summer. None of analytes showed any rhythm during winter.

Fractional urinary clearance of calcium, phosphorus and magnesium significantly varied between horses in this study. There are different fractional urinary clearances for

horses for FE_{Ca} ranging 1.3- 6.72, FE_P ranging 0.08-19.98 and FE_{Mg} ranging 2-50 (Lefebvre et al., 2008). Therefore, all the results that were obtained in this study were in the normal range for horses.

For iCa^{2+}, acrophases were recorded between 7 pm-1 am on day 1 and day 2 of sampling in summer and for PTH between 10 am-4 pm on day 1 and between 4 am-10 am on day 2 of the study in summer. These results show the direct diverse effect of these two analytes on each other, meanwhile the fluctuation of serum $1,25(OH)_2D$ concentration in summer suggests the important regulatory role of this hormone on iCa^{2+} and PTH in body (Lips, 2012).

Diurnal rhythms were seen in serum concentrations of Mg^{2+}, PO_4^{3-} and to some extent Ca^{2+} in summer. Interestingly Mg^{2+} and Ca^{2+} had an opposite rhythm to that of PO_4^{3-}, suggesting Mg^{2+} has a similar role in body towards PO_4^{3-} that Ca^{2+} does (Toribio, 2011).

Some previous studies on rhythmicity of serum concentrations of calcium, phosphorus and vitamin D metabolites in horses suggested calcium, phosphorus and $25OHD_3$ had a circadian rhythm (Piccione et al., 2008), which does not match with the findings in current study. Light has a direct effect on circadian rhythms in body, although some non-photic parameters such as exercise patterns (Turek, 1989) and food availability (Stokkan *et al.*, 2001) usually play an important role on these rhythms. It is suggested the reason that experimental horses and trained racehorses showed the most stable rhythms might be a product of the daily routine of horses' environment due to management regimes (feeding and exercise time), for example the rhythm of cortisol in horses only increased where they habituated to a management routine including stabling, feeding and sometimes exercise (Irvine & Alexander, 1994). The fact that horses in New Zealand are mainly kept in paddocks with limited stabling might reduce the management effects on them. In addition, during our study horses had unlimited access to food and this may explain the differences that were seen in the results of this studycompared with other studies. However, this suggests that any rhthym in serum analytes is the result of feeding and management practices, rather than changes in photoperiod.

This study could have significant relevance for New Zealand equine, as there was a significant difference between serum concentrations of measured analytes in this study

during the shortest and longest time of the year. It would be good to consider that equine atheletes due to the frequency of air travel to international equestrian competitions face a huge time and seasonal diversity that might affect their health, ability to adjust their circadian rhythms to time zone and their performances during competition events. Perhaps maintaining a regular feeding and handling schedule could mitigate some of these effects.

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