Vitamin D and Calcium Metabolism In Horses in New Zealand

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy

in

Veterinary Science

at

Institute of Veterinary, Animal and Biomedical Sciences (IVABS), Massey University, Manawatū, New Zealand

Sara Azarpeykan

2016

Abstract

The physiology of vitamin D in horses has not been studied in great depth. Few studies on vitamin D metabolites ($25OHD_2$, $25OHD_3$, and $1,25(OH)_2D$) and their relationship to other serum analytes exist. In addition, some studies suggest that equine vitamin D physiology may be different from other species. This thesis aimed to investigate aspects of vitamin D metabolism in horses.

The effect of blanketing on vitamin D synthesis and its relationship with other analytes involved in calcium homeostasis, including vitamin D metabolites (25-hydroxyvitamin D_2 ($25OHD_2$), and 25-hydroxyvitamin D_3 ($25OHD_3$), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$)), ionised calcium (iCa), total calcium (tCa), phosphorus (P), total magnesium (tMg) and parathyroid hormone (PTH) were studied in horses. Regardless of blanketing, $25OHD_3$ was undetectable in equine serum and $25OHD_2$ was the main form of 25OHD in circulation. A strong seasonal variation in serum $25OHD_2$, $1,25(OH)_2D$, iCa, tCa, P, tMg and PTH concentrations was detected, although no differences were seen between horses that were blanketed and those that were not. The circadian rhythms of serum vitamin D metabolites, iCa, tCa, P, tMg, and PTH concentrations in horses was studied over 48 h on the summer and winter solstices. A significant difference was seen between the serum concentrations of studied analytes between solstices, with no rhythm detected in winter. An *in vivo* study suggested that equine skin may be unable to convert 7-dehydrocholesterol (7-DHC) to vitamin D_3 after exposure to ultraviolet B (UVB) light. Quantitative PCR was performed on equine kidney to study the expression of vitamin D responsive and calcium transporting genes, which were then compared to genes in sheep and dogs.

The results suggested that TRPV6, $calD_{9k}$ /$calD_{28k}$, and PMCA were the main calcium transporting pathways in the kidney of these species, and there was a high correlation between VDR and other studied genes. It was concluded that $25OHD_2$ is the main metabolic precursor for $1,25(OH)_2D$ and should be considered the best available index of vitamin D status in unsupplemented horses, and that horses most likely rely on diet as their primary source of vitamin D.

Acknowledgements

I started this study three years ago as a fun horsey project, but it soon became a long battle against different analytes and genes. This thesis is the culmination of a long process, but it cannot fully speak of the many long days spent at the farm and in the lab, the careful preparation of experiments, the joy of good results and the disappointment of each failed attempt.

My deepest gratitude goes to my chief supervisor, Dr Keren Dittmer. I have been amazingly fortunate to have such a generous supervisor with her time and knowledge. Her patience and support helped me overcome many crisis situations and finish this thesis, not to mention her carefully reading and commenting on countless revisions of this manuscript.

My sincere thanks also go to my co-supervisors, Dr Erica Gee, Dr Els Acke, and Professor Keith Thompson, for their valuable guidance. I am extra thankful to Keith, for encouraging the use of correct grammar and consistent notation in my writings, and to Dr Jonathan Marshall for his statistical assistance and artwork!

I am also thankful to the laboratory staff at NZVP, Nutrition lab, and Canterbury Health lab, staff at VLATU and Massey University Veterinary Teaching Hospital, and Massey University veterinary students for their technical assistance. I am especially grateful to Mike Hogan and Craig Thomas at the Pathobiology post mortem room for their support throughout my study.

I also want to thank Debbie Hill, our lovely postgraduate and research administrator at IVABS, who has provided plenty of support, not only to me but to all postgrads, to produce and complete our theses.

I would like to acknowledge the Equine Trust and IVABS Postgraduate Student Research Fund, Institute of Veterinary, Animal and Biomedical Sciences, Massey University to provide funding for this body of work.

I have been blessed with a friendly and cheerful group of fellow students who have helped me stay sane these past three years. Your support, care, and friendship helped me overcome setbacks and stay focused as we deliberated on our problems and findings, and had broader discussion on life, the universe and all things in between. I greatly value your friendship.

Most importantly, none of this would have been possible without the love and patience of my partner, my Mom, my sisters and my brother. I would like to express my heart-felt gratitude to all of you and really thank you for supporting me through all my studies.

I would like to dedicate this dissertation to my beloved sister, Raha, who was a constant source of love, energy, concern, support and strength. You are forever in my mind and my heart.

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Glossary

$1,25(OH)_2D$	1,25-dihydroxyvitamin D
18S	ribosomal RNA
25OHD	25-hydroxyvitamin D
$25OHD_2$	25-hydroxyvitamin D_2
$25OHD_3$	25-hydroxyvitamin D_3
28S	ribosomal RNA
7-DHC	7-dehydrocholesterol
B2M	ß-2-microglobulin
Ba^{2+}	Barium
BLAST	Basic Local Alignment Search Tool
BMD	bone mineral density
Ca^{2+}	calcium
Calbindin	calcium-binding protein
$CalbindinD_{9k}$	vitamin-D-dependent 9k, calcium-binding protein
$CalbindinD_{28k}$	vitamin-D-dependent 28k, calcium-binding protein
cAMP	cyclic 3',5'-adenosine monophosphate
CaSR	calcium sensitive receptors
C cells	parafollicular cells
cDNA	complementary DNA
CGRP	calcitonin gene-related peptide
Cl^-	chloride
CNT	connecting tubule
CYP24A1	24-hydoxylase
CYP27A1	cytochrome P450, family 27, subfamily A, polypeptide 1
CYP27B1	1α-hydroxylase
CYP2R1	cytochrome P450, family 2, subfamily R, polypeptide 1
DAG	diacylglycerol
DBP	vitamin D-binding protein
DCAD	dietary cation-anion difference
DCs	dendritic cells

DCT	distal convoluted tubule
DHCR1	7-dehydrocholesterol reductase 1
DNA	deoxyribonucleic acid
Fe^{2+}	iron
FGF23	fibroblast growth factor 23
FE_{Ca}	fractional urinary clearance of calcium
FE_{Mg}	fractional urinary clearance of magnesium
FE_{P}	fractional urinary clearance of phosphorus
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GC	glucocorticoids
GC content	guanine-cytosine content
GCs	glucocorticoid hormones
GH	growth hormone
GIT	gastrointestinal tract
HKG	housekeeping gene
HMBS	hydroxymethylbilane synthase
HPO_4^{2-} /$H_2PO_4^-$	phosphorus anions
HPLC	high-performance liquid chromatography
HPRT1	hypoxanthine phosphoribosyltransferase 1
iCa	ionised calcium
CI	confidence interval
CV	coefficient of variation
IGFs	insulin-like growth factors
IL-1	interleukin 1
IL-6	interleukin 6
IVABS	Institute of Veterinary, Animal and Biomedical Sciences
K^+	potassium
LC-MS	liquid chromatography–mass spectrometry
MAPK	mitogen-activated protein kinase
Mg^{2+}	magnesium
mgcv	mixed generalized additive models computation vehicle
MS	multiple sclerosis

Na^+	sodium
Na^+/Pi	sodium-dependent phosphate
NCBI	national centre for biotechnology information
NCX1	sodium calcium exchanger 1
NH_4^+	ammonium ions
NH_4Cl	ammonium chloride
NIWA	National Institute of Water and Atmospheric research
NRC	nutrient requirements of horses
NZVP	New Zealand Veterinary Pathology
OPG	osteoprotegerin
P	phosphorus
PCR	polymerase chain reaction
PGK1	phosphoglycerate kinase 1
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PMCA	plasma membrane Ca^{2+}-ATPase
PTH	parathyroid hormone
PTHR1	parathyroid hormone receptor 1
PTHrP	parathyroid hormone -related peptide
RANK	receptor activator of the nuclear factor-kappaB
RANKL	receptor activator of the nuclear factor-kappaB ligand
RNA	ribonucleic acid
RPL13A	ribosomal protein L13a
RPL30	ribosomal protein L30
RPL32	ribosomal protein L32
RPS5	ribosomal protein S5
RPS19	ribosomal protein S19
RT-qPCR	real-time quantitative reverse transcriptase polymerase chain reaction
SCN	suprachiasmatic nucleus

SDHA	succinate dehydrogenase complex
SE	standard error
sFRP-4	secreted frizzled related protein-4
SLC17	type I sodium-phosphate co-transporters
SLC20/PiT2	type III sodium-phosphate co-transporters
SLC34	type II sodium-phosphate co-transporters
SLC34A1	type II sodium-phosphate co-transporters, member 1
SLC32A2	type II sodium-phosphate co-transporters, member 2
SLC34A3	type II sodium-phosphate co-transporters, member 3
SNPs	single nucleotide polymorphisms
SPF	sun protection factor
SST	serum separator tube
tCa	total calcium
TLR	toll-like receptors
tMg	total magnesium
TRPM	transient receptor potential cation channel, subfamily M
TRPM6	transient receptor potential cation channel, subfamily M, member 6
TRPM7	transient receptor potential cation channel, subfamily M, member 7
TRVP5	transient receptor potential cation channel, subfamily V, member 5
TRPV6	transient receptor potential cation channel, subfamily V, member 6
UBB	ubiquitin B
UV	ultraviolet
UVB	ultraviolet B
VDR	vitamin D receptor
VLATU	Veterinary Large Animal Teaching Unit
YWHAZ	zeta polypeptide

Chapter 1

Review of the Literature

1.1 Introduction

Calcitropic hormones comprise a group of hormones (Figure 1.1) that, through their combined actions on bone, kidney and the gastrointestinal tract, maintain plasma calcium and phosphorus concentrations within a narrow range in order to optimise the many calcium-requiring physiological functions of the body, including neural transmission and muscle contraction (Rizzoli & Bonjour, 2006). The traditional calcitropic hormones are the active form of vitamin D (1,25-dihydroxyvitamin D ($1,25(OH)_2D$)), parathyroid hormone (PTH), and calcitonin. Other hormones known to be involved in calcium and phosphorus homeostasis include fibroblast growth factor 23 (FGF23) (Figure 1.2).

While calcitropic hormones have been the subject of substantial research, some of the roles of these hormones are still poorly understood. For example, different studies have investigated the association between calcitropic hormones and bone mineral density (BMD) in different populations and species, but with conflicting results. Hypovitaminosis D is known to be a major public health problem worldwide; gender, ethnic group, menopausal status, and age are known factors that may affect

the relationship between calcitropic hormones, calcium and phosphorus metabolism, and BMD (Arabi *et al.*, 2010).

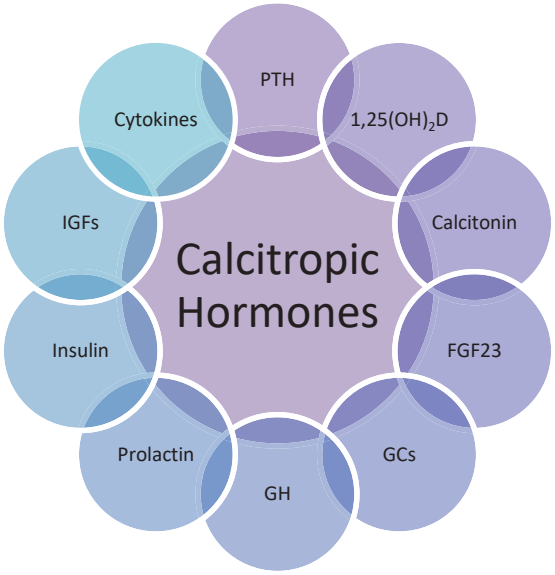


Figure 1.1 Calcitropic hormones. Calcitropic hormones are a group of hormones that maintain serum calcium and phosphorus concentration in the body, including vitamin D (1,25-dihydroxyvitamin D ($1,25(OH)_2D$)), parathyroid hormone (PTH), fibroblast growth factor 23 (GFG32), calcitonin, glucocorticoid hormones (GCs), growth hormone (GH), prolactin, insulin, insulin-like growth factors (IGFs) and a large number of cytokines (Bikle, 2008).

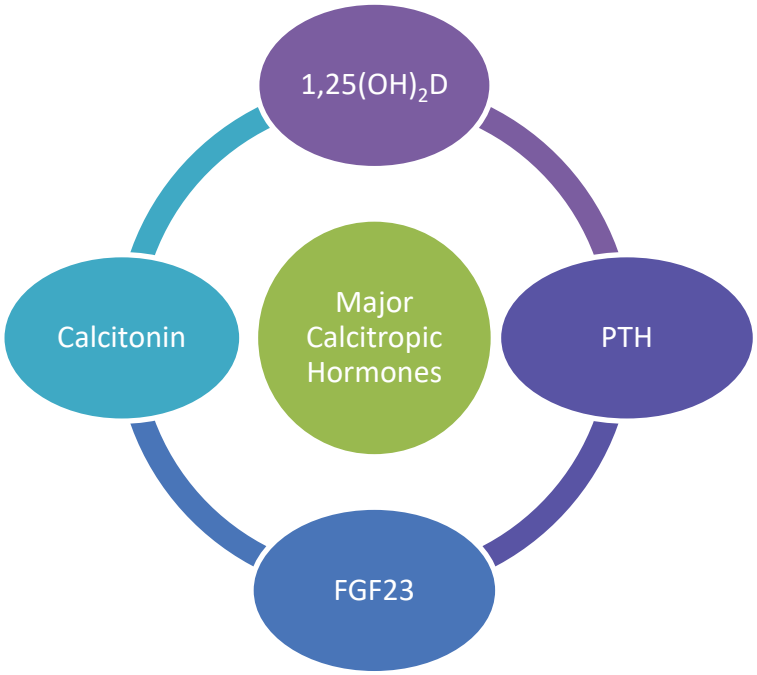


Figure 1.2 Major calcitropic hormones. Vitamin D (1,25-dihydroxyvitamin D ($1,25(OH)_2D$)), parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and calcitonin are considered the major calcitropic hormones in the body (Bikle, 2008).

1.2 Calcium metabolism

Calcium is a chemical element with the atomic number 20 and symbol Ca. It is an alkaline earth metal, and is the fifth most abundant element by mass in the Earth's crust, the fifth most abundant dissolved ion in seawater by both molarity and mass (Dickson & Goyet, 1994), and the fifth most abundant element in the human body (Peacock, 2010). One of the most important functions of calcium is skeletal mineralisation, but it also has essential roles in the physiology of several cellular processes such as bone formation, muscle contraction, neurotransmission, blood coagulation, enzyme activation, and hormone secretion (Boros, 2009; Peacock, 2010; Toribio, 2010).

The vast majority of total body calcium (approximately 99%) is present in the skeleton as calcium-phosphate complexes (hydroxyapatite crystals), with the remaining 1% distributed in the cell membrane, mitochondria, endoplasmic reticulum, and extracellular fluid (Wang *et al.*, 2006; Boros, 2009). Calcium is present in the circulation in three different forms: free or ionised calcium (50%–58%), calcium bound to proteins (40%-45%), and calcium complexed with anions such as bicarbonate, citrate, phosphate and lactate (5%-10%) (Figure 1.3) (Toribio *et al.*, 2001; Lopez *et al.*, 2006; Toribio, 2010). The main calcium binding proteins in the serum are albumin and globulin. Albumin has the highest calcium binding affinity and hypoalbuminaemia results in total hypocalcaemia, but this is a pseudohypocalcaemia with ionised calcium concentrations usually remaining within the reference range (Berlin & Aroch, 2009;

Peacock, 2010; Toribio, 2011). Ionised calcium concentration is pH dependent, with acidosis leading to higher serum ionised calcium, and alkalosis leading to lower serum ionised calcium (Berlin & Aroch, 2009; Toribio, 2011).

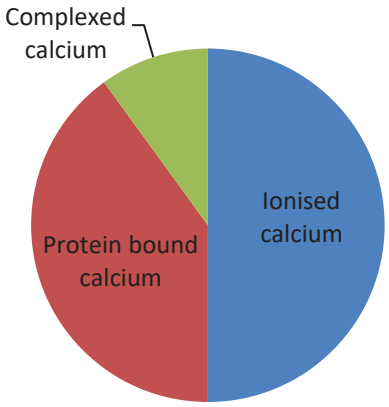


Figure 1.3 Distribution of calcium in the body: ionised calcium 50%–58%, bound to proteins 40%-45% and complexed with anions 5%-10% (Lopez *et al.*, 2006).

Calcium may be obtained from the diet or be made available by bone remodelling (Eckermann-Ross, 2008). The small intestine is the major site of calcium absorption in many species and involves two processes: i. passive diffusion, between the intestinal lumen and blood, and ii. active transport, a vitamin D-dependent process (Eckermann-Ross, 2008). Formation of calcium binding protein in intestinal epithelial cells is stimulated by $1,25(OH)_2D$ under the influence of PTH, and results in the transportation of calcium across the cell's cytoplasm to the bloodstream; this transportation is also influenced by the intestinal calcium concentration (Redrobe, 2002; Reece, 2009). Growth hormone enhances, while glucocorticoids inhibit the active intestinal transport of calcium (Figure 1.4) (Eckermann-Ross, 2008; Reece, 2009). The principal route of calcium loss from the body is urinary excretion, but faecal

excretion, sweat, tooth formation, pancreatic and biliary secretions, lactation, and pregnancy may also be routes of calcium loss and/or increased metabolic demand for calcium (Eckermann-Ross, 2008).

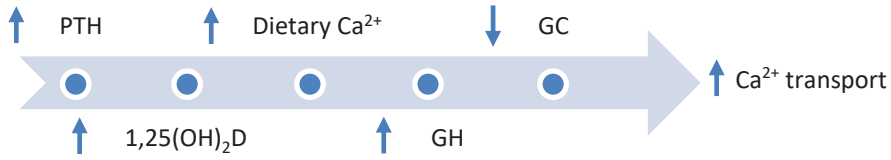


Figure 1.4 Active intestinal transport of calcium. The effect of parathyroid hormone (PTH), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), intestinal (dietary) calcium concentration, growth hormone (GH) and glucocorticoids (GC) on the active intestinal transport of calcium.

Calcium homeostasis is maintained through three major mechanisms (Figure 1.5); bone resorption and formation, intestinal absorption, and renal reabsorption, all regulated by an integrated hormonal system (Peacock, 2010).

1.3 Vitamin D

Humans have been aware of the substance we now know as vitamin D since early antiquity when they recognised the importance of sunlight in the promotion of human health (Mohr, 2009). The story of vitamin D parallels that of the metabolic bone diseases, rickets and osteomalacia (Table 1.1). Significant research demonstrated that cod liver oil, ultraviolet irradiated foods, and exposure to sunlight were beneficial in treating and preventing rickets (Mellanby, 1919; Hess *et al.*, 1925). In the 20th century, vitamin D and its chemical structure were discovered and the role of ultraviolet light in the activation of vitamin D was determined (Windaus, 1931;

Windaus *et al.*, 1936; Holick *et al.*, 1980). Research has shown that vitamin D plays a central role in the homeostasis of calcium and phosphorus, as well as maintaining bone health and other physiological processes (DeLuca, 2004, Norman, 2008, Zhang & Naughton, 2010).

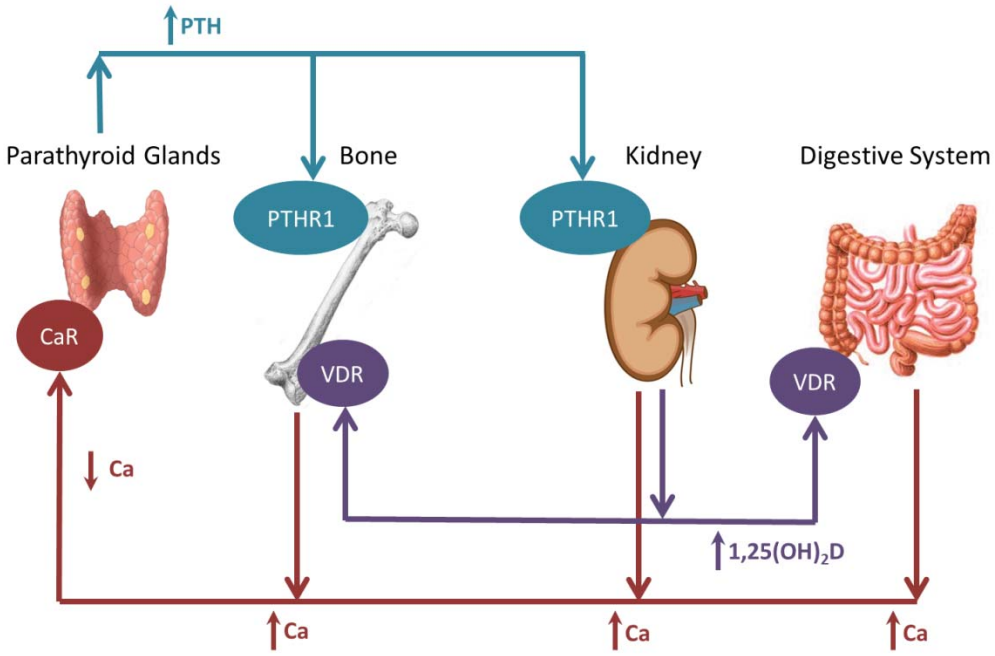


Figure 1.5 Regulation of serum calcium homeostasis. Serum calcium homeostasis is regulated by a rapid negative feedback hormonal pathway involving the concentration of ionised calcium in serum (Ca, maroon arrows) and the secretion of parathyroid hormone (PTH, aqua arrows) from the parathyroid glands. A fall in serum calcium inactivates the calcium receptor (CaR; maroon circle) in the parathyroid cell and increases PTH secretion, which restores serum calcium by activating the parathyroid hormone receptor (PTHR1; aqua circles) in bone, to increase calcium resorption, and in kidney, to increase tubular calcium reabsorption. In kidney, the increased PTH secretion also increases secretion of 1,25-dihydroxyvitamin D (1,25(OH)$_2$D; purple arrows), which, acting on the vitamin D receptor (VDR, purple circles) in digestive system, increases active calcium absorption and increases calcium resorption in bone (Peacock, 2010).

1.3.1 Metabolism of vitamin D

There are two main sources of vitamin D (Figure 1.6). The first is cholecalciferol (vitamin D_3), an inert secosteroid precursor either synthesised in the skin from 7-dehydrocholesterol (7-DHC) after exposure to sunlight/ultraviolet light or consumed

from natural (especially fatty fish) and supplemented foods. The second is ergocalciferol (vitamin D_2) (Figure 1.6), which is obtained from dietary intake of irradiated plants (Lips, 2006; Zhu & DeLuca, 2012).

Table 1.1 A selection of different types of rickets and osteomalacia in human (Nield *et al.*, 2006).

Various Types of Rickets in Human
Nutritional rickets or vitamin D-deficiency rickets
Vitamin D–dependent rickets ▪ Type I or pseudovitamin D-deficiency rickets ▪ Type II or hereditary 1-α,25-dihydroxyvitamin D-resistant rickets
Hypophosphataemic rickets ▪ Autosomal recessive hypophosphataemic rickets or X-linked hypophosphataemic rickets ▪ Hereditary hypophosphataemic rickets with hypercalciuria
Miscellaneous ▪ Chronic kidney disease-mineral and bone disorder (CKD-MBD or Renal osteodystrophy) ▪ Rickets of prematurity ▪ Tumour-induced or oncogenic osteomalacia

Cholecalciferol and ergocalciferol differ in their origin, chemical composition and biological activity (Norman, 2008). Both isomers are formed by photolytic breakdown of the C_9-C_{10} B ring bond of the steroid precursor (Norman, 2008). Vertebrates are able to produce cholecalciferol, whereas ergocalciferol is of plant origin. The side-chain of ergocalciferol contains an unsaturated bond at C_{22}-C_{23} and a methyl group at C_{24} that is absent in cholecalciferol (Holick, 2005). The difference in chemical structure and metabolism of cholecalciferol makes it two to three times more

active than ergocalciferol (Figure 1.6) (Armas *et al.*, 2004; Holick, 2005; Houghton & Vieth, 2006; Norman, 2008).

Dietary vitamin D (vitamin D_2 and vitamin D_3), which can be obtained from food or an oral supplement (Holick, 2004 a,b), is fat soluble and is absorbed through the intestinal wall, where it is incorporated into chylomicrons (lipoproteins) and transported in circulation to the liver where it undergoes hydroxylation (Radlović, 2009, DeLuca, 2004).

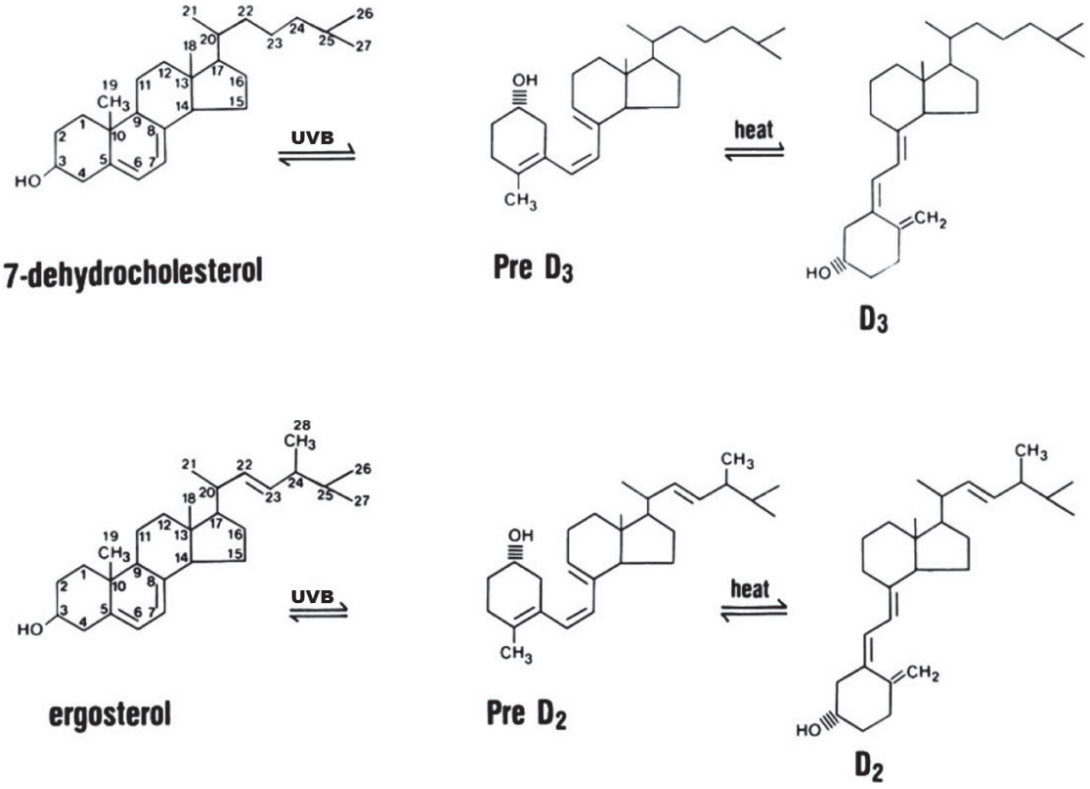


Figure 1.6 Production of vitamin D_2 and vitamin D_3. Ergosterol in plants and 7-dehydrocholesterol in skin are the precursors for vitamin D_2 and vitamin D_3, respectively. UVB light breaks the B chain of each molecule to form the pre-D isomer, which then undergoes isomerisation to vitamin D. Vitamin D_2 and D_3 differ only in the side chain in which D_2 has a double bond between C_{22}–C_{23} and a methyl group at C_{24}. These differences alter vitamin D_2 binding to DBP and its metabolism (Bikle, 2009).

1.3.1.1 Synthesis of vitamin D in skin

In animals, the physiological requirement for vitamin D is primarily fulfilled by photochemical and thermal conversion of cutaneous 7-DHC in the presence of solar ultraviolet B radiation. Epidermal keratocytes and dermal fibroblasts synthesise 7-DHC, which is converted by exposure to solar ultraviolet B (approximately 290-325 nm) radiation to previtamin D_3, followed by thermal isomerisation at 37˚C to vitamin D_3 (DeLuca, 2004; Holick, 2004 a,b; Tavera-Mendoza & White, 2007). Vitamin D_3 enters the circulation where it binds to vitamin D-binding protein (DBP) and is transported to the liver and other organs (DeLuca, 2004; Holick, 2004 a,b; Holick, 2006a).

The major source of vitamin D for most amphibians, reptiles, avian species, and mammals is exposure to sunlight (Holick, 2006a; Holick, 2007). Anything that inhibits the penetration of ultraviolet B radiation into the skin or reduces the transmission of solar ultraviolet B photons affects the photosynthesis of vitamin D_3 in the skin (Holick, 2003; Chen *et al.*, 2007). For instance, the skin of birds covered by feathers is not able to synthesise vitamin D_3, and the concentration of 7-DHC is 10-fold higher in non-feathered skin, such as the legs (Tian *et al.*, 1994).

In humans, the amount of 7-DHC in the skin is relatively constant until approximately 65 years old, when it begins to decline (MacLaughlin & Holick, 1985; Mosekilde, 2005). Exposure to the same amount of sunlight in a 70 year old person results in only 25% of the vitamin D_3 that a 20 year old person produces (Holick, 2004).

Melanin is a pigment that absorbs ultraviolet B photons, and has evolved as a natural sunscreen to protect animals and humans from sunburn and skin cancer (Kennedy *et al.*, 2003). High skin pigmentation markedly diminishes the production of vitamin D_3; skin with higher amounts of melanin therefore requires longer exposure to sunlight in order to make the same amount of vitamin D_3, compared with skin that has lower melanin pigmentation (Clemenset *et al.*, 1982). Sunscreens with a sun protection factor (SPF) of 8 act similarly to skin pigmentation by absorbing around 92-95% of ultraviolet B photons before they enter the skin, thus reducing the synthesis of vitamin D_3 (Matsuoka *et al.*, 1987).

The zenith angle of the sun is altered by time of day, season, geographic latitude and altitude, and has substantial effects on vitamin D_3 synthesis (Webb *et al.*, 1988; Holick, 1994; Holick *et al.*, 1995; Holick, 2003). During winter, although the sun is closer to the earth, the rays of the sun enter at a more oblique angle, resulting in a greater number of ultraviolet B photons being absorbed by the ozone layer. This means that little, if any, synthesis of vitamin D_3 occurs in the skin (Webb *et al.*, 1988; Holick *et al.*, 1995). A similar effect occurs in the early morning and late afternoon in summer (Holick, 2004 a,b). There is little known about the effect of cloud cover on the synthesis of vitamin D_3 in the skin (Holick *et al.*, 2007).

Vitamin D intoxication by sun exposure does not occur due to melanin's protective effects, continuous skin desquamation, limited vitamin D binding protein (DBP) transport capacity, and photo isomerisation of vitamin D_3 to non-toxic metabolites such as lumisterol, tachysterol, suprasterol I and II, and 5,6-trans-

cholecalciferol (Holick, 2004 a,b). Any excess vitamin D, from dietary intake or cutaneous synthesis, is stored in the liver, fat tissue, skeleton and muscles (DeLuca, 2004).

Vitamin D_3 is fat soluble; it is stored in the adipose cells and used during periods when little vitamin D_3 is synthesised in the skin, such as winter. Obese individuals, however, have decreased serum vitamin D concentration as their abdominal fat can provide an irreversible sink for vitamin D, and thus have an increased risk of vitamin D deficiency (Bell *et al.*, 1985; Wortsman *et al.*, 2000). Obese people given the same dose of vitamin D_2 and exposed to the same amount of radiation for the same period of time as non-obese people, had only 50% of the increase in blood vitamin D concentrations that occurred in non-obese people (Wortsman *et al.*, 2000).

In humans, vitamin D status is affected by the sum of exposure to ultraviolet B photons and dietary intake, but is also influenced by lifestyle, culture, traditional diets, behavioural parameters, geographical pattern (latitude and altitude), season, time of day, cloud cover/pollution, amount of sun exposure, melanin concentration in the skin, age, the degree of protection from sunlight, clothing traditions, and sun exposure behaviour (Holick, 1995; Holick & Chen, 2008). Due to the limited sources of nutritional vitamin D (sea fish, liver, egg yolk, mushrooms and milk products), human physiological requirements for vitamin D are mostly fulfilled by cutaneous synthesis (Holick & Chen, 2008; Norman, 2008). The ozone layer, ultraviolet B absorbing pollutants in the air, and the amount of the skin covered by clothes, all reduce the amount of solar

ultraviolet B photons that reach the skin, thus reducing dermal synthesis of vitamin D_3 (Holick *et al.*, 2007). As a result, vitamin D deficiency and insufficiency, low serum 25OHD concentrations that might be associated with adverse health outcomes (Thacher & Clarke, 2011), are becoming increasingly diagnosed in developed countries.

1.3.1.2 Activation of vitamin D

Vitamin D is transported to the liver, where it undergoes hydroxylation to 25-hydroxyvitamin D (25OHD). This step is dependent on the serum concentration of 25OHD (Dusso *et al.*, 2005; Holick, 2008; de Paula & Rosen, 2012). The activation process is initiated in the hepatocyte microsome with hydroxylation at C_{25} to generate 25OHD, the main form of vitamin D in circulation. The hydroxylation is catalysed by CYP27A1 and CYP2R1, with minor roles for other enzymes (Cheng *et al.*, 2004; Prosser & Jones, 2004; Shinkyo *et al.*, 2004; Dusso *et al.*, 2005; Holick, 2007).

After formation of 25OHD in the liver, it is bound to DBP and has a half-life in circulation of 10-20 days. This is the major circulating form of vitamin D and can be measured by several techniques, including high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS) and competitive protein binding assays such as radioimmunoassay and enzyme-linked immunoassays. Measurement of 25OHD in serum reflects the overall vitamin D status in the body (Holick, 2007; Morris & Anderson, 2010; White, 2012).

Bound to DBP, 25OHD is transported to the kidneys. The transport of DBP-25(OH)D complex into the renal cells mediates with the megalin/cubulin system

(Nykjaer et al., 2001). Megalin and cubilin are two structurally different endocytic receptors that interact to serve the selective transport of substances from one compartment to another (Christensen & Birn, 2002). The final step in activation of vitamin D takes place in the mitochondria of the renal proximal tubular cells of the kidney, where 25OHD is 1α-hydroxylated by CYP27B1 (1α-hydroxylase) to the hormonally active 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Holick, 2004 a,b; Hewison *et al.*, 2007; Morris & Anderson, 2010). This form of vitamin D ($1,25(OH)_2D$) is the most potent vitamin D metabolite and its synthesis and serum concentration is tightly controlled by PTH, FGF23, calcium, phosphorus, $1,25(OH)_2D$, and the vitamin D status of the animal (Figure 1.7) (Cheng *et al.*, 2004; Liu & Quarles, 2007; Tavera-Mendoza & White, 2007). The half-life of $1,25(OH)_2D$ in circulation is 4-7 hours, after which it becomes inactivated (Hewison *et al.*, 2007). Inactivation of $1,25(OH)_2D$ is carried out by hydroxylation at C_{24} by 24-hydoxylase (CYP24A1) in the kidney, intestine, bone and other organs/tissues, resulting in the formation of calcitroic acid and C_{23} carboxylic derivatives, which are then removed in the urine and bile (Morris & Anderson, 2010).

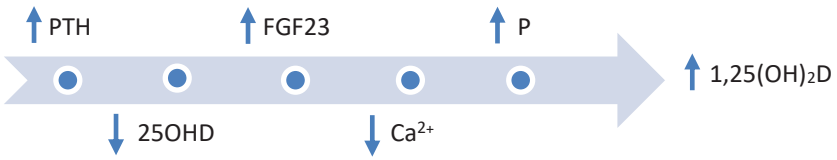


Figure 1.7 $1,25(OH)_2D$ synthesis. The effect of parathyroid (PTH), fibroblast growth factor 23 (FGF23), vitamin D metabolites in circulation (25OHD) and calcium and phosphorus concentration on $1,25(OH)_2D$ synthesis.

In vitamin D target cells, $1,25(OH)_2D$ binds to the vitamin D receptor (VDR). VDR is a ligand-dependent transcription factor belonging to the superfamily of

steroid/thyroid hormone receptors (Pinette *et al.*, 2003; Haussler *et al.*, 2008). The 1,25$(OH)_2$D and VDR complex forms a heterodimer with the retinoid receptor and binds to vitamin D-response elements on a responsive gene. This is followed by transcription and formation of proteins such as calcium binding protein, osteocalcin and other proteins associated with maintenance of calcium homeostasis (Lips, 2006). Vitamin D receptors are present in a variety of cells, and have biological effects on more than mineral metabolism (Zhang & Naughton, 2010). The VDR and vitamin D are also involved in cell proliferation, differentiation, and immunomodulation (Nagpal *et al.*, 2005).

1.3.2 Physiological function of vitamin D

The importance of vitamin D is emphasised by the consequences of vitamin D deficiency, which results in rickets in the young, and osteomalacia and increased risk of fractures in adults (Lips, 2001; Holick, 2006a; Holick, 2007; Bouillon *et al.*, 2008). The principal action of the hormonally active form of vitamin D (1,25$(OH)_2$D) is intestinal absorption of calcium and phosphorus (Wasserman, 2004) and without it, only 10-15% of dietary calcium and approximately 60% of dietary phosphorus is absorbed in humans (DeLuca, 2004).

Transport of calcium in the intestine mediates by both the active transcellular and passive paracellular pathways (Hoenderop *et al.*, 2005). The passive paracellular pathway functions through the entire length of the intestine, although it predominates in the more distal regions (Hoenderop *et al.*, 2005). The passive diffusion is driven by

the luminal electrochemical gradient and the integrity of the intercellular tight junctions (Tuskita *et al.*, 2001). It has been suggestd that transjunctional transport of calcium by the passive pathway occurs in a regulated fashion, and may be tied to the active transcellular movement of calcium in a harmonized manner (Fujita *et al.*, 2008). Tight junctions are specialized membrane domains located between the apical and basolateral membranes of the enterocyte, which form a barrier to the movement of ions, proteins, and other macromolecules across the intestine (Tuskita *et al.*, 2001). The major transmembrane components of tight junctions are Claudins (Tuskita *et al.*, 2001), and it has been suggested that $1,25(OH)_2D$ can promote paracellular calcium diffusion by increasing junction ion permeability (Fujita *et al.*, 2008). The process of active ingestion and absorption of dietary calcium is dependent on an adequate active vitamin D concentration in the body (Christakos, 2012). Calcium transport is stimulated when $1,25(OH)_2D$ binds to VDR in the intestinal epithelial cell, resulting in increased synthesis of vitamin D-dependent calcium-binding protein (calbindin), especially calbindin-D_{9K}, and the calcium channel TRPV6 (Figure 1.8) (Bouillon *et al.*, 2008).

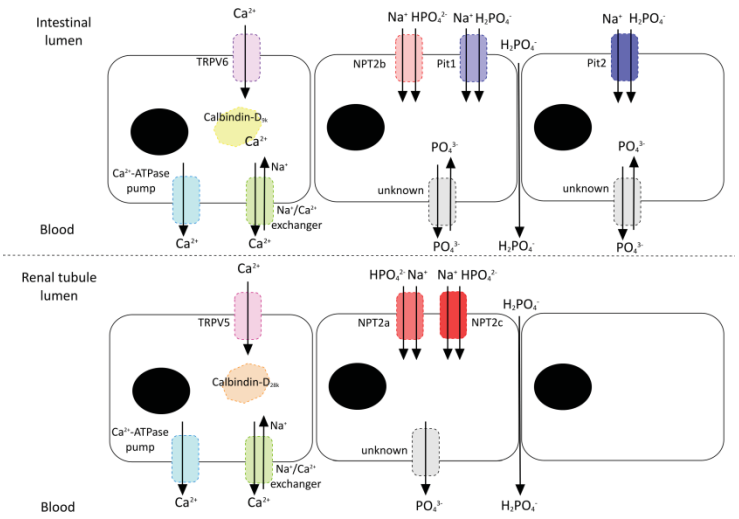


Figure 1.8 Mechanism of transepithelial transport of calcium. The Individual steps of transepithelial transport of calcium are stimulated by 1,25(OH)$_2$D and PTH, resulting in increased calcium absorption. A three step process occurs which includes entry of calcium through the epithelial channels, TRPV5 in kidney and TRPV6 into intestine, in to the epithelium, binding of calcium to the specialised intracellular carrier protein calbindin-D$_{9K}$ in intestine and calbindin-D$_{28K}$ in kidney, and diffusion of calcium through the basolateral membrane via an ATP-dependent Ca^{2+}-ATPase (PMCA) and Na^+/Ca^{2+} exchanger (NCX1). Transepithelial phosphorus transport also consists of three steps, including uptake at the border membrane, translocation across the cell, and efflux at the basolateral membrane channels (Figure courtesy of Keren Dittmer).

Kidneys have an important role in the reabsorption of calcium and contribute to the maintenance of calcium homeostasis. In the renal tubule, 1,25(OH)$_2$D and PTH stimulate reabsorption of filtered calcium. Around 45% of the plasma free ionised calcium is filtered through the glomerulus and then enters the proximal tubule segment of the nephron, where approximately 65% of the filtered calcium is passively reabsorbed (Reilly & Ellison, 2000). An additional 20% of the calcium is reabsorbed through the passive paracellular pathway in the thick ascending loop of Henle (Biner *et al.*, 2002). The regulation of calcium reabsorption in these segments depends on gradients established by Na^+, Cl^-, and water reabsorption (Suki, 1979). According to the physiological needs of the body, the final regulation of calcium excretion occurs in two segments of the distal part of the nephron, the late part of the distal convoluted

tubule (DCT2) and the connecting tubule (CNT) (Costanzo & Windhager, 1978). Expression of vitamin D responsive proteins, Na^+/Ca^{2+} exchanger (NCX1), plasma membrane ATPase type 1b (PMCA1b), TRVP5 and calbindin-D_{28K} has been found along the DCT2/CNT region (Boros, 2009). Transcellular calcium transport occurs against an electrochemical gradient in the DCT2/CNT and therefore occurs by active transepithelial transport (Boros, 2009). Transepithelial transport of calcium, regulated by 1,25$(OH)_2$D and PTH, is a three step process: i. influx of calcium across the apical membrane mediated by TRPV5 (Hoenderop *et al.*, 2005) ii. entrance of calcium and diffusion between the apical and basolateral membrane, sequestered by the specialised intracellular carrier protein calbindin-D_{28K} and iii. efflux of calcium across the basolateral membrane through Na^+/Ca^{2+} exchange mechanism (NCX1) and Ca^{2+}-ATPase (PCMA1) (Hoenderop *et al.*, 1999). Calbindin-D_{28k}, in association with TRPV5 facilitates high calcium transport rates by preventing calcium-dependent inactivation of channels (Figure 1.8) (Lambers *et al.*, 2006).

Vitamin D has important effects on bone, but must first be activated (Figure 1.9). The renal 1α-hydroxylase enzyme plays an important role in the production of 1,25$(OH)_2$D, which is mainly regulated by PTH, FGF23 and the status of vitamin D in the body (Suda *et al.*, 2012). It has been postulated that vitamin D directly stimulates osteoblastic bone formation and mineralisation, but even now there is no direct evidence for this (Suda *et al.*, 2012). Active vitamin D via the VDR maintains calcium homeostasis (DeLuca, 1988), although results of studies on VDR knockout mice showed that 1,25$(OH)_2$D also has VDR-independent action, in addition to the indirect effect on

bone formation and mineralisation through increasing intestinal absorption of calcium (Yoshizawa *et al.*, 1998; Tanaka & Seino, 2004; Lieben & Carmeliet, 2013). Apart from the classical physiological role of $1,25(OH)_2D$ on calcium and phosphate homeostasis, studies on mice with conditional inactivation of VDR in chondrocytes revealed non-essential roles for $1,25(OH)_2D$ in growth plate chondrocytes (Masuyama *et al.*, 2006; St-Arnaud, 2008). Serum phosphate concentration increased in chondrocyte-specific VDR-ablated mice due to decreased circulating levels of FGF23. This appears to be due to involvement of a $1,25(OH)_2D$-induced secreted factor from chondrocytes which affects FGF23 production by neighbouring osteoblasts (Panda *et al.*, 2004; St-Arnaud, 2008).

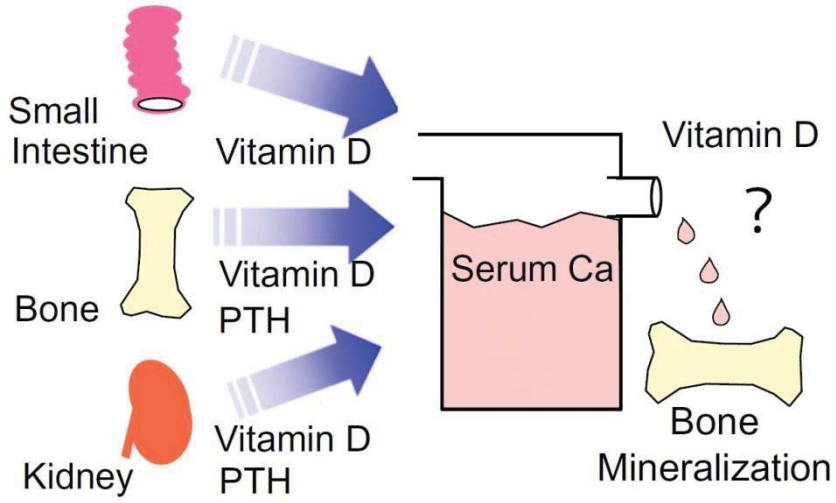


Figure 1.9 Classical actions of vitamin D. A diagrammatic representation of the classical actions of vitamin D to maintain serum calcium homeostasis. While vitamin D is the sole substance to stimulate intestinal absorption of calcium, vitamin D and PTH working in concert are necessary to mobilise calcium from bone and conserve calcium from urine. (Suda *et al.*, 2012).

To release calcium to the extracellular fluid, $1,25(OH)_2D$ induces the mobilisation of calcium from bone (Suda *et al.*, 2012); when serum calcium

concentration is low, bone resorbing factors such as 1,25(OH)$_2$D, PTH and interleukin 6 (IL-6) stimulate expression of the receptor activator ligand of nuclear factor-kappaB (NF-κB or RANKL) on osteoblasts. RANKL recognises RANK receptors present on osteoclast progenitors, stimulating them to differentiate into mature osteoclasts (Suda *et al.*, 2012). Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL; its role is to suppress osteoclast differentiation and activity through interrupting the binding of RANKL to RANK (Figure 1.10) (Yasuda *et al.*, 1998; Wada *et al.*, 2006). Many calcitropic hormones, including 1,25(OH)$_2$D and PTH inhibit production of OPG and stimulate and up-regulate RANKL expression in osteoblasts (Suda *et al.*, 2012).

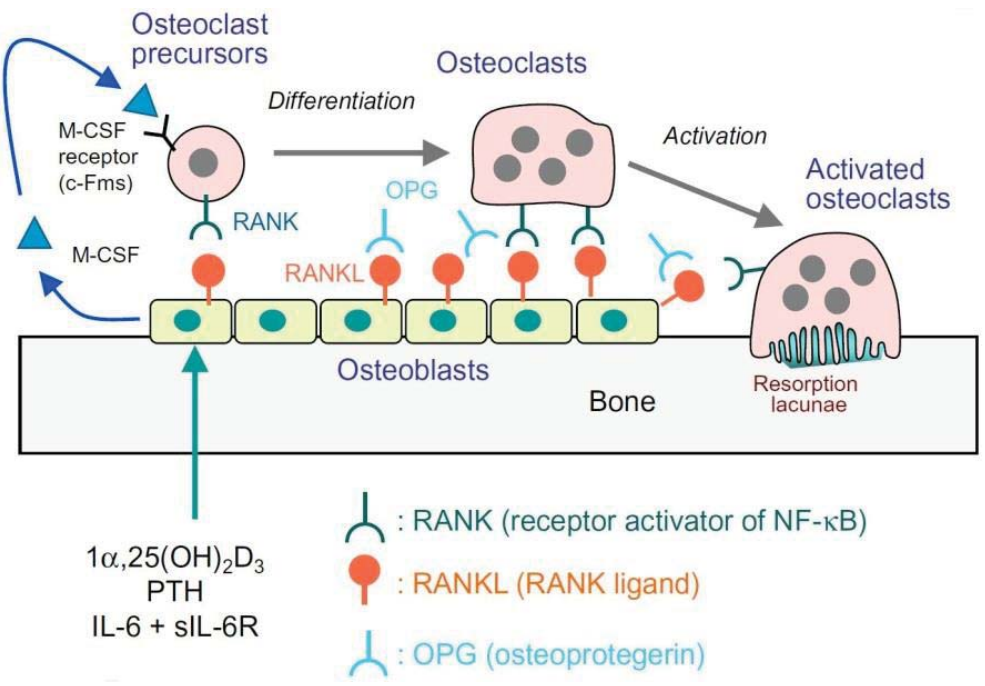


Figure 1.10 Osteoclast differentiation and activation. A model illustrating the mechanism by which osteoblastic cells regulate osteoclast differentiation and activation. Three distinct signals stimulated by 1,25(OH)$_2$D$_3$, PTH and IL-6 + sIL-6R induce RANKL expression on osteoblastic cells. RANKL mediates a signal for osteoclast differentiation and activation through its receptor RANK expressed on osteoclast progenitors. OPG, a decoy receptor of RANKL, inhibits osteoclast differentiation and activation by interrupting the binding of RANKL to RANK. M-CSF produced by osteoblastic cells is also required for proliferation and differentiation of osteoclast progenitors (Suda *et al.*, 2012).

1.3.3 Vitamin D and single nucleotide polymorphisms

There have been many studies on the association between vitamin D status and disease over the last few decades. Recently, attention has turned to genetics and environmental interactions which could influence various vitamin D-related disorders (McGrath *et al.*, 2010).

Single nucleotide polymorphisms (SNPs) are the most abundant form of genetic variation and are a useful resource for mapping complex genetic traits. Different studies investigating heritable factors have confirmed the influence of different genes on serum vitamin D concentrations in different individuals (Snellman *et al.*, 2009; Engelman *et al.*, 2008).

Genes that influence serum concentrations of vitamin D are often involved in the production and elimination of vitamin D (McGrath *et al.*, 2010; Dastani *et al.*, 2013), including:

i. genes involved in production of vitamin D
ii. genes involved in activation/deactivation of vitamin D
iii. genes involved in vitamin D carrier proteins that bind to vitamin D
iv. genes involved in the ligand bound receptor that influences gene expression
v. genes that impact calcium and PTH concentrations

Some of these candidate genes have been identified, including group-specific component, 7-dehydrocholesterol reductase 1 (DHCR1), Cytochrome P450, family 2,

subfamily R, polypeptide 1 (CYP2R1), vitamin D receptor (VDR), and Cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24A1)(Dastani *et al.*, 2013).

Several aspects of vitamin D metabolism remain unclear despite the fact that vitamin D deficiency is associated with many adverse health outcomes (Kulie *et al.*, 2009). Certain environmental factors, especially nutrition and UVB radiation, have been considered major determinants of vitamin D status, although they have only explained a modest proportion of the variation in vitamin D status to date (Snellman *et al.*, 2009). There is little known about the impact of genetic epidemiology on vitamin D and its metabolites in the body and further research is needed to identify the specific genes impacting on vitamin D status.

1.3.4 Vitamin D and fractures

Vitamin D deficiency causes rickets in children and osteomalacia in adults, both of which are associated with increased risk of bone fractures (Holick, 2007; Bouillon *et al.*, 2008). Vitamin D deficiency leads to hypocalcaemia, hypophosphataemia, impaired bone mineralisation, muscle weakness, low bone density and pathological fractures (Need, 2006).

During the last 50 years, the prevalence and number of fractures has risen among adults over 50 years (Rosengren *et al.*, 2012). Falls and bone fragility are considered the two main risk factors for fractures, especially among women (Tinetti, 1987). Fractures occur most commonly in the hip, followed by the forearm and vertebral bones (Karlsson *et al.*, 2013). Postmenopausal women with vitamin D

deficiency, compared to those with sufficient vitamin D, are more prone to osteoporosis and fractures due to low serum concentration of 25OHD, high serum concentration of PTH, increased bone turnover and low serum ionised calcium (Jesudason *et al.*, 2002). People who do not obtain adequate vitamin D through their diet, and do not have enough exposure to sunlight, also have a higher risk of vitamin D deficiency, osteoporosis and fractures (Gaugris *et al.*, 2005).

There is controversy among human medical experts as to the appropriate serum concentration of vitamin D and as to what constitutes insufficient and adequate serum vitamin D concentrations. It had been believed that obtaining sufficient vitamin D through supplementation and increased sun exposure would increase bone mineral density and decrease the risk of fractures (Jesudason *et al.*, 2002; Gaugris *et al.*, 2005; Karlsson *et al.*, 2013). However, some recent studies indicate that vitamin D supplementation in adults does not influence bone density, except in those who have osteomalacia, which represents very severe vitamin D deficiency (Bolland et al., 2016b; Reid, 2016). The later research demonstrated through multiple meta-analyses that vitamin D supplementation does not reduce the risk of fracture (Avenell et al., 2016; Bolland et al., 2016a).

1.3.5 Vitamin D and the immune system

Vitamin D receptors are not only present in tissues involved in calcium homeostasis and bone metabolism, but also in many other tissues (Bouillon & Suda, 2014). During the last 30 years, non-classical roles of vitamin D have been investigated.

One of the non-classical effects of vitamin D is its interaction with the immune system. It has been shown that vitamin D plays an important role in the regulation of both the innate and adaptive immune systems (White, 2012).

Macrophages, dendritic cells (DCs) and activated T and B cells express VDRs and CYP27B1, and $1,25(OH)_2D$ can regulate the proliferation and function of these cells (O'Brien & Jackson, 2012). Vitamin D in the form of $1,25(OH)_2D$, stimulates the differentiation of monocytes into mature tissue macrophages, and stimulates the innate immune system (Mangelsdorf *et al.*, 1984). The activation of macrophage toll-like receptors (TLR) up-regulates the expression of VDR and CYP27B1, and the conversion of 25OHD to $1,25(OH)_2D$, leading to induction of the antimicrobial peptide cathelicidin and killing of microbes via vitamin D-dependent induction of antimicrobial peptide genes (Liu *et al.*, 2006). Therefore, vitamin D status is an important link between activation of TLR and cells of the innate immune system (O'Brien & Jackson, 2012).

Vitamin D also plays an important role in antigen presentation by forming a link between the innate and adaptive immune systems. Proliferation and production of immunoglobulin is suppressed by $1,25(OH)_2D$, which prevents the expression of co-stimulatory molecules, decreases maturation of dendritic cells (DCs) and their ability to present antigen and activate T cells (Szeles *et al.*, 2009). In vitamin D deficient situations, macrophages function abnormally with changes to chemotaxis, phagocytosis, maturation, and the production of pro-inflammatory cytokines (O'Brien & Jackson, 2012). The physiological role of vitamin D as an immune modulator is

epidemiologically supported by the correlation between vitamin D deficiency/insufficiency and increased susceptibility to respiratory infections and tuberculosis (Hewison, 2012). *Mycobacterium tuberculosis* organisms primarily infect pulmonary macrophages and, as mentioned above, $1,25(OH)_2D$ acts as an immunoregulator that stimulates the differentiation of monocytes to macrophages (Liu & Modlin, 2008). As a link between vitamin D deficiency and susceptibility to infections of the respiratory system is well documented, it is important to obtain sufficient vitamin D for resistance to respiratory infection (Haider *et al.*, 2010).

There is no doubt that genetic susceptibility is involved in autoimmune diseases but epidemiologic studies suggest that environmental factors that cause vitamin D insufficiency also influence the incidence of these diseases (Kulie *et al.*, 2009). Knowledge of the effects of supplementary vitamin D on autoimmune and infectious diseases is limited, but several trials (Hewison, 2012) have shown beneficial effects of vitamin D supplementation on patients with autoimmune disease. Despite strong support for an association between vitamin D status and autoimmune diseases, the role of vitamin D signalling in controlling innate immunity may be complicated by the fact that animal models such as mice may not recapitulate many of the vitamin D-dependent molecular-genetic mechanisms seen in humans (White, 2012).

There are different theories regarding the supposed immune-modulatory actions of vitamin D. Even though vitamin D concentrations have been shown to be associated with a large number of medical conditions, the evidence that vitamin D replacement changes the incidence or presentation of these conditions is almost

entirely lacking. Therefore, further studies are required to clarify the relationship between vitamin D status and autoimmune diseases such as multiple sclerosis (MS), type 1-diabetes, Crohn's disease, and psoriasis.

1.4 Parathyroid hormone

Parathyroid hormone is a single chain polypeptide with 84 amino acids and a molecular weight of approximately 9400 Daltons. It functions as a key regulator of serum calcium homeostasis and is the major mediator of bone remodelling in mammals (Gensure *et al.*, 2005; Potts, 2005). PTH is responsible for the regulation of calcium concentration in blood and extracellular fluids in combination with other calcitropic hormones (Habener *et al.*, 1984). The parathyroid chief cells respond to changes in extracellular calcium concentrations. PTH secretion is increased by a reduction in extracellular calcium concentration, whereas small increases in extracellular calcium concentration inhibit its secretion (Brown *et al.*, 1993). Calcium sensitive receptors (CaSR) on PTH chief cells are G protein-coupled receptors which control calcium concentration via an intracellular signalling cascade (Ward, 2004). CaSR are inactivated by decreased serum calcium concentration, resulting in increased PTH secretion. PTH restores serum calcium concentration by activating parathyroid hormone receptor (PTHR1) in bone and kidney, leading to increased osteoclastic resorption of bone and increased renal tubular reabsorption of calcium (Peacock, 2010). CaSR are also expressed in other tissues that have roles in calcium homeostasis, such as the intestine, kidney and bone (Topala *et al.*, 2009).

A single G protein-coupled receptor, the PTH/ PTH-related peptide (PTHrP) receptor/ type 1 PTH receptor (PTHR1), is responsible for most of the traditional actions of PTH in mineral ion homeostasis and is critical to its actions on bone and kidney (Jüppner *et al.*, 1991; Abousamra *et al.*, 1992). The PTHrP molecule shares significant structural homology and even some overlap in function with PTH by using the same G protein-linked receptor (Jüppner *et al.*, 1991; Potts, 2005). PTHR1 is highly expressed in bone, kidneys and a variety of other tissues (Gensure *et al.*, 2005; Potts, 2005). It is activated equally by PTH and PTHrP, resulting in activation of G-alpha proteins, stimulation of adenylate cyclase and formation of cyclic 3',5'-adenosine monophosphate (cAMP) (Swarthout *et al.*, 2002; Murray *et al.*, 2005). The cAMP molecule binds to and activates protein kinase A (PKA), which then activates a variety of transcription factors and cascades, such as the pro-proliferative mitogen-activated protein kinase (MAPK) pathway (Swarthout *et al.*, 2002). PTHR1 also activates other pathways, including the phospholipase C (PLC) pathway, leading to formation of diacylglycerol (DAG), which activates protein kinase C (PKC), phospholipase D (PLD) and phospholipase A2 (PLA2) (Gensure *et al.*, 2005; Murray *et al.*, 2005). In bone, PKC mediates the cAMP-independent effects of PTH/ PTHrP (Gensure *et al.*, 2005; Murray *et al.*, 2005).

The principal target organs for PTH are the kidneys and bone. In the kidneys, the interaction of PTH with PTHR1 on the plasma membrane results in activation of the adenylate cyclase-cAMP-PKA pathway, which activates calcium influx across the plasma membrane through cAMP and PKA-dependent activation of voltage-operated

calcium channels and the phospholipase C-diacylglycerol PKC pathways (Picotto *et al.*, 1997). In the kidneys, PTH enhances calcium reabsorption in the proximal tubules, excretion of phosphorus and stimulation of CYP27B1, leading to the formation of $1,25(OH)_2D$ (Poole & Reeve, 2005).

Bone remodelling is a dynamic process consisting of osteoblastic bone formation, and osteoclastic bone resorption (Suda *et al.*, 2012). PTH is known to have both anabolic and catabolic effects on bone remodelling (Silva & Bilezikian, 2015). It increases bone formation through its ability to enhance osteoblastogenesis, which is partially mediated by a decrease in SOST/Sclerostin expression gene in osteocytes. As a result, PTH may be used as a treatment for osteoporosis (Figure 1.11) (Silva *et al.*, 2011).The RANK–RANKL–OPG axis is regulated by PTH, interleukin-1 (IL-1) and $1,25(OH)_2D$, all of which may stimulate osteoclast formation (Takeda *et al.*, 1999; Silva *et al.*, 2011). PTH stimulates both bone resorption and formation through its catabolic or anabolic effects respectively, depending on the dosage levels and periodicity of the PTH signal (Silva & Bilezikian, 2015).

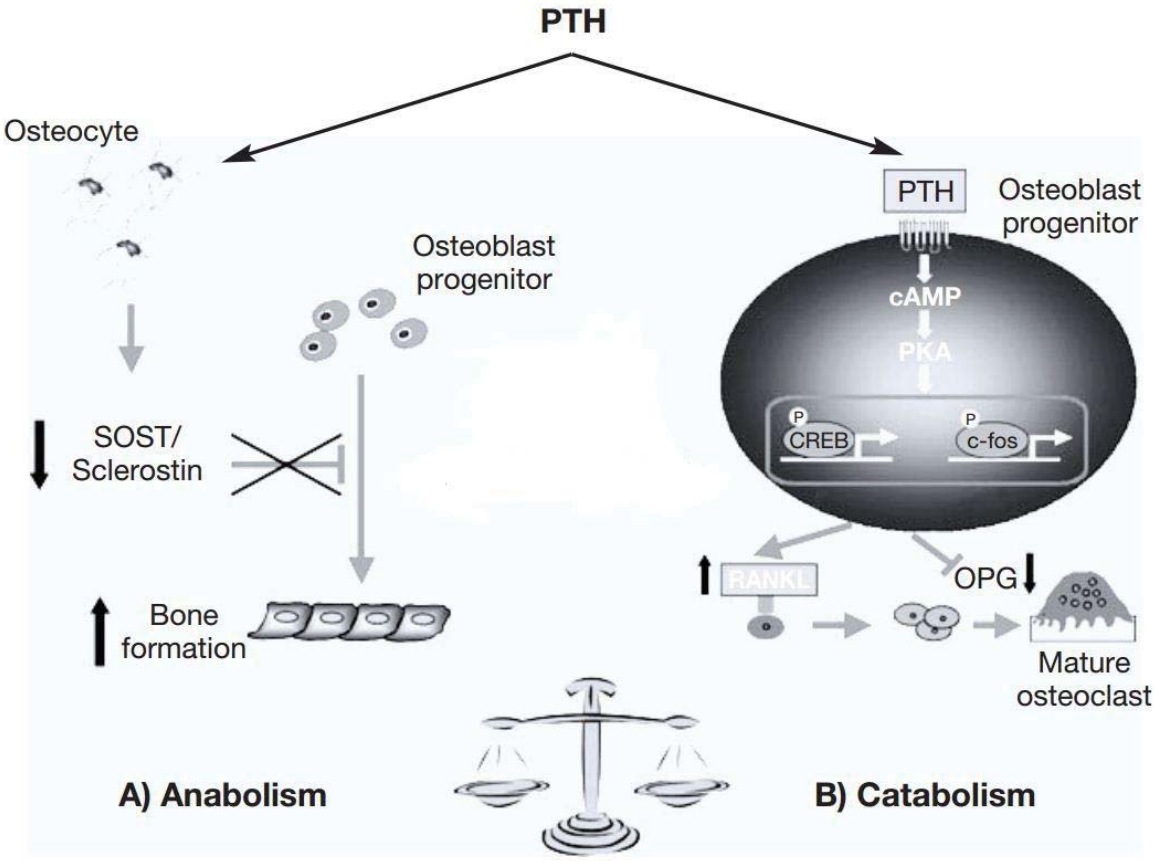


Figure 1.11 Anabolic and catabolic pathways of PTH on the skeleton. A) PTH decreases SOST/sclerostin expression in osteocytes. Sclerostin functions as a negative regulator of bone formation, and its downregulation by PTH contributes for the PTH-induced osteoanabolism. B) PTH favours bone resorption, mostly by increasing receptor activator of nuclear factor kappa-B ligand (RANKL) and decreasing osteoprotegerin (OPG) expression in osteoblasts, which ultimately leads to an increase in osteoclast formation and activity (Silva et al., 2011).

1.5 Calcitonin

Calcitonin is a 32 amino acid linear polypeptide hormone produced by parafollicular cells (C cells) of the thyroid gland, and is known as a blood calcium-lowering hormone (Zaidi *et al.*, 2002). The group of peptide hormones that compose the calcitonin family, includes calcitonin gene-related peptide (CGRP), amylin, adrenomedullin, and adrenomedullin 2, all share a similar structure to calcitonin (Naot & Cornish, 2008). The calcitonin family of hormones are produced by a number of different tissues: αCGRP mainly in neural tissue, amylin predominantly in β-islet cells of

the pancreas, adrenomedullin in many tissues and cell types, and adrenomedullin 2 primarily in the pituitary and gastrointestinal tract (Kitamura *et al.*, 1994; Ogawa *et al.*, 1999; Roh *et al.*, 2004). Bone is a common target for all the calcitonin family peptides; amylin, adrenomedullin and αCGRP are effective in inhibiting osteoclast activity and bone resorption, and may also affect cells of the osteoblast lineage, inducing osteoblast proliferation and promoting bone formation (Naot & Cornish, 2008).

Calcitonin inhibits bone-resorbing activity of osteoclasts by increasing osteoclast apoptosis, decreasing PTH stimulation of osteoclasts, and lowering the cytosolic calcium concentration in the cells of bone (Plotkin *et al.*, 1999; Eckermann-Ross, 2008). Calcitonin also inhibits tubular reabsorption of calcium and phosphorus by increasing renal calcium excretion (McKeever, 2011). Calcitonin enhances chloride (Cl^-) conductance in the distal convoluted tubule (DCT) of the kidney, which results in hyperpolarisation and activation of calcium entry through calcium channels (Gesek & Friedman, 1993). The gene promoter of renal 1α-hydroxylase gene expression is regulated by PTH as a positive regulator and active vitamin D_3 as a negative regulator (Takeyama & Shigeaki, 2011), therefore any depression in 1α-hydroxylase gene activity results in decreased $1,25(OH)_2D$ concentration and decreased renal and intestinal calcium absorption/resorption (Kato, 1999).

1.6 Fibroblast growth factor 23

FGF23 is a member of the fibroblast growth factor family that consists of 22 members with different functions (Yamashita *et al.*, 2000). It is a protein of 251 amino

acids in length with a weight of approximately 32-kD (Yamashita *et al.*, 2000) and acts mainly as a phosphaturic factor and suppressor of 1α-hydroxylase activity in the kidney (Shimada *et al.*, 2001). In bone, FGF23 is mainly expressed in osteocytes, which regulate osteoblastic function and bone mineralisation, as well as in pericyte-like cells that surround the venous sinuses in the bone marrow, the ventrolateral thalamic nucleus, thymus and lymph nodes (Liu *et al.*, 2006). Klotho, a single-pass transmembrane protein, acts as binding protein for FGF23. It is mainly synthesised in the distal tubule of the kidney and is considered an essential mediator of FGF23 receptor activity (Liu & Quarles, 2007).

Target organs for FGF23 activity included the kidney, parathyroid gland, choroid plexus, and possibly the pituitary gland (Li *et al.*, 2004). The kidneys are the principal target for FGF23, where it regulates production of $1,25(OH)_2D$ and reabsorption of calcium and phosphorus (Shimada *et al.*, 2001). In the proximal tubule of the kidney, FGF23 inhibits 1α-hydroxylase activity, decreases renal Na^+/Pi co-transporters, and increases 24-hydroxylase activity, resulting in decreased production of $1,25(OH)_2D$ and reduced serum concentration of $1,25(OH)_2D$ in addition to phosphaturia and hypophosphatemia (Figure 1.12) (Liu & Quarles, 2007; Hardcastle & Dittmer, 2015). Another target organ for FGF23 is the parathyroid gland, where FGF23 inhibits PTH secretion (Urakawa *et al.*, 2006; Hardcastle & Dittmer, 2015).

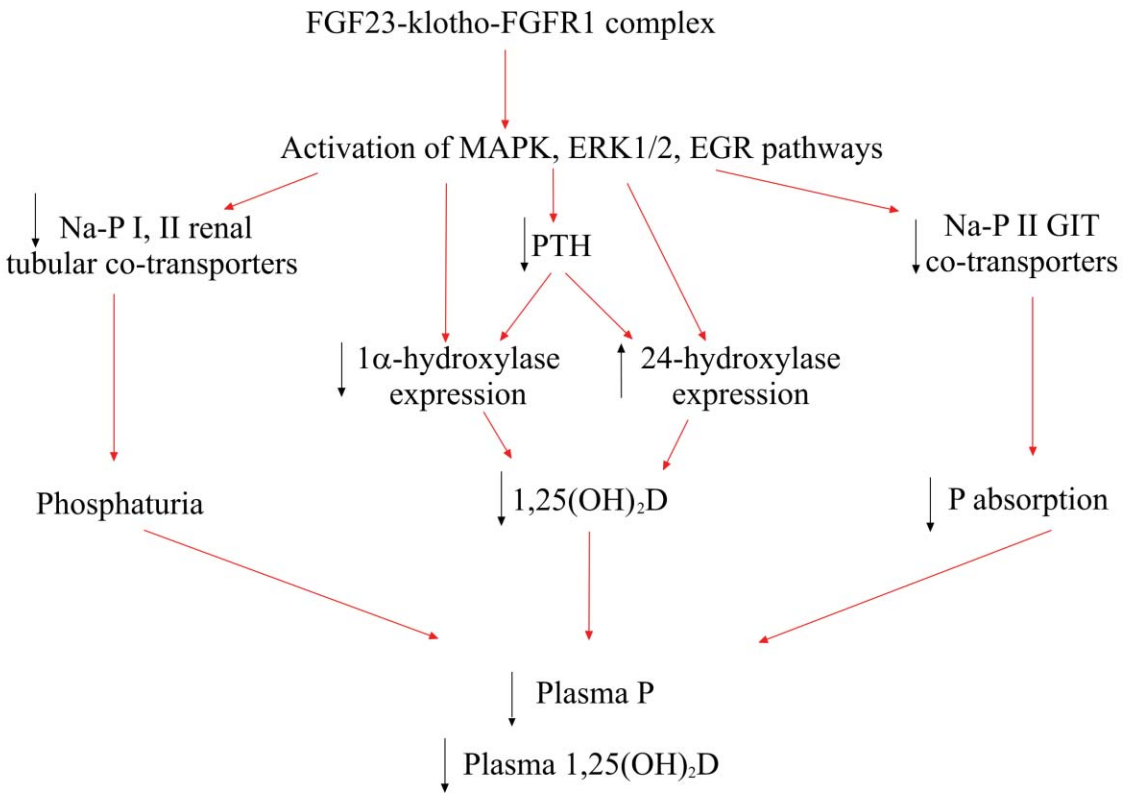


Figure 1.12 Functions of fibroblast growth factor 23 (FGF23) and FGF23 signalling pathways. The FGF23-klotho-fibroblast growth factor receptor 1 (FGFR1) complex activates the mitogen-activated protein kinase (MAPK) and extracellular signal-related kinase-1/2 (ERK1/2) and early growth response (EGR) signalling pathways. This results in a decrease in Na^+/Pi renal and gastrointestinal tract (GIT) co-transporters, leading to phosphaturia and decreased GIT phosphate (P) absorption, respectively. At the same time, plasma PTH concentrations decrease, leading to decreased expression of renal 1α-hydroxylase and increased expression of 24-hydroxylase, resulting in decreased plasma $1,25(OH)_2D$ concentrations. The overall result of activation of the FGF23 signalling pathway is a decrease in plasma phosphorus concentration and decreased plasma $1,25(OH)_2D$ concentration (Hardcastle & Dittmer, 2015).

1.7 Phosphorus metabolism

Phosphorus is a chemical element with the atomic number 15 and symbol P. Since phosphorus is highly reactive, it is never found as a free element on Earth. Approximately 1% of the human body weight is phosphorus; 85% is located in bone matrix as hydroxyapatite, 15% in blood and soft tissue as the major intracellular anion, and less than 0.1% in the extracellular fluid (Toribio, 2011). Phosphorus exists as

organic and inorganic forms in the circulation; organic phosphorus in the form of phospholipids represents 70% of phosphorus in the circulation, however, only the inorganic phosphorus (HPO_4^{2-} and $H_2PO_4^-$) is measurable by routine methods (Toribio, 2011).

Inorganic phosphorus plays essential roles in muscle contraction, neurologic function, enzyme activity, electrolyte transport, oxygen transport, gene transcription, and in the metabolism of proteins, carbohydrates and fats (Endres *et al.*, 2006). Compared with calcium, serum phosphorus shows a greater fluctuation in its concentration, depending on diet, age, physiologic status, activity, disease, glycaemic hormones, and also the quality of the sample (Toribio, 2011).

There are intracellular and extracellular pools of phosphorus in the body. The intracellular phosphorus concentration is relatively high and is electronegative, therefore when required, a rapid translocation through diffusion of phosphorus can occur between the two pools (Toribio, 2011). Serum phosphorus concentration is regulated by intestinal absorption and renal reabsorption, with the kidneys being the major regulator of the plasma concentration (Toribio, 2010). Most of the filtered phosphorus is reabsorbed in the proximal tubules of the kidney (Takeda *et al.*, 2000). Transepithelial phosphorus transport is effectively unidirectional and consists of three steps: i. uptake at the brush border membrane of the renal tubule cell, ii. translocation across the cell, and iii. efflux at the basolateral membrane. The brush border membrane is the major site of phosphorus regulation, where transport is mediated by

sodium-dependent phosphate (Na^+/Pi) co-transporters (Figure 1.12) (Takeda *et al.*, 2000).

There are three different families of Na^+/Pi co-transporters in body including Na^+/Pi-I (SLC17), Na^+/Pi-II (SLC34), and Na^+/Pi-III (SLC20 or PiT-2) (Werner *et al.*, 1998). The Na^+/Pi-II (SLC34) family contains three members, Na^+/Pi -lla (SLC34A1), Na^+/Pi-llb (SLC34A2) and Na^+/Pi-llc (SLC34A3) (Murer *et al.*, 2004). The regulation of proximal reabsorption of phosphorus in the kidney is controlled by Na^+/Pi-IIa (SLC34A1), Na^+/Pi-IIc (SLC34A3), and the SLC20 family (SLC20A2 or PiT-2) (Biber *et al.*, 1996; Biber *et al.*, 2013).

SLC34A1, SLC34A3 and PiT-2 are located at the apical membrane of the proximal tubule. Na^+/Pi-IIc and PiT-2 are expressed mainly in the S1 segment whereas the amount of Na^+/Pi-IIa gradually reduces along the proximal tubule (Biber *et al.*, 2013). Uptake of phosphorus at the brush border membrane of the proximal tubular cells depends on the presence or absence of Na^+ (Biber *et al.*, 2009). Na^+/Pi-llb (SLC34A2) is not present in renal tissue but is expressed in a number of epithelial tissues such as small intestine, lung, mammary glands, testis, and liver (Murer *et al.*, 2004). In the small intestine, Na^+/Pi-llb (SLC34A2) is responsible for dietary phosphorus absorption (Marks *et al.*, 2010).

Different hormonal and non-hormonal factors (e.g. diet) influence the reabsorption of phosphorus through the Na^+/Pi co-transporters (Berndt & Kumar, 2009). The major hormones controlling phosphate homeostasis are FGF23 and PTH. Increased plasma phosphorus concentration stimulates FGF23 secretion from bone,

resulting in decreased renal phosphorus reabsorption by the Na^+/Pi co-transporters (Figure 1.13) (Quarles, 2008). FGF23 has a phosphaturic action and decreases the apical expression of Na^+/Pi-IIa, Na^+/Pi-IIc, and PiT-2 (Martin *et al.*, 2012). FGF23 activates the mitogen-activated protein kinase (MAPK) pathway through the FGFR1 receptor and klotho complex (Kato *et al.*, 2000; Yamashita *et al.*, 2002; Gattineni *et al.*, 2009). As mentioned earlier, FGF23 also decreases renal production of $1,25(OH)_2D$ by inhibiting 1α-hydroxylase, leading to decreased intestinal phosphorus absorption and, ultimately normalising serum phosphorus concentration (Peacock, 2010).

Klotho has two forms: i. the membrane bound form that is expressed in the distal and also proximal renal tubules, and regulates the action of FGF23 on phosphorus reabsorption through the renal tubules (Hu *et al.*, 2010; Martin *et al.*, 2012), and ii. the soluble form that is present in blood, urine and the proximal renal tubule lumen (Hu et al., 2010). It is suggested that klotho also has a phosphaturic action that is independent of FGF23 which may happen via modification of Na^+/Pi-IIa glycosylation (Hu *et al.*, 2010).

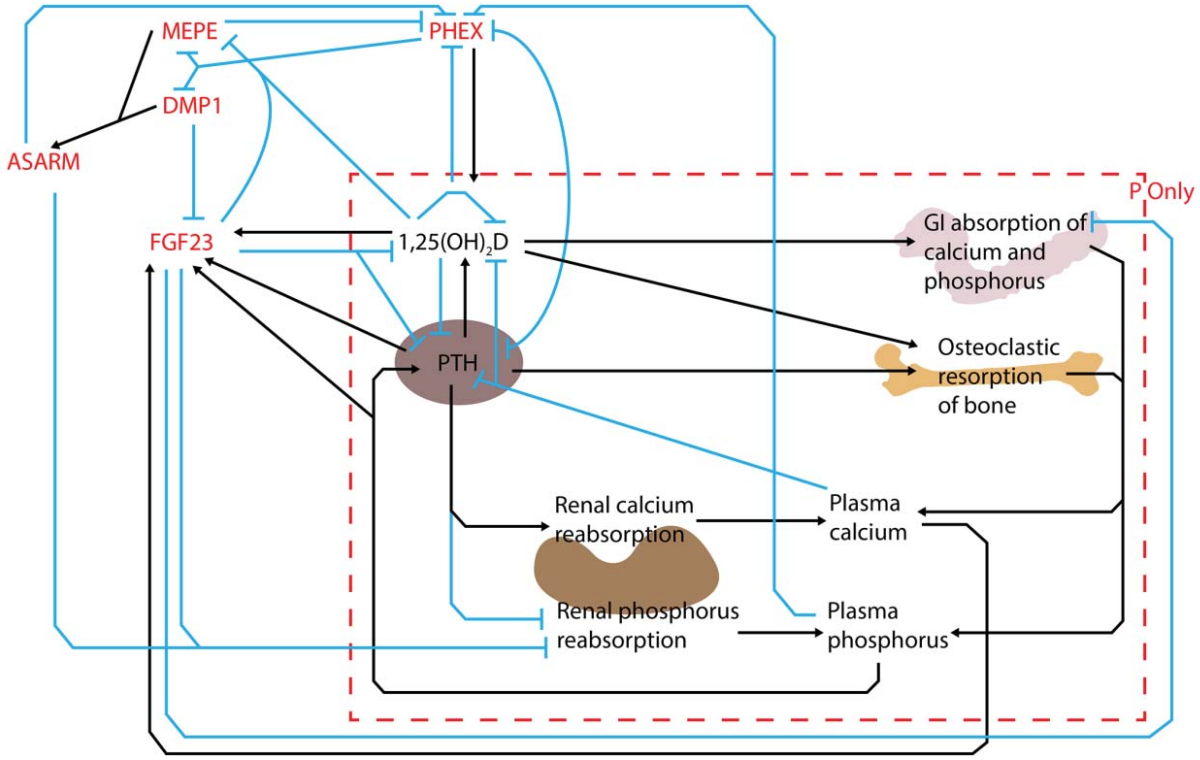


Figure 1.13 Summary of the main players in phosphorus (P) metabolism. Minor regulatory pathways and hormones or factors peripheral to calcium-phosphorus control are not depicted (arrow ¼ upregulates/increases, bar ¼ downregulates/decreases). The central player in phosphorus metabolism is fibroblast growth factor 23 (FGF23), and it inhibits renal phosphorus reabsorption, decreases active vitamin D ($1,25(OH)_2D$) production, and inhibits production of parathyroid hormone (PTH) by the parathyroid gland. Plasma FGF23 concentrations are increased by $1,25(OH)_2D$, PTH, and long-term hypercalcemia and hyperphosphatemia, and are regulated by other components of the phosphatonin pathways including matrix extracellular phosphoglycoprotein (MEPE), dentin matrix protein 1 (DMP1), phosphate-regulating gene with homology to endopeptidases on the X chromosome (PHEX), and acidic serine–aspartate-rich MEPE associated motif (ASARM). GI, gastrointestinal. (Hardcastle & Dittmer, 2015).

PTH also regulates the renal Na^+/Pi co-transporter. Increases in PTH lead to decreased renal phosphorus reabsorption and plasma phosphorus concentration (Figure 1.14) (Kronenberg, 2002). FGF23 acts directly on the parathyroid glands leading to decreased PTH secretion (Ben-Dov *et al.*, 2007). Although the actions of FGF23 and PTH on the renal Na^+/Pi co-transporters result in phosphaturia, they have antagonistic effects on $1,25(OH)_2D$ metabolism where FGF23 inhibits the 1α-hydroxylase enzyme while PTH stimulates it (Peacock, 2010).

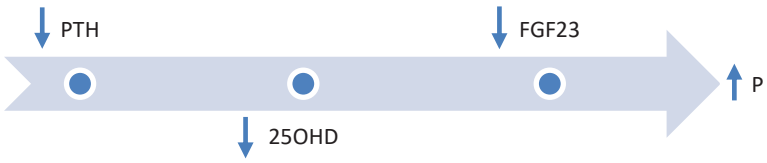


Figure 1.14 Phosphorus concentration. The effect of PTH and fibroblast growth factor 23 (FGF23) on $1,25(OH)_2D$ secretion and serum phosphorus concentration.

1.8 Magnesium metabolism

Magnesium is a chemical element with the atomic number 12 and symbol Mg. It is the most abundant intracellular ion in plants, the second most common divalent ion in the ocean, the third most common on land, and the fourth most abundant mineral in the body (Wester, 1987; Rude, 1998). In the mammalian body, magnesium is the fourth most abundant divalent cation and the second most prevalent intracellular cation after potassium (Stewart, 2011). Magnesium has been referred to as 'the forgotten cation' because its importance was recognised much later than that of calcium (Salem *et al.*, 1991).

Around 55-60% of total body magnesium is found in bone, 38-43% in soft tissues (intracellular) and 1-2% in extracellular fluid (Rude, 1998; Stewart, 2011). Only 1% of total body magnesium is found in the blood (Stewart, 2011). To maintain the extracellular concentration of magnesium, 30% of the bone magnesium is readily exchangeable and functions as a reservoir. The remaining 70% of bone magnesium has a structural function and is found in the hydroxyapatite lattice (Rude, 1998; Stewart, 2011).

Plasma magnesium is divided into three fractions: i. free or ionised magnesium (55%), ii. protein-bound magnesium (30%) and iii. magnesium complexed to anions (15%). Ionised magnesium is the biologically active form (Altura, 1994).

The physiological role of magnesium is related to its importance in the support of enzyme activity. Magnesium is an essential co-factor for more than 300 biochemical reactions in the body (Stewart, 2011). All enzymes utilizing ATP require magnesium for substrate formation, including critical enzyme systems such as adenylate cyclase, phospholipase C, Na^+/K^+-ATPase, and regulation of calcium channel activity (Ca^{2+}-ATPase) (Dorup & Clausen, 1993). Classically, magnesium is considered nature's physiologic calcium blocker and when depleted, intracellular calcium (Ca^{2+}) and sodium (Na^+) increase while potassium (K^+) decreases (Rude, 1998; Shechter et al., 2000).

Magnesium has important effects on bone and mineral metabolism, the neuromuscular system and the cardiovascular system, through its ability to influence movement of ions such as sodium, potassium and calcium across the plasma membrane (Rude, 1998; Noronha & Matuschak, 2002). The other key role of magnesium is participating in many important biological processes, for instance protein synthesis, cell replication, oxidative phosphorylation, glycolysis, DNA transcription, and cellular energy metabolism (Rude, 1998; Noronha & Matuschak, 2002).

Magnesium homeostasis is regulated in the gastrointestinal tract and the kidneys. In the intestine, magnesium is absorbed along the entire intestinal tract via an active magnesium transport system and/or passive absorption based on dietary intake

(Fine *et al.*, 1991; Kayne & Lee, 1993). The kidneys regulate magnesium metabolism via a filtered magnesium threshold, with any excess magnesium being excreted (Quamme & Dirks, 1986; Rude & Ryzen, 1986).

Magnesium efflux across the cell membrane occurs by Na^+-dependent and Na^+-independent pathways (Na^+/Mg^{2+} exchanger), while magnesium influx occurs by many different mechanisms including Mg^{2+}/anion co-transport, and counter-transport pathways utilising the electro-chemical gradient of Na^+ or other cations with transient receptor potential ion (TRPM) channels (TRPM6 and TRPM7) (Figure 1.15) (Schlingmann & Gudermann, 2005; Romai, 2007).

The principal sites of magnesium reabsorption in the kidneys are the proximal tubule with 5-15% of absorption occurring at this site and 70-80% of absorption occurring in the thick ascending limb of the loop of Henle (Quamme & Dirks, 1986). Different factors may affect renal magnesium excretion by decreasing magnesium reabsorption in the proximal tubules. For instance, depletion of phosphate significantly increases urinary magnesium excretion and hypercalcaemia results in increased urinary excretion of magnesium and calcium (Swaminathan, 2003).

Microfluorescent studies in mice showed that magnesium reabsorption is concentration and voltage-dependent. Increasing the transepithelial voltage gradient increases paracellular magnesium reabsorption (Derouffignac & Quamme, 1994; Dai *et al*., 2001). Peptide hormones such as PTH increase absorption of magnesium in the distal renal tubules by stimulating distal convoluted tubule cells via a cAMP-mediated process (Dai *et al.*, 2001).

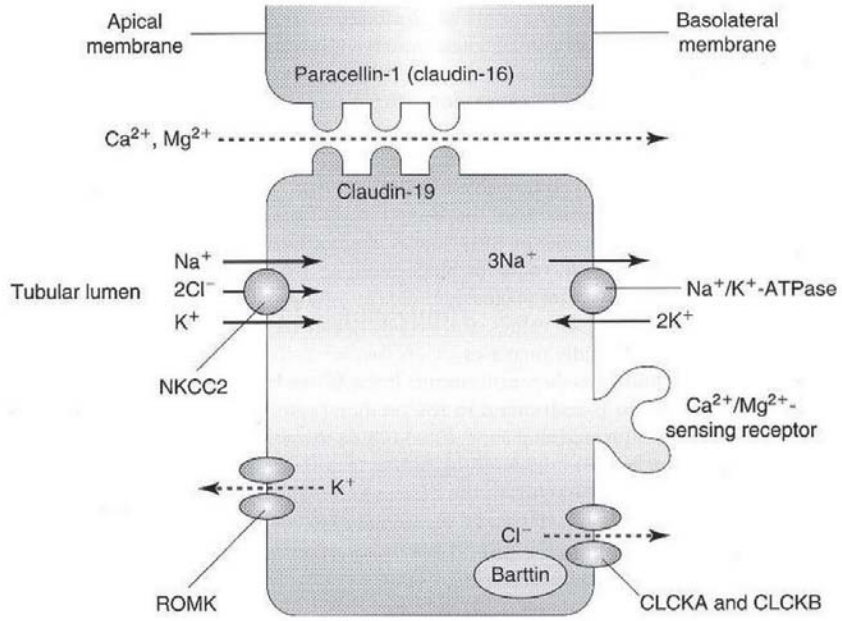


Figure 1.15 Magnesium reabsorption in the thick ascending limb of the loop of Henle. Absorption is conducted via the paracellular pathway which requires generation of a voltage gradient by movement of sodium from the lumen to the capillary side, as well as and presence in the paracellular space of specific magnesium channels. (Kaplinsky & Alon, 2013).

There is a significant functional link between calcitropic hormones and magnesium. Physiologically, serum calcium concentration regulates the secretion of PTH, but magnesium may also play a similar role in the body as the serum concentration of magnesium and PTH are related (Rude *et al.*, 1978). There is a complex relationship between PTH and magnesium, and although the secretion of PTH is controlled by plasma calcium concentration, magnesium has similar effect on PTH (Vetter & Lohse, 2002).

Very low plasma concentrations of magnesium induce a paradoxical block by activation of the alpha-subunits of heterotrimeric G-proteins, which mimic activation of the calcium sensing receptor and inhibit of PTH secretion, leading to hypocalcaemia

(Vetter & Lohse, 2002). The lack of PTH response affects renal reabsorption of calcium and magnesium, decreases bone resorption and reduces renal synthesis of $1,25(OH)_2D$ (Fatemi *et al.*, 1991). Magnesium influences the utilisation of vitamin D through its action on the renal 1 α-hydroxylase, whereby hypomagnesaemia can result in decreased production of $1,25(OH)_2D$ (Carpenter, 1988; Zofková & Kancheva, 1995).

1.9 Calcium and vitamin D metabolism in horses

Horses belong to the taxonomic family of *Equidae* which includes horses, zebras, donkeys and mules, all of which are in the genus *Equus*. The domestic horse (*Equus ferus caballus*) is one of two surviving subspecies of wild horse (*Equus ferus*) (Oakenfull *et al.*, 2000).

Horses are large land mammals notable for their speed, strength, endurance, and beauty. They are considered to be unique; as they differ from other mammalian species in many aspects, for instance they are monogastric, hindgut fermenters (Leblanc, 2013).

1.9.1 Equine calcium homeostasis

Horses are thought to have unique regulation of calcium balance and calcium homeostasis, although there is still limited information available in the literature.

The calcium proportions in equine blood are similar to those in other mammals (50-58% ionised, 40-50% bound to proteins and 5-10% complexed with anions). However, similar to human neonates, serum calcium concentrations are reported to be lower in neonatal foals (1-7 days) than in adult horses (Berlin & Aroch, 2009). In other

mammals, neonates typically have higher plasma calcium concentrations than adults (Kovacs, 2003).

The proximal small intestine, especially the duodenum, is considered to be the major site of transcellular calcium transport in horses (Sprekeler *et al.*, 2011). In the kidneys, transcellular calcium transport occurs in the distal convoluted tubules and connecting tubules (Hoenderop *et al.*, 2005; van de Graaf *et al.*, 2007). The fractional excretion of calcium in horses is high (5-10%) compared to other species, such as sheep and cattle (Toribio *et al.*, 2007).

TRPV6 is reported to be the predominant calcium channel in the small intestine and kidney of horses, while TRPV5 expression is higher in the duodenum than in other parts of the intestine and kidney (Rourke *et al.*, 2010). Calbindin-D_{9k} is the main calcium binding protein in the intestine (duodenum and proximal jejunum), while calbindin-D_{28k} is the main calcium binding protein in the kidneys (Rourke *et al.*, 2010). NCX1 is considered to have a uniform pattern of expression throughout the intestines and kidneys. PMCA1 is also present throughout the gastrointestinal tract but renal expression of PMCA1 is 50-fold higher in the kidney than intestine (Rourke *et al.*, 2010). Expression of the VDR is higher in the large colon than small intestine and kidneys (Rourke *et al.*, 2010). According to the expression pattern of genes in the equine intestine, TRPV6 and calbindin-D_{9k} are the major equine intestinal calcium transporters (Schryver *et al.*, 1970; Rourke *et al.*, 2010). The finding that TRPV6 has high renal expression in horses compared to TRPV5, in contrast to other species, could explain the low renal reabsorption/high renal excretion of calcium in horses (Toribio *et*

al., 2001; Mensenkamp *et al.*, 2007). The high expression of calbindin-D_{28k} in the equine kidney indicates that similar to other species, this calcium binding protein is more important than calbindin-D_{9k} in the kidney (Hoenderop *et al.*, 2005).

The similar expression pattern of NCX1 and PMCA1 in the equine intestine implies that they both have equal responsibility for movement of calcium from the epithelium to the blood, although the high renal expression of PMCA1 suggests that it is the primary transport mechanism in the equine kidney (Rourke *et al.*, 2010).

There is no information regarding the role of PTH and 1,25$(OH)_2$D in the regulation of calcium transporting genes in horses, although in other species it is well documented that 1,25$(OH)_2$D mediates transcellular calcium transport via the VDR by stimulating TRPV5, TRPV6, calbindin-D_{28k} and calbindin-D_{9k} (Hoenderop *et al.*, 2005). The finding that equine VDR expression is low in the small intestine (Rourke *et al.*, 2010) may explain the poor regulation of intestinal calcium absorption in horses compared to other species (Schryver *et al.*, 1970; Toribio, 2010). Another possible theory is that the low VDR results in low TRPV5 and TRPV6 expression in equine kidneys, resulting in low renal reabsorption of calcium by the horse. Low VDR expression may also explain why the serum concentration of vitamin D metabolites in horses is very low (Breidenbach *et al.*, 1998).

The synthesis of 1,25$(OH)_2$D and activation of 1α-hydroxylase are dependent on body calcium status (Lips, 2012). Horses absorb a larger proportion of their dietary calcium (up to 75%) (Schryver *et al.*, 1983) and have higher serum calcium concentrations than other species (Toribio, 2010). Renal 1α-hydroxylase expression is

low in horses, most likely because they already absorb a large amount of calcium from their diet (Schryver *et al.*, 1970; Breidenbach *et al.*, 1998).

Horses are reliant on their diet to fulfil their calcium requirements (Toribio, 2010; Sprekeler *et al.*, 2011). The small intestine is the major site of active calcium absorption in horses with absorption of approximately 90% of total dietary calcium; less than 10% of calcium absorption takes place in the large intestine (Schryver *et al.*, 1970; Perez *et al.*, 2008; Rourke *et al*., 2010). It is suggested that paracellular calcium transport plays a more important role in horses compared to other species (Rourke *et al.*, 2010). The high intestinal calcium absorption and low serum vitamin D concentration means that the parathyroid glands maybe less sensitive to extracellular calcium than in other species (Toribio *et al.*, 2003).

Compared to other mammals, horses have a number of unique features in their calcium homeostasis (Table 1.2), including high serum total and ionised calcium concentration (Toribio, 2010), low serum 25OHD and 1,25$(OH)_2$D concentrations (Breidenbach *et al.*, 1998), high intestinal calcium absorption (Schryver *et al.*, 1970; Rourke *et al.*, 2010), high urinary calcium excretion, decreased renal reabsorption of calcium (Toribio *et al.*, 2007), and decreased parathyroid gland sensitivity to calcium (Toribio *et al.*, 2003).

Table 1.2 Serum biochemical reference range - Normal serum concentration of ionised calcium (iCa) (mmol/L), calcium (tCa) (mmol/L), phosphorus (P) (mmol/L), and magnesium (Mg) (mmol/L) in horse, cattle, sheep, pig, goats, dog, cat, rabbit, and llama (Latimer, 2011; Kaneko *et al.*, 2008).

Species	iCa (mmol/L)	Ca (mmol/L)	PO_4 (mmol/L)	Mg (mmol/L)
Horse	1.58 – 1.68	2.5 – 3.3	0.5 – 1.5	0.6 – 0.9
Cattle	1.2 -1.6	2.0 – 2.8	1.08 – 2.6	0.6 – 1.2
Sheep	1.4 – 1.6	2.88 – 3.2	1.62 – 2.36	0.9 – 1.31
Pig	0.9 – 1.4	1.78 – 2.90	1.71 – 3.10	1.11 – 1.52
Goats	1.12 – 1.32	2.23 – 2.93	1.4 – 2.9	0.31 – 1.48
Dog	1.26 – 1.39	2.3 – 2.9	0.9 – 1.7	0.7 – 1.0
Cat	1.25 – 1.47	2.2 – 2.9	1.0 – 2.0	0.7 – 1.1
Rabbit	1.13 – 1.71	2.7 – 3.5	1.3 – 2.1	0.8 – 1.2
Llama	N/A	2.2 – 2.58	1.0 – 3.49	0.75 – 1.55

1.9.1.1 Calcium disorders in horses

Disorders of the regulation of calcium in the body result in either hypocalcaemia or hypercalcaemia. These disorders may be acute or chronic and may also be associated with phosphorus and magnesium imbalances (Toribio, 2011). In horses, disorders including hypoparathyroidism, primary hyperparathyroidism, nutritional secondary hyperparathyroidism, renal failure, vitamin D toxicity, exercise-induced hypocalcaemia, and sepsis have been associated with abnormal calcium concentrations (Toribio, 2010).

Table 1.3 Causes of hypocalcaemia and hypercalcaemia in horses (Toribio 2011)

Hypocalcaemia	Hypercalcaemia
Colic	Primary hyperparathyroidism
Sepsis and endotoxemia	Secondary hyperparathyroidism
Endurance exercise (Exercise-induced hypocalcaemia)	Chronic renal failure
Lactation and transport	Hypervitaminosis D
Acute renal failure	Hypercalcaemia of malignancy
Rhabdomyolysis	Idiopathic systemic granulomatous disease
Pancreatitis	Calcinosis
Hypoparathyroidism	Neonatal hypercalcemia and asphyxia
Hypomagnesaemia	
Hypocalcaemia tetany/seizures	
Ileus	
Oxalate toxicity	

1.9.2 Equine phosphorus homeostasis

In horses, phosphorus absorption occurs in both the small and large intestines, with absorption ranging from 30 to 55% of dietary phosphorus ingested (Schryver *et al.*, 1972; Schryver, 1975). The proximal tubules are considered the main site for renal reabsorption of phosphorus (approximately 80%) (Toribio, 2010) and interestingly compared to other ions, the urinary fractional excretion of phosphorus is low in horses (less than 0.5%) (Toribio, 2011).

Hormones controlling phosphorus homeostasis include vitamin D ($1,25(OH)_2D$), PTH and phosphatonins (Berndt & Kumar, 2009; Toribio, 2011). The phosphatonins are a family of substances including FGF23 and secreted frizzled related protein-4 (sFRP-4), which directly inhibit renal phosphorus reabsorption in the proximal tubule and

indirectly inhibit the synthesis of 1,25$(OH)_2$D, resulting in reduction of the intestinal absorption of phosphorus (Berndt et al., 2005; Berndt & Kumar, 2009).

1.9.2.1 Phosphorus disorders in horses

Diseases that cause hypophosphataemia and hyperphosphataemia in the horse are closely associated with disorders of the calcium and magnesium metabolism (Toribio, 2011).

Table 1.4 Causes of hypophosphatemia and hyperphosphatemia in horses (Toribio 2011)

Hypophosphataemia	Hyperphosphataemia
Renal failure	Nutritional secondary hyperparathyroidism
Malignancies secreting phosphatonins or PTHrP (HHM)	Acute renal failure
Hyperparathyroidism	Massive tissue necrosis
Parenteral nutrition	Rhabdomyolysis
Hyperinsulinism (Hyperglycaemia)	Haemolysis
Severe sepsis	Intestinal infarction
Malnourishment	Hypervitaminosis D
Alkalosis	Hypoparathyroidism

1.9.3 Equine magnesium homeostasis

Magnesium absorption in horses takes place in the proximal (25%) and distal small intestine (35%), and the caecum and colon (5%) (Hintz & Schryver, 1972). Intestinal absorption of magnesium and the amount of magnesium in the diet are directly correlated (Hintz & Schryver, 1973). Horses have higher average absorption of magnesium from feed, compared with ruminants (Rook, 1969).

The regulation and maintenance of serum magnesium concentration in horses is balanced by renal reabsorption and/or excretion of magnesium in the kidneys, which is directly influenced by dietary magnesium intake (Stewart, 2011). Renal glomeruli

filter approximately 70% of circulating magnesium, mainly as ionised and anion bound magnesium, and 70-90% of this magnesium is reabsorbed in the various segments of the nephron (Stewart, 2011). The thick ascending limb of the loop of Henle is the main site for magnesium reabsorption (70-80%), followed by the proximal tubule (5-15%) (Quamme & Dirks, 1983). The distal convoluted tubule is mainly responsible for determining the amount of magnesium excreted in the urine (Stewart, 2011). Horses obtain their magnesium requirements from their diet and different factors such as growing, lactating and exercising status, result in increased requirements of magnesium (Stewart, 2011).

1.9.3.1 Magnesium disorders in horses

Disorders of magnesium most commonly lead to hypomagnesaemia. Hypermagnesaemia is regarded as rare and usually only induced experimentally (Stewart, 2011). Any magnesium disorders can be linked to disorders of other electrolytes, including sodium and calcium (Stewart, 2011).

Table 1.5 Causes of hypomagnesaemia and hypermagnesaemia in horses (Stewart 2011)

Hypomagnesaemia	Hypermagnesaemia
Inflammation	Iatrogenic Mg overdose
Colic	Excessive supplementation to a patient with renal failure
Endotoxemia	Severe cellular damage
Gastrointestinal disease	Rhabdomyolysis
Infectious respiratory disease	Tumour lysis syndrome
Multiorgan disease	Haemolysis
Synchronous diaphragmatic flutter	Severe sepsis
Sepsis	

1.10 Equine metabolic bone disease

Metabolic bone diseases are systemic bone disorders of various aetiologies including dietary/nutritional, hormonal and toxic causes (Weisbrode, 2012). Rickets, osteomalacia, osteoporosis and fibrous osteodystrophy are among the well-known metabolic bone diseases.

1.10.1 Rickets

Rickets is a disorder of growth plate mineralisation; therefore it is unique to adolescents and young animals prior to skeletal maturity. Rickets is caused by vitamin D deficiency and/or phosphorus deficiency (Weisbrode, 2012; Craig *et al.*, 2015). Horses are regarded as being relatively resistant to phosphorus deficiency, and the most common nutritional imbalance is excessive phosphorus rather than phosphorus deficiency (Shore & Chesney, 2013).

Regardless of low serum vitamin D concentrations, horses seem to be relatively resistant to developing rickets. El Shorafa *et al.* (1979) experimentally created rickets by depriving ponies of dietary vitamin D and sunlight. They studied 12 Shetland ponies through putting them in three different groups, one group being deprived of dietary vitamin D and sunlight, a second group going without sunlight but receiving 1000 IU supplemental vitamin D per day, and a third group kept outdoors without supplemental vitamin D. The results suggested that horses require dietary vitamin D for normal bone development, but that with adequate sunlight dietary vitamin D is not needed (El Shorafa *et al.*, 1979). However, other authors have made different

conclusions about rickets in horses, including that true rickets in horses may not occur (Nieberle & Cohrs, 1954), or that rickets occurs less frequently in horses than in other species (Park, 1923). There has been huge debate among scientists as to whether rickets truly occurs in horses.

1.10.2 Osteoporosis

Osteoporosis is a chronic condition that occurs when there is an imbalance between bone formation and resorption, leading to a reduction in bone mass/mineral density, resulting in fragile bones and increased likelihood of fracture (Ni Chroinin *et al.*, 2013). Osteoporosis is part of the aging process in all mammals, although some disorders such as nutritional deficiencies (calcium, phosphorus or copper deficiency), severe gastrointestinal parasitism, corticosteroids, oestrogen deficiency, and physiological conditions can accelerate this process and lead to osteoporosis in younger animals (Weisbrode, 2012; Craig *et al.*, 2015).

Osteoporosis most severely affects trabecular bone and is most obvious in the metaphyses and epiphyses of long bones and the vertebrae. Osteoporosis is accompanied by reduction in soft tissue mass and muscular power which results in bones being more prone to fracture (Craig *et al.*, 2015). A bone fragility disorder has been diagnosed in California horses which develop osteoporosis, silicosis, and pathologic fractures. Radiographic findings revealed that all affected horses had osteopenia and histologic evidence of osteoporosis characterised by osteopenia, numerous resorption cavities, cement lines, and a mosaic lamellar pattern indicative of

multiple remodelling events (Arens *et al.*, 2011). It was found that horses with this bone fragility disorder had systemic osteoporosis accompanied by fibrosing pulmonary silicosis, however, the aetiopathogenesis of the bone fragility has not yet been determined (Arens *et al.*, 2011).

1.10.3 Fibrous osteodystrophy

Fibrous osteodystrophy (*osteodystrophia fibrosa*) is the most common metabolic bone disease of horses and is caused by prolonged and excessive secretion of PTH (hyperparathyroidism). Any nutritional deficiency that results in an imbalanced Ca:P ratio (reduced calcium and increased phosphorus concentration) can result in secondary hyperparathyroidism (Craig *et al.*, 2015).

Secondary hyperparathyroidism historically occurs in horses and other equidae on diets consisting largely of corn and cereal grains, such as wheat bran (low in calcium and high in phosphorus), leading to what is known as bran disease, big head or miller's disease (Aslani *et al.*, 2001). The approximate ratio of calcium to phosphorus in the diet of horses should be 1:1 and if the ratio is 1:3 or higher fibrous osteodystrophy results (Secombe & Lester, 2012; Craig *et al.*, 2015). Fibrous osteodystrophy is characterised by marked bone resorption and replacement by fibrous tissue and poorly mineralised woven bone (Palmer, 1993). The mandible and maxilla are particularly affected, resulting in expansion of these bones and “big head” (Aslani *et al.*, 2001).

1.10.3.1 Oxalate toxicity

The major dietary source of oxalate in livestock and horses is forage plants, including South African pigeon grass (*Setaria sphacelata*), buffelgrass (*Cenchrus ciliaris*), pangola grass or elephant grass (*Digitaria decumbens*), kikuyu grass (*Pennisetum clandestinum*), and napier grass (*Pennisetum purpureum*). All these plants may contain toxic concentrations of oxalates (Rahman & Kawamura, 2011).

Oxalates are common constituents of plants and play several roles, such as calcium regulation, ion balance and osmoregulation (Libert & Franceschi, 1987). Oxalate accumulates in two forms: i. soluble oxalate that usually binds with sodium (Na^+), potassium (K^+) and ammonium ions (NH_4^+), and ii. insoluble oxalate that binds with calcium (Ca^{2+}), magnesium (Mg^{2+}) and iron (Fe^{2+}) (Savage & Dubois, 2000).

Dietary oxalate binds with calcium to form insoluble calcium oxalate, thus reducing calcium absorption from diet leading to chronic hypocalcaemia, excessive mineral mobilisation from bones, and negative calcium balance (Rahman & Kawamura, 2011; Toribio, 2011). Therefore, oxalate ingestion results in chronic calcium deficiency, nutritional secondary hyperparathyroidism, fibrous osteodystrophy (big head), and lameness (Swartzman *et al.*, 1978; McKenzie *et al.*, 1981; Secombe & Lester, 2012).

1.11 Aims of the thesis

As discussed above, there is limited knowledge on vitamin D metabolites ($25OHD_2$, $25OHD_3$ and $1,25(OH)_2D$) and their relation to the metabolism of other serum mineral ions such as Ca^{2+}, PO_4^{3-} and Mg^{2+}, and PTH in horses. The main aim of this study was to

investigate different aspects of vitamin D metabolism and calcium homeostasis in horses, in particular the ability of horses to synthesise vitamin D in their skin and its role in calcium metabolism.

The primary aims were to:

i. Assess whether horses that are blanketed for substantial periods of time would have lower serum concentrations of vitamin D than non-blanketed horses
ii. Investigate if serum vitamin D concentrations fluctuate seasonally and/or daily based on the amount of sunshine/UV light
iii. Determine the relationship between calcitropic hormones (25OHD, 1,25$(OH)_2$D and PTH) and Ca^{2+}, $PO_4^{3-}$and Mg^{2+} in horses
iv. Investigate if horses are able to synthesise vitamin D in their skin under the influence of UVB light
v. Determine the most stably expressed HKGs in the horse kidney, in order to use these for normalisation of gene expression in subsequent RT-qPCR experiments.
vi. Investigate which vitamin D-responsive genes are present in the equine kidney and compare them to those in sheep and dogs.

Chapter 2

Influence of blanketing and season on vitamin D and parathyroid hormone, calcium, phosphorus, and magnesium concentrations in horses in New Zealand

2.1 Introduction

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

Humans obtain most of their vitamin D from casual exposure to sunlight (Chen *et al.*, 2007). Solar UVB radiation of wavelength 290-315 nm results in synthesis of vitamin D in the human skin (Holick *et al.*, 1980; Maclaughlin *et al.*, 1982). Vitamin D may also be obtained through the diet (e.g. fatty fish, cod liver oil, and egg yolk), and/or dietary supplements (DeLuca, 2004; Holick, 2007). The production of vitamin D in the skin starts with the photolytic conversion of 7-DHC to previtamin D_3 under the influence of UVB photons from the 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 (vitamin D_2 and/or D_3) are transported by vitamin D-binding protein (DBP) to the liver where vitamin D is hydroxylated by 25-hydroxylases (CYP27A1 and CYP2R1) forming 25-hydroxyvitamin D_2 and D_3 (25OHD) (Deluca, 2004). This form of vitamin D is stable and is the main form of vitamin D in the circulation; its concentration commonly being used to determine an individual's vitamin D status (Dusso *et al.*, 2005; Radlovic *et al.*, 2012). When required, 25OHD is transported to the kidneys where it undergoes 1α-hydroxylation by the renal 25-hydroxyvitamin-D-1α-hydroxylase (CYP27B1), resulting in the production of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), the biologically active form of vitamin D (Holick, 2004a; Hewison *et al.*, 2007; Morris & Anderson, 2010).

The active form of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D_2$ and $1,25(OH)_2D_3$), 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 concentration within the range required for calcium-dependent physiological functions in the body. The calcium regulating functions of vitamin D include increased absorption of dietary calcium, increased mobilization of calcium from bone and increased reabsorption of calcium from the kidney (Bouillon *et al.*, 2008; Lips, 2012).

The other major calcitropic hormone is parathyroid hormone (PTH), whose secretion is regulated by serum calcium and vitamin D metabolite concentrations.

Decreased serum calcium concentration stimulates the parathyroid glands, increasing PTH secretion (Lips, 2012). PTH stimulates the production of $1,25(OH)_2D$ in the kidney and increases calcium mobilisation from bone (Bouillon *et al.*, 2008; Lips, 2012). PTH also results in increased reabsorption of calcium and increased excretion of phosphorus in the kidney (Kumar *et al.*, 2012). Active vitamin D, ($1,25(OH)_2D$), has a negative feedback on PTH secretion (Kumar & Thompson, 2011).

When compared to other domestic grazing animals, horses have several unique features with regard to calcium balance, including a high serum total and ionised calcium concentration (Toribio, 2004), poorly regulated intestinal absorption of calcium (Schryver *et al.*, 1970), high urinary excretion of calcium (Toribio *et al.*, 2001), low serum concentration of vitamin D metabolites (Maenpaa *et al.*, 1988a,b; Breidenbach *et al.*, 1998), and a high calcium setpoint with decreased parathyroid gland response to hypocalcaemia (Toribio *et al.*, 2003).

Many factors have an influence on the ability of the 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 the lower the percentage of ultraviolet (UV) radiation transmitted (Loomis, 1967). Sunscreen usage with a high sun protection factor (SPF8) has the same effect and prevents the penetration of ultraviolet B (UVB) 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 (Matsuoka *et al.*, 1992;

Salih, 2004), aging (Maclaughlin & Holick, 1985; Holick *et al.*, 1989), time of day and length of sunlight exposure (Holick, 1987).

The influence of clothing on vitamin D_3 synthesis in the skin is well documented in humans. The effect of UVB radiation on the photosynthesis of vitamin D_3 using different fabrics covering the skin (cotton, wool, and polyester) with different skin types (from racial pigmentation) has been investigated. Clothing significantly prevented the formation of vitamin D_3 in the skin in a study by Matsuoka *et al.* (1992). Similarly, 15 different types of fabrics were tested in another study for their effect on the efficiency of solar conversion of 7-DHC to vitamin D_3. The results showed that the number of threads per square inch of fabric had a direct effect on sunlight transmission and therefore the amount of vitamin D_3 production in skin (Salih, 2004). A recent study on Danish Holstein dairy cows showed that blanketed cows had lower serum 25-hydroxyvitamin D_3 ($25OHD_3$) concentration compared to non-blanketed cows, and this change occurred within 28 days (Hymoller & Jensen, 2010).

The effect of blanketing in horses has not been previously investigated. The majority of horses in New Zealand have dark skin and spend a large proportion of their time outside in paddocks wearing a blanket. This may adversely affect the ability of horses to synthesise vitamin D_3 in their skin. A thirteen-month study was conducted to investigate whether horses that are blanketed for substantial periods of time will have lower serum vitamin D concentrations than those that are not blanketed. In addition, we aimed to study the effect of season and UV exposure on vitamin D concentration, and to assess the normal baseline concentrations and the annual rhythm of calcitropic

hormones (vitamin D metabolites and PTH), calcium, phosphorus, and magnesium in New Zealand horses.

2.2 Materials and methods

2.2.1. Sample population

Twenty-one healthy adult horses (*Equus caballus*) (7 Standardbred and 14 Thoroughbred) with a mean age of 11.9 ± 4.7 year (Table 2.1) were included in the study. The horses were permanently kept in paddocks at the Veterinary Large Animal Teaching Unit (VLATU), Massey University, Palmerston North, New Zealand. Five of the horses were blanketed with standard combined horse rugs, including a neck rug (Shires StormBreaker, Shires Equestrian, 600 denier), for 13 months (Figure 2.1). The criteria for selecting the horses to wear blankets was based on their temperament and individual tolerance towards wearing a blankets for a long period of time. Blankets were removed at least weekly and the horses groomed while they were indoors. Horses were kept in three main herds and rotationally grazed in eight different paddocks during the study (Figure 2.2). All horses were fed *ad libitum* grass pasture, and hay when pasture availability was limited, and had free access to water at all times.

2.2.2. Sample collection and analysis

2.2.2.1. Serum

Blood samples were collected by jugular venipuncture monthly for 13 months (January 2013 - January 2014) at the same time of day and same time of month

throughout the study. The serum was separated by centrifugation at 3500 x g for 15 min within 4 h of collection, and transferred to 1.5 mL tubes for storage at -80°C until further analysis.

Serum 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. This PTH assay was originally intended for use in humans, but validated for use in horses. PTH assays were performed in duplicate and had an intraassay coefficient of variation (CV) of <6.2%, and interassay CV of <7.3%. Measured concentrations obtained for dilutional parallelism had CVs <11.1%. All the CVs mentioned in the text are for equine samples.

Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry (LC/MS) was used to measure $25OHD_3$ and $25OHD_2$ concentrations at Endolab, Christchurch (Lankes *et al.*, 2015). Samples were analysed in duplicate with intraassay CVs of less than 12.7%, and an interassay CV for $25OHD_3$ of 11.7% and 17.9% for $25OHD_2$. Measured concentrations obtained for dilutional parallelism had CVs < 11.8%. Serum $1,25(OH)_2D$ concentrations (both $1,25(OH)_2D_2$ and $1,25(OH)_2D_3$) were measured using the DiaSorin 1,25-dihydroxyvitamin D RIA kit (Stillwater, Minnesota, USA) as per manufacturer's instructions at the Nutrition laboratory, Massey University, Palmerston North, New Zealand. Samples were analysed in duplicate and intraassay and interassay CVs were < 10%, measured concentrations obtained for dilutional parallelism had CVs < 10%.

Serum tCa, P and tMg 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). Serum iCa was measured within 4 h of collection using a Radiometer ABL800 FLEX analyser (Radiometer Medical ApS, Denmark) at MedLab Central, Palmerston North Hospital, New Zealand.

Table 2.1 List of horses in the study with breed, gender, age in years, colour and blanket status.

ID	Breed	Gender	Age	Colour	Blanket
1	Thoroughbred	Mare	18	Bay	Blanketed
2	Standardbred	Mare	25	Bay	Blanketed
3	Standardbred	Mare	8	Black	Blanketed
4	Standardbred	Mare	12	Bay	Blanketed
5	Thoroughbred	Mare	11	Bay	Blanketed
6	Standardbred	Mare	16	Bay	Non-blanketed
7	Standardbred	Mare	7	Bay	Non-blanketed
8	Standardbred	Mare	11	Bay	Non-blanketed
9	Thoroughbred	Mare	9	Bay	Non-blanketed
10	Standardbred	Gelding	9	Bay	Non-blanketed
11	Thoroughbred	Mare	14	Bay	Non-blanketed
12	Standardbred	Mare	7	Bay	Non-blanketed
13	Standardbred	Gelding	9	Bay	Non-blanketed
14	Standardbred	Gelding	8	Brown	Non-blanketed
15	Standardbred	Mare	13	Bay	Non-blanketed
16	Standardbred	Mare	6	Bay	Non-blanketed
17	Standardbred	Gelding	14	Bay	Non-blanketed
18	Thoroughbred	Mare	19	Chestnut	Non-blanketed
19	Thoroughbred	Mare	18	Chestnut	Non-blanketed
20	Thoroughbred	Mare	13	Bay	Non-blanketed
21	Standardbred	Mare	9	Bay	Non-blanketed

Figure 2.1 Blanketed horses. Horses blanketed with standard combined horse blankets, including a neck rug.

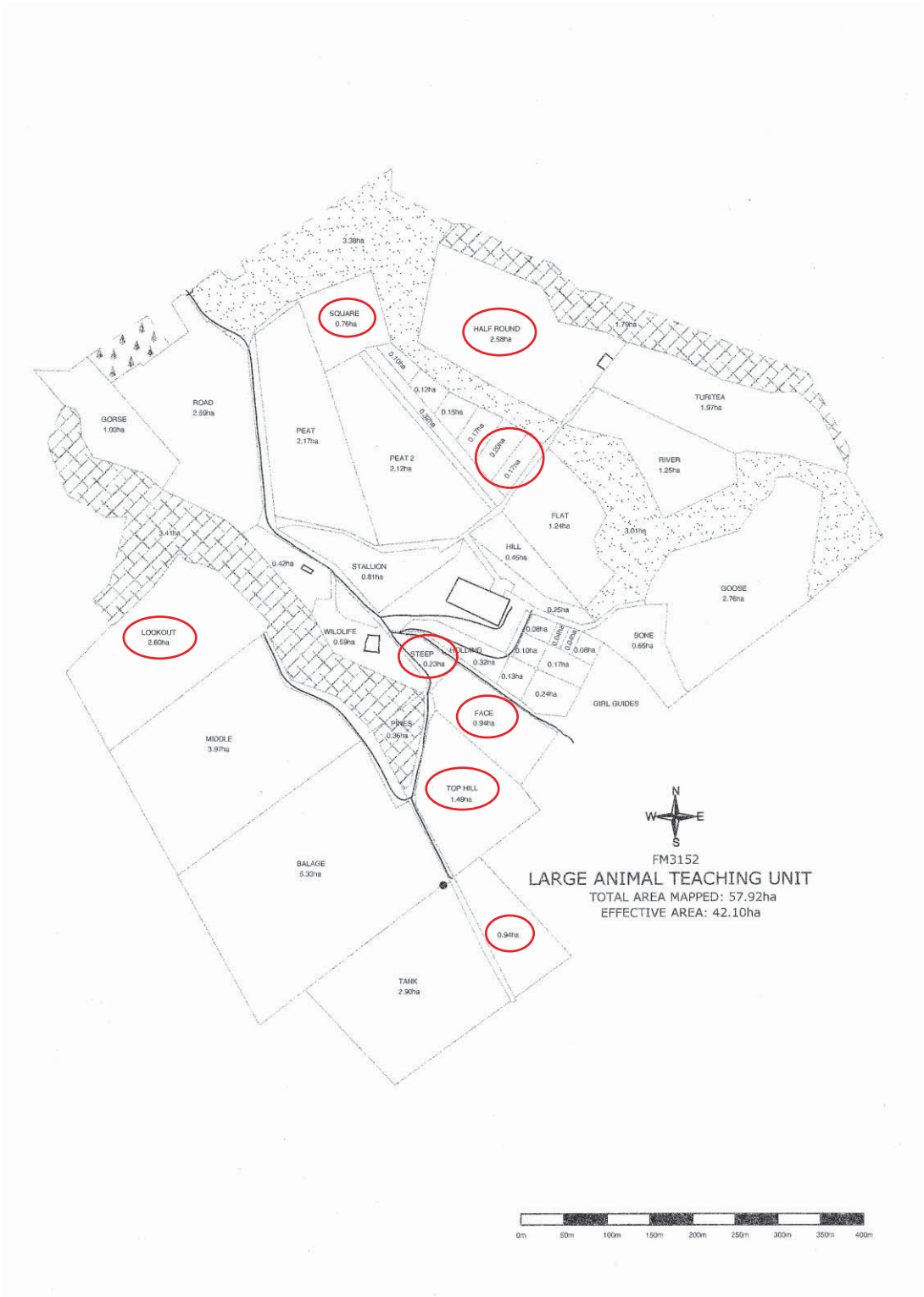


Figure 2.2 Map of Veterinary Large Animal Teaching Unit (VLATU), Massey University, Palmerston North, New Zealand. Horses were kept in three main herds and rotationally grazed in eight different paddocks (marked with red oval shapes) during the study.

2.2.2.2. Pasture

Pasture (grass and hay) samples were collected before 12 noon, at the same time of month on the day prior to blood sample collection, from paddocks the horses were kept in during the month prior, in order to determine the Ca, P, Mg and vitamin D content of the herbage consumed by the horses. Horses are selective grazers, and actively avoid areas where they have defecated; therefore samples were collected from areas where horses were actively grazing (i.e. short 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 line 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 0.5 kg fresh weight of pasture from each paddock.

Pasture samples from different paddocks were mixed, freeze-dried and ground to pass through a 1 mm screen. The same procedure was performed on hay samples. The ground sample was analysed for calcium, phosphorus and magnesium using Plasma Emission Spectrometry subcontracted method, and for vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) using AOAC 982.29, modified and

AOAC 2002.05 method at the Nutrition laboratory, Massey University, Palmerston North, New Zealand [16]. Samples were run in duplicate and had an intraassay and interassay CV of <10% for vitamin D_2 and <12% for vitamin D_3.

2.2.2.3. Climate data

The daily sunshine hours and amount of UV radiation (January 2013 – January 2014) was obtained from the Palmerston North climate station at Palmerston North airport operated by the National Institute of Water and Atmospheric Research (NIWA/Taihoro Nukurangi), New Zealand. The daily sunshine hours refer to the visible bright part of sunlight which is not blocked by clouds and UVB light/radiation refers to the invisible part of sunlight

2.2.3. Statistical analysis

Results from the serum iCa, tCa, P, tMg, PTH, and vitamin D metabolite ($25OHD_2$, $25OHD_3$, $1,25(OH)_2D$) analyses are expressed in absolute concentrations. It is conceivable that analytes concentrations may be influenced by age, sex, breed, and time of year, thus to account for this we included these as covariates in our model, alongside a variable for whether horses were blanketed or not. We modelled the analytes concentrations Y_{*ijt*} for horse *i* from group *j* (blanketed/non-blanketed) at time t using a generalized additive mixed model,

$$Y_{ijt} = \beta_C Cover_i + \beta_A Age_{ij} + \beta_B Breed_{ij} + \beta_G Gender_{ij} + s(t) + Horse_j + \varepsilon_{ijt} \quad (1)$$

where $\beta_C, \beta_A, \beta_B$ and β_G are coefficients for blanket, age, breed and gender, $s(t)$ is a smooth trend through time, $Horse_j$ are random effects per horse and ε_{ijt} are

residuals which we assume are normally distributed and correlated in time. We may then assess whether there is a difference between blanketed and non-blanketed horses by testing whether $\beta_C = 0$.

To investigate the relationship between analytes, environmental effects and food, the equation above was extended to include terms for the average monthly UV radiation and sunshine; the concentration of Ca, P, Mg and vitamin D in feed (pasture and hay); and serum concentrations of other analytes that may be directly related to the analyte of interest.

All statistical models and plots were produced in R (R Development Core Team, 2014). Generalized Additive Mixed Models were fitted using the mgcv package (Wood, 2011). For all statistical comparisons a value of $P<0.05$ was considered statistically significant.

2.3 Results

The serum concentrations for each analyte of the whole study population were plotted per month as boxplots and a smooth trend with confidence intervals was overlaid using a generalized additive model. Spearman's pairwise correlation and scatterplots were used to study the correlation between different analytes.

2.3.1. Effects of blanketing, sunlight, UV radiation and diet on serum analytes

The data obtained for each serum analyte for blanketed and non-blanketed horses are presented in Figure 2.3 (Mean (± SE) serum concentrations of different analytes are presented in Appendix 1). No statistically significant differences were detected in

serum concentration of iCa, tCa, P, tMg, PTH, $25OHD_3$, $25OHD_2$ and $1,25(OH)_2D$ between blanketed and non-blanketed horses after adjusting for blanket, age, breed, gender and month, using the generalized additive mixed model described in Equation 1 above. Serum concentrations of $25OHD_3$ for all horses, both blanketed and non-blanketed, were below the detection limits of the assay (78%), with the remainder being close to the limit of detection, so with little variability, statistical analysis was not performed. The data gathered from pasture/hay analytes including Ca, P, Mg and vitamin D through the study are presented in Table 2.2.

The mean monthly sunshine hours and UV radiation (MJ/m^2) recorded in Palmerston North over the 13 months of the study are shown in Table 3. Climate data, as would be expected, showed that the amount of UV radiation was higher during spring (September-November) and summer (December-February) than in autumn (March-May) and winter (June-August) (Table 2.3).

Table 2.2 Food analysis. Concentration of calcium (%), phosphorus (%), magnesium (%) and vitamin D (ng/g) on the dry matter basis in the pasture and hay consumed by the 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 2.3 Climate data. Climate variables of mean (± SE) monthly sunshine (h) 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)

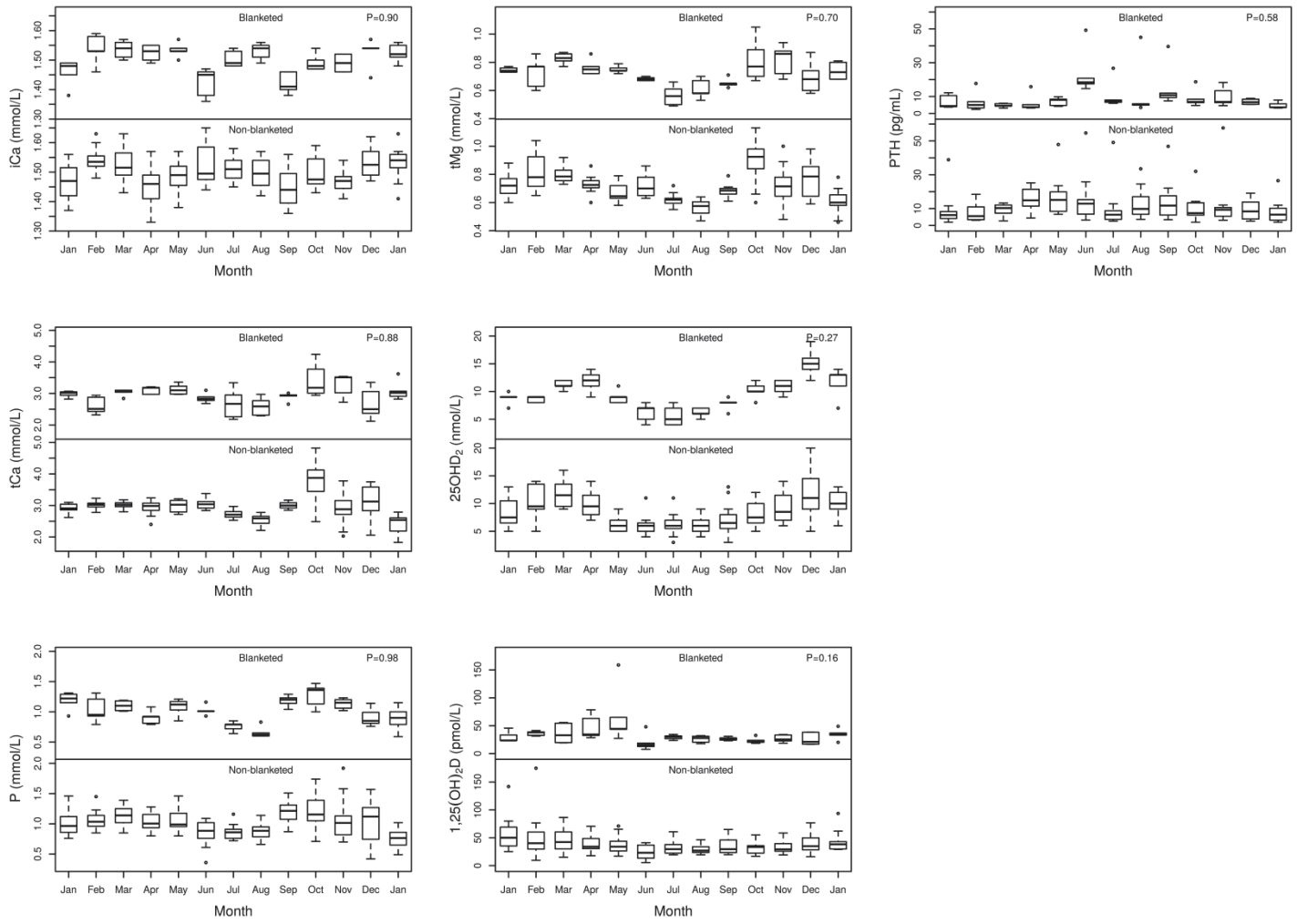


Figure 2.3 Serum analyte concentrations from blanketed and non-blanketed horses. Variation in serum ionised calcium (iCa), total calcium (tCa), 25-hydroxyvitamin D_2 ($25OHD_2$), total magnesium (tMg), phosphorus (P), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) and parathyroid hormone (PTH) from January 2013 to January 2014 in blanketed (5) and non-blanketed (16) adult horses in New Zealand. Data are shown using boxplots (box from quartiles 1 to 3, median in bold, individual points indicating potential outliers). P-values are for differences between blanketed and non-blanketed groups after adjusting for months.

2.3.2. Trend of serum analytes through the year

As there were no statistically significant differences between blanketed and non-blanketed groups, the full data were grouped and analysed to determine trends in the measured serum analytes across the year. The results were plotted to illustrate the shape and rhythm of each analyte through the year and are presented in Figure 2.4. All analytes showed significant differences between months (P<0.05).

The concentration of $25OHD_2$ in serum was higher in spring and summer (November-March) than in autumn and winter (April-October). Similarly the lowest concentration of $1,25(OH)_2D$ was measured during winter (July-September) and the concentration of $1,25(OH)_2D$ was higher during spring and summer (November-March).

Seasonal changes in iCa concentration were more distinct than for the other analytes. The highest concentrations of iCa were measured in February, July, December and January whereas the lowest concentrations were measured in April and September. The trends of tCa, tMg and to some extent P were similar (Figure 2.4). PTH and $1,25(OH)_2D$ showed opposing trends, where PTH had a trough in summer, while $1,25(OH)_2D$ peaked at the same time of year.

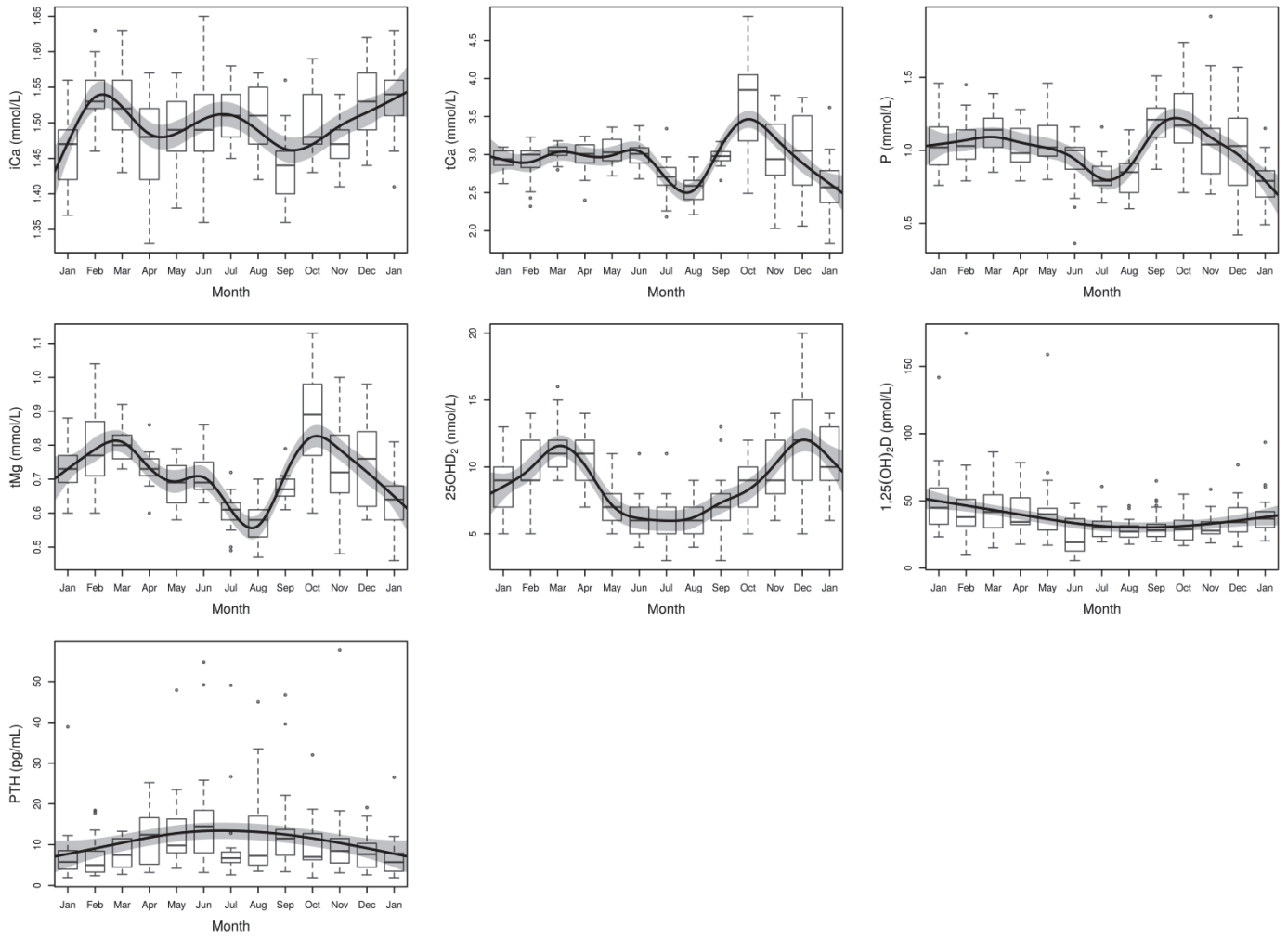


Figure 2.4 Rhythm of serum analytes through the year. Variation in serum ionised calcium (iCa), total calcium (tCa), 25-hydroxyvitamin D_2 ($25OHD_2$), total magnesium (tMg), phosphorus (P), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) and parathyroid hormone (PTH) from January 2013 to January 2014 in 21 adult horses in New Zealand. Data are shown using boxplots (box from quartiles 1 to 3, median in bold, individual points indicating potential outliers), overlaid with average trends through time with 95% confidence bands from a generalised additive model.

2.3.3. Pairwise correlation of serum analytes

As there were no statistically significant differences between horses in blanketed and non-blanketed groups, the full data were analysed and plotted to determine the pairwise correlation of serum analytes towards each other. Serum iCa and PTH showed the highest negative correlation, whereas tCa and tMg showed the highest positive correlation. PTH showed a significant negative association with $25OHD_2$, tMg and $1,25(OH)_2D$. The full results are presented in Figure 2.5.

All results including serum analytes concentrations for blanketed and non-blanketed horses, pasture/hay analytes and climate data were entered into the generalized additive mixed models, in order to account for all related analytes in addition to other covariates. The results are presented in Table 2.4.

Average monthly sunshine had a statistically significant positive association with serum $25OHD_2$, $1,25(OH)_2D$, and iCa concentrations. In contrast, while average monthly radiation had a significant positive association with serum $25OHD_2$, $1,25(OH)_2D$, tCa and, tMg concentrations it had a significantly negative association with serum iCa concentration (effect sizes and confidence intervals are given in Table 2.4).

Serum iCa concentration had a significant negative association with serum P and PTH concentrations, while serum iCa and tMg concentrations had a positive association with serum $25OHD_2$ concentration. Serum $1,25(OH)_2D$ concentration had a significant negative association with serum PTH concentration. The full results including confidence intervals are shown in Table 2.4.

The concentration of magnesium in pasture had a significant positive association with serum tMg concentration. Similarly, the concentration of vitamin D in pasture and hay had a significant positive association with serum $25OHD_2$ and $1,25(OH)_2D$ concentrations, after adjusting for different parameters in the statistical model (Table 2.4).

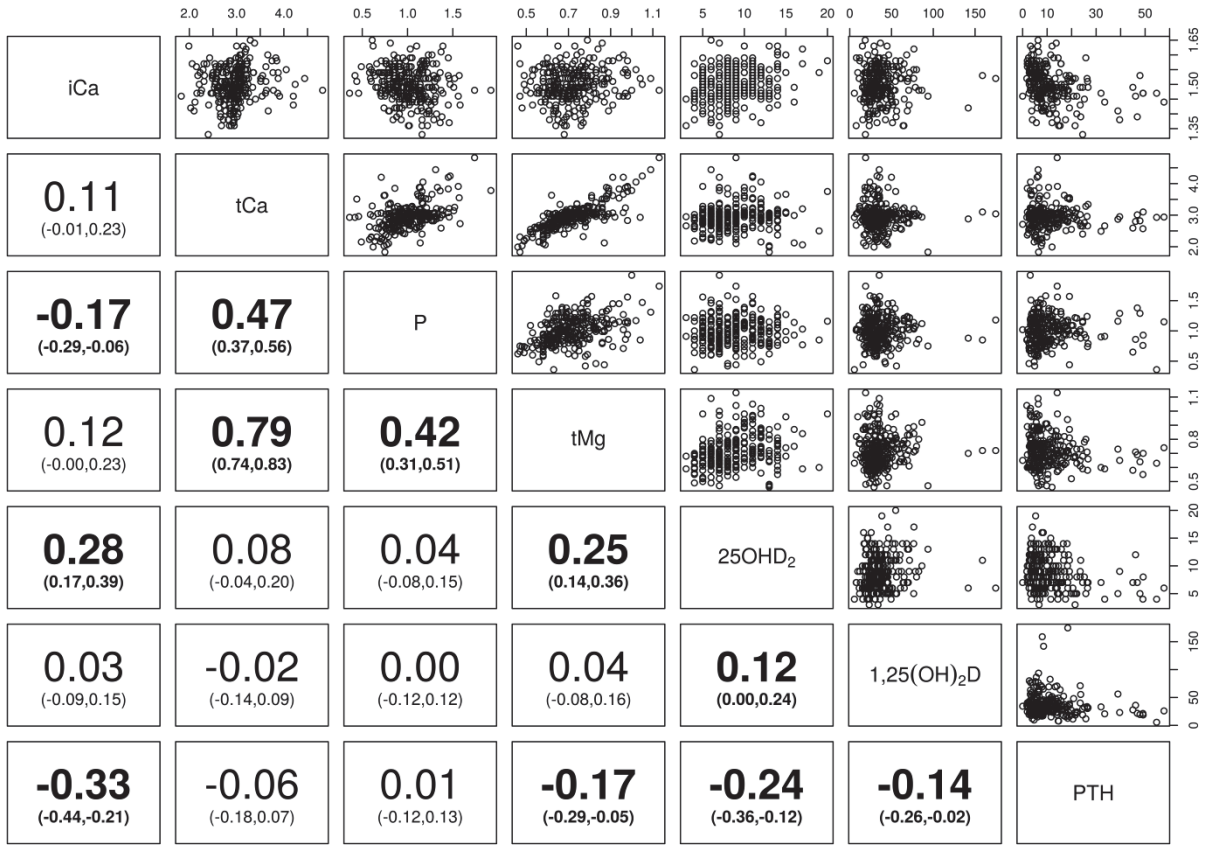


Figure 2.5 Pair-wise and Spearman's correlation between serum analytes. Pair-wise plots (upper triangle) and Spearman's correlation (lower triangle, with 95% Confidence Intervals) between serum ionised calcium (iCa), total calcium (Ca), phosphorus (P), total magnesium (tMg), 25-hydroxyvitamin D_2 ($25OHD_2$) and 1,25-dihydroxyvitamin D ($1,25(OH)_2$) and parathyroid hormone (PTH) in horses. Bolded numbers indicate statistically significant positive and/or negative correlation of different serum analytes towards each other ($P<0.05$).

Table 2.4 Statistical analysis performed by using generalised additive mixed models to test the influence of coverage, age, gender, breed, the amount of calcium, phosphorus, magnesium and vitamin D in pasture and hay, and the average monthly UV radiation and sunshine on serum concentration of ionised calcium (iCa), total calcium (tCa), phosphorus (P), total magnesium (tMg), 25-hydroxyvitamin D_2 ($25OHD_2$), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) and, parathyroid hormone (PTH) incorporating a smooth trend for these, with a random effect for horses in order to find any differences between blanketed and non-blanketed horses. Bolded numbers indicate statistically significant results along with their 95% confidence intervals. The proportion of variance explained by the model (R^2) and the *P*-value for whether there is a trend through time after adjusting for other covariates are also given.

	$25OHD_2$	$1,25(OH)_2D$	tCa^{2+}	iCa^{2+}	P	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	**-11.35 (-19.08, -3.61)**	42.12 (-36.08, 120.32)	**1.27 (0.79, 1.75)**	**1.56 (1.45, 1.67)**	**1.31 (0.53, 2.08)**	-0.21 (-0.47, 0.04)	**97.03 (66.70, 127.36)**
Blanket	1.01 (-0.30, 2.32)	-3.86 (-11.40, 3.68)	-0.05 (-0.16, 0.06)	-0.01 (-0.05, 0.03)	0.04 (-0.02, 0.11)	0.01 (-0.02, 0.04)	-3.83 (-10.26, 2.59)
Age	0.06 (-0.07, 0.19)	0.00 (-0.74, 0.74)	0.00 (-0.01, 0.01)	0.00 (-0.00, 0.00)	**-0.01 (-0.01, -0.00)**	0.00 (-0.00, 0.00)	0.48 (-0.15, 1.10)
Gender	**-2.38 (-3.88, -0.87)**	-7.07 (-16.53, 2.39)	-0.00 (-0.13, 0.13)	-0.01 (-0.05, 0.04)	-0.03 (-0.11, 0.06)	0.01 (-0.03, 0.04)	-2.32 (-9.55, 4.91)
Breed	1.09 (-0.22, 2.41)	2.10 (-5.54, 9.74)	0.08 (-0.03, 0.19)	0.01 (-0.02, 0.05)	-0.04 (-0.11, 0.03)	-0.02 (-0.06, 0.01)	0.98 (-5.51, 7.46)
tCa	–	–	–	–	**0.28 (0.16, 0.39)**	0.22 (0.19, 0.24)	-3.22 (-7.79, 1.35)
iCa	**7.46 (2.72, 12.20)**	-1.33 (-51.25, 48.60)	–	–	**-0.92 (-1.44, -0.40)**	-0.10 (-0.28, 0.07)	**-53.08 (-73.71, -32.46)**
P		-0.09 (-12.37, 12.19)	**0.30 (0.17, 0.42)**	**-0.03 (-0.06, -0.01)**	–	0.01 (-0.03, 0.05)	3.15 (-1.49, 7.79)
tMg	**3.48 (1.33, 5.62)**	-9.90 (-34.82, 15.02)	**2.59 (2.33, 2.86)**	**0.10 (0.04, 0.15)**	-0.03 (-0.43, 0.36)	–	7.18 (-8.22, 22.58)
$25OHD_2$	–	-0.20 (-1.49, 1.08)	–	**0.00 (0.00, 0.01)**	–	–	-0.14 (-0.57, 0.30)
$1,25(OH)_2D$	0.00 (-0.01, 0.01)	–	–	-0.00 (-0.00, 0.00)	–	–	**-0.07 (-0.11, -0.02)**
PTH	-0.02 (-0.05, 0.01)	**-0.34 (-0.65, -0.04)**	-0.00 (-0.01, 0.00)	**-0.00 (-0.00, -0.00)**	0.00 (-0.00, 0.00)	-0.00 (-0.00, 0.00)	–
Pasture Ca	–	–	-0.15 (-1.51, 1.22)	**-0.38 (-0.68, -0.08)**	–	–	–
Hay Ca	–	–	**-0.15 (-0.26, -0.03)**	-0.02 (-0.04, 0.01)	–	–	–
Pasture P	–	–	–	–	0.00 (-0.24, 0.25)	–	–
Hay P	–	–	–	–	0.11 (-0.13, 0.36)	–	–
Pasture Mg	–	–	–	–	–	**1.73 (1.18, 2.28)**	–
Hay Mg	–	–	–	–	–	-0.11 (-0.23, 0.00)	–
Pasture Vit D	**0.60 (0.39, 0.82)**	0.63 (-1.65, 2.91)	–	–	–	–	–
Hay Vit D	**0.18 (0.13, 0.22)**	0.26 (-0.29, 0.81)	–	–	–	–	–
Monthly Sunshine	**0.78 (0.6, 0.9)**	**2.46 (0.48, 4.44)**	**-0.11 (-0.18, -0.04)**	**0.04 (0.02, 0.05)**	0.04 (-0.01, 0.08)	**-0.05 (-0.07, -0.02)**	0.05 (-1.53, 1.63)
Monthly Radiation	**0.33 (0.20, 0.45)**	**0.63 (0.1, 1.15)**	0.01 (-0.00, 0.03)	**-0.01 (-0.01, -0.01)**	0.01 (-0.00, 0.03)	**0.02 (0.01, 0.03)**	-0.33 (-0.76, 0.10)

	$25OHD_2$	$1,25(OH)_2D$	tCa	iCa	P	tMg	PTH
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

Table 2.5 Vitamin D concentration in horse - Reported plasma or serum 25OHD$_2$, 25OHD$_3$, and 25OHD concentrations in horses.

Reference	Location	Breed	Number of Horses	Feed Additive (mg/d)	Sun Exposure	Measurement Method	25 (OH)D$_2$ (ng/mL)	25 (OH)D$_3$ (ng/mL)	25 (OH)D (ng/mL)
Pozza *et al.*, 2014	Thailand	Thoroughbred	20	0	✓	Multi-species enzyme immunoassay (EIA)	-	-	18.4–30.5
	USA	Thoroughbred	17	0	✓		-	-	14.3–37.2
	Thailand	Thoroughbred	10	0	✓		-	-	18.1–31.1
		Thoroughbred	13	0	✓		-	-	9.3–22.0
	USA	Thoroughbred	10	0	✓		-	-	9.5–19.2
	Thailand	Pony	21	0	✓		-	-	5.7–13.1
Hymøller & Jensen, 2011	Denmark,	Danish Warmblood	2	NRC	✓	High performance liquid chromatography (Dionex UltiMate 3000 vacuum degasser)	0.6 ± 0.1	-	0.1 ± 0.03
Piccione *et al.*, 2008	Italy	Thoroughbred	5	-	✗	High performance liquid chromatography (HPLC - Agilent 1100, BIO-RAD)	-	-	6.0 ± 0.2
Harmeyer & Schlumbohm, 2004	Germany	Standardbred	2	NRC	✗	High performance liquid chromatography and Calf thymus radioreceptor assay	-	-	0.6
Breidenbach *et al.*, 1998	Germany	Thoroughbred, Coldblood and Warmblood	16	-	-	Radioimmunoassay	-	-	<1.9–18.0
		Pony	8	-	-		-	-	<2.5–36.0
Mäenpää *et al.*, 1988	Finland	Finnhorse	30	50 – 100	✗	Competitive protein-binding assay	-	-	2.75 ± 0.28
		Finnhorse	30	37.5 – 7.5	✗		-	-	2.66 ± 0.36
		Finnhorse	30	-	✓		-	-	3.21 ± 0.20
		Finnhorse	30	-	✓		-	-	2.74 ± 0.23
		Finnhorse	30	50 – 100	✗		-	-	3.39 ± 0.28
		Finnhorse	30	37.5 – 75	✗		-	-	3.59 ± 0.33
		Finnhorse	30	125	✗		-	-	1.73 ± 0.21
		Finnhorse	30	50 – 100	✗		-	-	2.16 ± 0.25
		Finnhorse	30	-	✓		-	-	2.22 ± 0.24
		Finnhorse	30	-	✓		-	-	1.76 ± 0.13
		Finnhorse	30	-	✓		-	-	2.2–7.2
		Finnhorse	15	125	✗		-	-	2.24 ± 0.24
		Finnhorse	30	50 – 100	✗		-	-	2.54 ± 0.30
Mäenpää *et al.*, 1988	Finland	Finnhorse	20	50 - 100	✗	Competitive protein-binding assay	-	-	4.20 ± 0.34
		Finnhorse	20	-	✓		-	-	6.20 ± 0.36

2.4. Discussion

This is the first study to assess changes in serum Ca, Mg, P, vitamin D metabolites and PTH in horses kept at pasture over a year-long period. A seasonal trend was seen for iCa, tCa, tMg, P, 25OHD$_2$ and 1,25(OH)$_2$D, all of which reached a nadir towards the end of winter when ultraviolet radiation was lowest. In contrast, PTH peaked at the end of winter. Interestingly, the concentration of minerals (Ca, Mg, P) in the pasture remained relatively constant throughout the year, however the digestibility of the pasture and availability of nutrients may vary during the year and perhaps had an effect on the serum concentrations of these analytes. However, the low point for the concentration of vitamin D$_2$ in pasture also coincided with the end of winter and beginning of spring suggesting that despite adequate Ca, P and Mg present in the pasture (NRC, 2007), horses likely rely on the action of 1,25(OH)$_2$D to enhance intestinal absorption of Ca, P and Mg (Dittmer &Thompson, 2011). In addition, PTH ostensibly increased towards the end of winter, possibly to maintain plasma calcium concentrations within normal limits, and perhaps due to reduced negative feedback from lower serum 1,25(OH)$_2$D concentrations (Holick *et al.*, 2007).

One of the aims of the study was to determine if wearing a blanket had a detrimental on the ability of horses to synthesise vitamin D$_3$ in the skin. Skin coverage has a direct impact on the synthesis of 25OHD$_3$ in mammalian skin, particularly in humans (Hymoller & Jensen, 2010; Salih, 2004; Matsuoka *et al.*, 1992). However, the results from this study showed no differences in either serum 25OHD$_3$ or 25OHD$_2$ concentrations between blanketed and non-blanketed horses. In fact, a surprising finding was that the measured serum 25OHD$_3$ concentrations were either below the

detection limit of the assay or barely detectable in the horses, $25OHD_2$ being the principal form of 25OHD found in equine serum.

Vitamin D may be obtained from two sources, either isomerization of 7-dehydrocholesterol (7-DHC) in the skin after exposure to ultraviolet light, or from ingestion of vitamin D_2 in grass/hay or D_3 in cod liver oil and fatty fish in non-grass grazing species (Holick *et al.*, 2007). The circulating vitamin D then undergoes a hydroxylation reaction in the liver to form $25OHD_2$ or $25OHD_3$, and measurement of these metabolites is considered to be proportional to the amount of vitamin D consumed in the diet or obtained from skin production as this step in vitamin D metabolism is not closely regulated (Holick *et al.*, 2007, Lips, 2006). The final step of vitamin D activation occurs in the kidney, whereby the renal 1α-hydroxylase adds an additional hydroxyl group to form $1,25(OH)_2D$, the active form of vitamin D (Dittmer & Thompson, 2011).

The principal source of vitamin D in horses has previously been assumed to be through synthesis of vitamin D_3 in their skin when exposed to sunlight/UV light, as in cattle and sheep (Hymoller & Jensen, 2010; Smith & Wright, 1984), however serum $25OHD_3$ concentrations in the serum of horses in this study were minimal or undetectable. Several studies have shown that the serum concentration of $25OHD_3$ in horses was much lower than other species but was still detectable, which is in contrast to the results of the current study (Pozza *et al.*, 2014; Piccione *et al.*, 2008; Breidenbach *et al.*, 1998; Maenpaa *et al.*, 1988a; Maenpaa *et al.*, 1987; Smith & Wright, 1984; El Shorafa *et al.*, 1979). However, one study suggested that $25OHD_3$ might not be a good index of vitamin D status in the horse as its concentration was

almost undetectable and clearly showed that the main form of circulating vitamin D metabolite in horse serum was $25OHD_2$ (Smith & Wright, 1984).

Generally, studies on horses and ponies have reported a limited response in $25OHD_3$ status to sunlight exposure (Table 2.5). Studies performed in Finland, using competitive protein-binding assay as their method of measurement, all reported very low serum concentrations of 25OHD in mares with very small seasonal variation (Mäenpää *et al.*, 1988 a,b). Another study in Denmark, using high performance liquid chromatography (Dionex UltiMate 3000 vacuum degasser) as their measurement method, found no effect of access to sunlight during summer grazing on the serum concentration of 25OHD in adult Danish Warmblood horses (Hymøller & Jensen, 2011).

A more recent study in Thoroughbred horses in both Thailand and the USA investigated the effect of season and latitude on the 25OHD status of horses and found higher plasma 25OHD contents in their Throughbred horses than previously reported by others (Pozza *et al.,* 2014) (Table 2.5). One possible explanation for the differences found could be the lower latitudes this study was performed at compared with the previously mentioned studies. However, the enzyme immunoassay (Multi-species enzyme immunoassay (EIA)) used for measuring $25OHD_3$ in this study is reported to crossreact with both $1,25(OH)_2D_3$ (100% crossreactivity) and 24,25-dihydroxyvitamin D_3 (50% crossreactivity) (Lind *et al.*, 1997) and may have contributed to the variation observed.

These results, together with the results from the current study, suggest that the measured concentration of 25OHD in previous research may have included $25OHD_2$, $25OHD_3$, and other vitamin D metabolites due to the lack of specificity in the measurement method.

The fact that 25OHD$_3$ was undetectable in the serum from horses in this study could suggest that horses are not able to produce much, if any, vitamin D$_3$ in their skin, whether they are kept outside in paddocks wearing blankets or not; raising the prospect that horses may rely on vitamin D$_2$ obtained from pasture or hay to fulfil their need for vitamin D. However, despite LC-MS being considered the most reliable method to measure serum 25OHD, an analytical problem cannot be definitively ruled out (Snellman *et al.*, 2010). Further studies involving irradiation of horse skin with ultraviolet light and other functional studies are necessary to determine if this hypothesis is true.

The predominance of 25OHD$_2$ in equine serum raises questions with regards to the effectiveness of vitamin D$_3$ vs vitamin D$_2$ in horses. For example, could the equine vitamin D receptor have adapted to manifest a greater affinity for vitamin D$_2$? In humans the opposite occurs; 25OHD$_2$ has a shorter half-life in serum compared with 25OHD$_3$ (Jones *et al.*, 2014), and is considered to be half as potent as 25OHD$_3$ (Romagnoli *et al.*, 2008). In addition, higher serum 25OHD$_2$ concentrations are associated with lower concentrations of 25OHD$_3$ and 1,25(OH)$_2$D$_3$ (Swanson *et al.*, 2014; Lehmann *et al.*, 2013). A proportion of vitamin D$_2$ (but not vitamin D$_3$) may undergo an alternative activation pathway and be converted to 24-hydroxyvitamin D$_2$ in the liver (Horst *et al.*, 1990), the resulting activated metabolite 1,24-dihydroxyvitamin D$_2$ has less affinity for the vitamin D receptor, and therefore less biological activity (Horst *et al.*, 2000; Horst *et al.*, 1990). If horses have adapted to preferentially use vitamin D$_2$, are they at less risk of vitamin D toxicity if the diet is supplemented with vitamin D$_2$ rather than vitamin D$_3$? There does not appear to be data on vitamin D$_2$ toxicity in horses but when a toxic dose of vitamin D$_3$ was given to

horses, while serum $25OHD_3$ concentrations increased more than 350-fold, serum $1,25(OH)_2D_3$ concentrations remained within the physiological range, serum calcium concentrations only slightly increased, but serum phosphate concentration showed a three-fold increase, a feature that was thought to be unique to horses and possibly rabbits (Harmeyer & Schlumbohm, 2004).

A periodic fluctuation change was clearly noticeable in iCa. In addition, as would be expected, iCa was negatively correlated with PTH. However, comparison of the fluctuation of iCa and tCa through the year revealed that tCa had an often opposing rhythm suggesting it is not a good indicator of the amount of biologically active calcium and iCa, as the physiologically active form of calcium, should be considered the true regulator and measured more often in practice (Peacock, 2010), although this may not always be practical.

Our results showed a significant positive correlation between tMg and $25OHD_2$, and a negative correlation with PTH. In addition, serum tCa and tMg were strongly correlated. There is a significant functional link between calcitropic hormones and magnesium; however there is little scientific literature on magnesium metabolism in horses. In humans, a significant correlation between serum magnesium and 25OHD concentrations has been reported (Kelishadi *et al.*, 2014) and when combined with our results could suggest that the absorption of Mg in the horse, like that of Ca, may also be stimulated by vitamin D. Plasma calcium concentrations are known to regulate the secretion of PTH, and magnesium may play a similar role, as the serum concentrations of magnesium and PTH are also related (Rude & Ryzen, 1986), similar to the findings in our study.

Serum P concentration was significantly associated with serum tCa and tMg concentration; however the relationship between either P and Ca or P and Mg was not as strong as that between Ca and Mg. Traditionally, control of body phosphorus was thought to be indirect and secondary to the tighter control of calcium in the body by PTH and vitamin D, and thus it could be expected that P and Ca would be strongly correlated. However, over the last 15 years the so-called phosphatonin pathway for the control of body phosphorus, of which fibroblast growth factor 23 (FGF23) is the lynchpin, has been elucidated (Hardcastle, & Dittmer, 2015; Bergwitz & Jueppner, 2010). Therefore, it could be suggested that the relationship between Ca and P is not as strong as once thought due to the existence of this separate but linked physiological pathway.

This study does have a number of limitations, the main one of which is the non-random assignation of horses to the two groups, resulting in differences between the groups in terms of age and gender. The blanketed group consisted of only mares, and these horses were on average 3 years older than the non-blanketed group. However, we did account for these differences by including age, gender and breed as covariates in the statistical model. In addition because there were only 5 horses included in the blanketed group and therefore low statistical power, thus we cannot definitively rule out that blanketing did not have an effect.

The results of this study are the first in the Southern hemisphere to show that there is seasonal variation in vitamin D metabolites, PTH, Ca, P and Mg in horses at pasture, but there were no differences detected in these analytes between blanketed and non-blanketed horses. Regardless of coverage, $25OHD_2$ was the main precursor of active vitamin D in equine serum, and analysis of vitamin D status in horses should include

measurement of $25OHD_2$ in addition to $25OHD_3$. Based on these results, dietary vitamin D appears to be necessary for horses to fulfil their vitamin D requirements. Further work is required to determine the contribution of vitamin D_3 produced in the skin to the overall vitamin D status of horses.

Sections of this chapter have been published:

S. Azarpeykan, K.E. Dittmer, E.K. Gee, J.C. Marshall, J. Wallace, P. Elder, E. Acke, K.G. Thompson (2016). Influence of blanketing and season on vitamin D and parathyroid hormone, calcium, phosphorus and magnesium concentrations in horses in New Zealand. *Domestic Animal Endocrinology*. doi: 10.1016/j.domaniend.2016.03.003

[Appendix 3]

Chapter 3

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

3.1 Introduction

Life is a cycle regulated by a variety of internal chemical processes and biological rhythms including annual, seasonal, weekly, circadian (24 h), and diurnal (day/night) rhythms. Chronobiology is the science of these biological rhythms (Smolensky & Peppas, 2007). The term circadian is derived from Latin and means “around the day”. A circadian rhythm is a biological process that is driven by an “internal clock” and this has been observed in animals, plants, and even bacteria and fungi (Dunlap, 1999). Although it is an endogenous self-sustaining rhythm, the external environment has a huge impact on it. The circadian clock has a direct link with the perception of light and temperature, which provide 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 algae through to mammals, depend on sunlight, and use sunlight exposure 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 possess a biochemical system driven by sunlight and known as the internal, biological, and/or circadian clock

(Whitmore *et al.*, 2000; Buijs *et al.*, 2003). 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 daily and seasonal environmental changes, such as physical activity and sleep, hormonal changes, body temperature, and digestive activity (Aton & Herzog, 2005; Bernard *et al.*, 2007).

Sunlight has a direct influence on the synthesis of vitamin D_3 in the skin of most mammals. The number of ultraviolet B photons that reach the skin is directly correlated with the photoconversion of 7-dehydrocholesterol (7-DHC) to vitamin D_3 (Holick, 2003; Chen *et al.*, 2007). 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 the action of 1α-hydroxylase (CYP27B1) (Morris & Anderson, 2010). Calcitropic hormones (vitamin D and PTH) play an important role in the regulation of calcium homeostasis (Peacock, 2010).

Circadian rhythms have 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 different patterns in their sleep-wake time compared to other species. Their sleep periods are not limited to dark hours, and their average sleeping

time is 2.9 hours every 24 hours, occurring in short 15 minutes bursts. This likely affects the circadian and circannual rhythms of horses (Dallaire, 1986).

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

The aim of this study was to determine the circadian rhythm and trends of calcitropic hormones ($25OHD_3$, $25OHD_2$, $1,25(OH)_2D$ and PTH), in addition to serum calcium, phosphorus and magnesium concentrations, in a population of horses on the shortest (21st July 2013) and longest (22nd December 2013) days of the year in New Zealand.

3.2 Materials and methods

All procedures involving animals were approved by the Massey University Animal Ethics Committee (approval no. 12/93).

3.2.1 Animals

Five healthy adult mares (*Equus caballus*) with a mean age of 13 (±1.7) years (Table 3.1) were included in the study. Prior to commencement of the study, all horses were housed in outside paddocks at the Veterinary Large Animal Teaching Unit (VLATU), Massey University, for more than five months. At the beginning of the study, all horses were transported from VLATU to the Massey University Veterinary Hospital and housed in individual stalls close to each other that allowed them to have visual contact

with other horses. All horses spent the daylight hours outside the stalls in a large turnout yard to allow visual and physical contact with each other. A standard horse wellbeing environment was provided to reduce stress. Horses were fed *ad libitum* pasture that was cut daily (approximately 95% perennial ryegrass (*Lolium perenne*) and ~5% white clover (*Trifolium repens*)), and red clover (*Trifolium pratense*) and perennial ryegrass (*Lolium perenne*) hay, and had free access to water.

Table 3.1 Signalment of horses included in the study.

ID	Breed	Gender	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

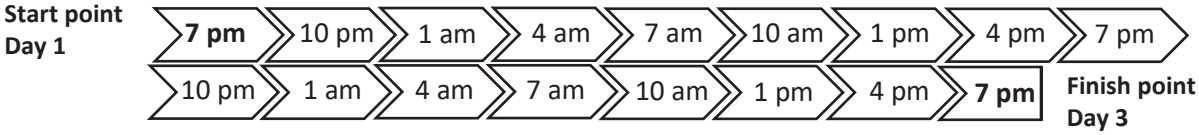
3.2.2 Sample collection

3.2.2.1 Blood sample collection

Samples were collected on June 25th-27th 2013, during the shortest days (winter solstice) of the year (sunrise 7:41 am and sunset 4:59 pm) and on December 16th-18th 2013, during the longest days (summer solstice) of the year (sunrise 5:42 am and sunset 8:45 pm). One serum separator tube (SST) and two plain tubes of blood were collected (10 mL, Vacutainer, Becton–Dickinson) from each horse at 3 h intervals over a 48 h period (starting at 7:00 pm on day one and finishing at 7:00 pm on day three) via an intravenous catheter inserted into the jugular vein (Table 3.2). To prevent catheters from blocking, they were flushed with a heparinised saline solution before and after each blood collection. Up to 10 mL blood was withdrawn and discarded

before samples were taken for analysis. 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 3.2 Blood collection time-frame. Blood samples were collected at 3 h intervals over a 48 h period (starting at 7:00pm on day one and finishing at 7:00 pm on day two) during the shortest days and longest days of the year 2013.



3.2.2.2 Feed sample collection

Samples were taken from the grass and hay that the horses were fed. Approximately 0.5 kg of grass and hay were collected after performing the first blood collection of the day (7 am on day two and three). All samples were wrapped immediately in a dark plastic cover to protect them from sunlight, weighed, and transported to the Nutrition Laboratory, Institute of Food, Nutrition and Human Health (IFNHH), Massey University, Palmerston North, New Zealand.

3.2.2.3 Urine sample collection

Urine samples were collected by catheterisation for urinalysis and urine biochemical analyses on the second day of sampling in June at 10 am. Blood samples for serum biochemical analyses were collected at the same time as urine samples.

3.2.3 Sample analysis

3.2.3.1 Serum biochemistry

Ionised calcium (iCa) concentrations were measured, using the Radiometer ABL800 FLEX analyser (Radiometer Medical ApS, Denmark) at Medlab Central, Palmerston North Hospital, New Zealand.

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 assay was performed in duplicate, and had an intraassay coefficient of variability (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 (25OHD2) concentrations, also at the Endolab (Canterbury Health Laboratories, Christchurch).

Total calcium (tCa), phosphorus (P) and total magnesium (tMg) 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 (Stillwater, Minnesota, USA) was used to measure total 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) as per the manufacturers' instructions (Nutrition Lab, IFNHH). Samples were analysed in duplicate, intraassay and interassay CV was always <10%.

As some tests were not available at Massey University, serum samples were sent overnight packed in dry ice to other laboratories as required.

3.2.3.2 Feed analysis

The pooled samples (one set of pasture and hay samples on day two and one set of pasture and hay samples on day three) 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 (ergocalciferol) and vitamin D_3 (cholecalciferol) using AOAC 982.29, modified and AOAC 2002.05 method at the Nutrition laboratory, Massey University, Palmerston North, New Zealand [16]. Samples were run in duplicate and had an intraassay and interassay CV of <10% for vitamin D_2 and <12% for vitamin D_3.

3.2.3.3 Urinalysis

Urine was acidified with ammonium chloride (NH_4Cl) 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 (Lefebvre *et al.*, 2008).

3.2.4 Data analysis

Results from the iCa, tCa, P, tMg, PTH, and vitamin D metabolite ($25OHD_2$, $25OHD_3$ and, $1,25(OH)_2D$) analyses are expressed in absolute concentrations, and all results are expressed as means (± SE) (Table 3.3). In order to identify any differences between seasons for 'summer solstice' (the longest days measurements in December) and 'winter solstice' (the shortest days measurements in June), smooth trends through the 48 h of sampling were calculated by fitting a generalised additive model for each analyte using the mixed generalised additive models computation vehicle (mgcv)

package (Wood, 2011) in R (R Development Core Team 2014). The models allowed testing for differing trends over and above average level differences on the shortest and longest days of the year, while accounting for variation between horses through the use of a random offset. For each analyte, we modelled the concentration *Y_{ist}* for horse *i* at time *t* of season *s* (s = 1, Summer, s=2, Winter) using

$$Y_{ist} = \beta_S Season_s + f_s(t) + Horse_i + \epsilon_{ist}$$

where β_S represents the average analyte concentration for season *S*, $f_s(t)$ represents a smooth trend through time for each season, $Horse_i$ is a random level for each horse, and ϵ_{ist} are residuals. We assessed whether there is a difference in trend between the seasons by determining whether $f_{Winter}(t) = f_{Summer}(t)$.

For all statistical comparisons a value of *P* <0.05 was considered significant.

3.3 Results

The daily mean (±SE) serum concentrations of tCa, iCa, P, tMg, 25OHD$_2$, 1,25(OH)$_2$D and PTH in horses during the shortest days of the year (June 25th-27th 2013) were significantly different from those measured during the longest days of the year (December 16th-18th 2013) (Table 3). Serum concentration of 25OHD$_3$ was very low and mostly undetectable during both the shortest and longest days of the year.

Serum 25OHD$_2$ was much higher during the longest days of the year than the shortest days (*P*<0.0001) (Table 3.3). In both measurement periods (the shortest and longest days) a constant pattern was seen, indicating no daily rhythm in 25OHD$_2$. The smooth trend for this analyte though was significantly different between the shortest and longest days of the year (*P*=0.003) (Figure 3.1).

Table 3.3 Serum analytes concentrations. Mean (± SE) serum concentration of total calcium (tCa) (mmol/L), ionised calcium (iCa) (mmol/L), phosphorus (P) (mmol/L), total magnesium (tMg) (mmol/L), 25-hydroxyvitamin D_2 ($25OHD_2$) (nmol/L), $25OHD_3$ (nmol/L), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (pmol/L), parathyroid hormone (PTH) (pg/mL) during the shortest days of the year (S) (June 25th-27th 2013) and the longest days of the year (L) (December 16th-18th 2013) in the study population of five horses in New Zealand. The P-value is assessing whether mean concentration differs between L and S.

		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	P-value
tCa	S	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.)	0.90
	L	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	S	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)	0.0006
	L	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)	
P	S	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)	< 0.0001
	L	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)	
tMg	S	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)	< 0.0001
	L	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)	
$25OHD_2$	S	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)	< 0.0001
	L	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)	
$25OHD_3$	S	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)	N/A
	L	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)	
$1,25(OH)_2D$	S	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)	< 0.0001
	L	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	S	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)	0.006
	L	3.64 (± 0.58)	10.86 (± 3.6)	6.32 (± 1.29)	7 (± 1.93)	7.3 (± 1.65)	6.4 (± 1.36)	18.02 (± 6.85)	5.28 (± 0.8)	5.3 (± 2.69)	3.4 (± 0.82)	7.14 (± 1.63)	13.5 (± 7.67)	11.04 (± 6.34)	13.6 (± 7.15)	4.34 (± 1.33)	6.1 (± 1.43)	4.6 (± 0.99)	

Table 3.4 Urinalysis. Fractional urinary clearance of calcium (FE_{Ca}), phosphorus (FE_P) and magnesium (FE_{Mg}) in the study population of five horses in New Zealand on the shortest day of the year.

	Serum Creatinine	Urine Creatinine	Serum Ca^{2+} (mmol/L)	Urine Ca^{2+} (mmol/L)	FE_{Ca} %	Serum PO_4^{3-} (mmol/L)	Urine PO_4^{3-} (mmol/L)	FE_P %	Serum Mg^{2+} (mmol/L)	Urine Mg^{2+} (mmol/L)	FE_{Mg} %
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 3.5 Values for fractional urinary clearance of calcium (FE_{Ca}), phosphorus (FE_P) and magnesium (FE_{Mg}) in horses, cattle, sheep, dogs and cats.

Species	FE_{Ca} %	FE_P %	FE_{Mg}%	Refrences
Horse	1.3- 6.72	0.08-19.98	2-50	Lefebvre *et al.*, 2008
Cattle	1.53± 0.44	0.05 ± 0.04	7.9± 1.6	Hartmann et al., 2001
	0.38 ± 1.399	0.33 ± 0.64	8.50 ± 18.55	Ulutas et al., 2003
Sheep	0010± 0.001	0.0010 ± 0.0001	0.08± 0.01	Godwin &Williams, 1986
	0.03 – 8.90	0.01 – 3.39	1.21 – 22.40	Bickhardt & Dungelhoef, 1994
Dogs	0.04 – 0.36	2.14 -29.7	1.822 – 5.9	Lulich et al., 1991
	0.08 (0.03-0.22)	5.1 (0.4-20.1)	N/A	Bennett et al., 2006
Cat	0.05 – 0.14	0.36 – 0.48	1.84 – 3.90	Finco et al., 1997

Table 3.6 Feed analysis. Pooled samples (one set of pasture and hay samples on day two and one set of pasture and hay samples on day three) were used for feed analysis. Calcium (%), phosphorus (%), magnesium (%) and vitamin D (ng/g) concentrations on the dry matter basis in the pasture and hay consumed by the five horses during the longest and shortest days of year.

	The shortest days of year		The longest days of year	
	Pasture	Hay	Pasture	Hay
Calcium (%)	0.39	0.78	0.51	1.07
Phosphorus (%)	0.61	0.32	0.18	0.18
Magnesium (%)	0.22	0.19	0.25	0.31
Vitamin D (ng/g)	0.14	3.86	1.03	3.72

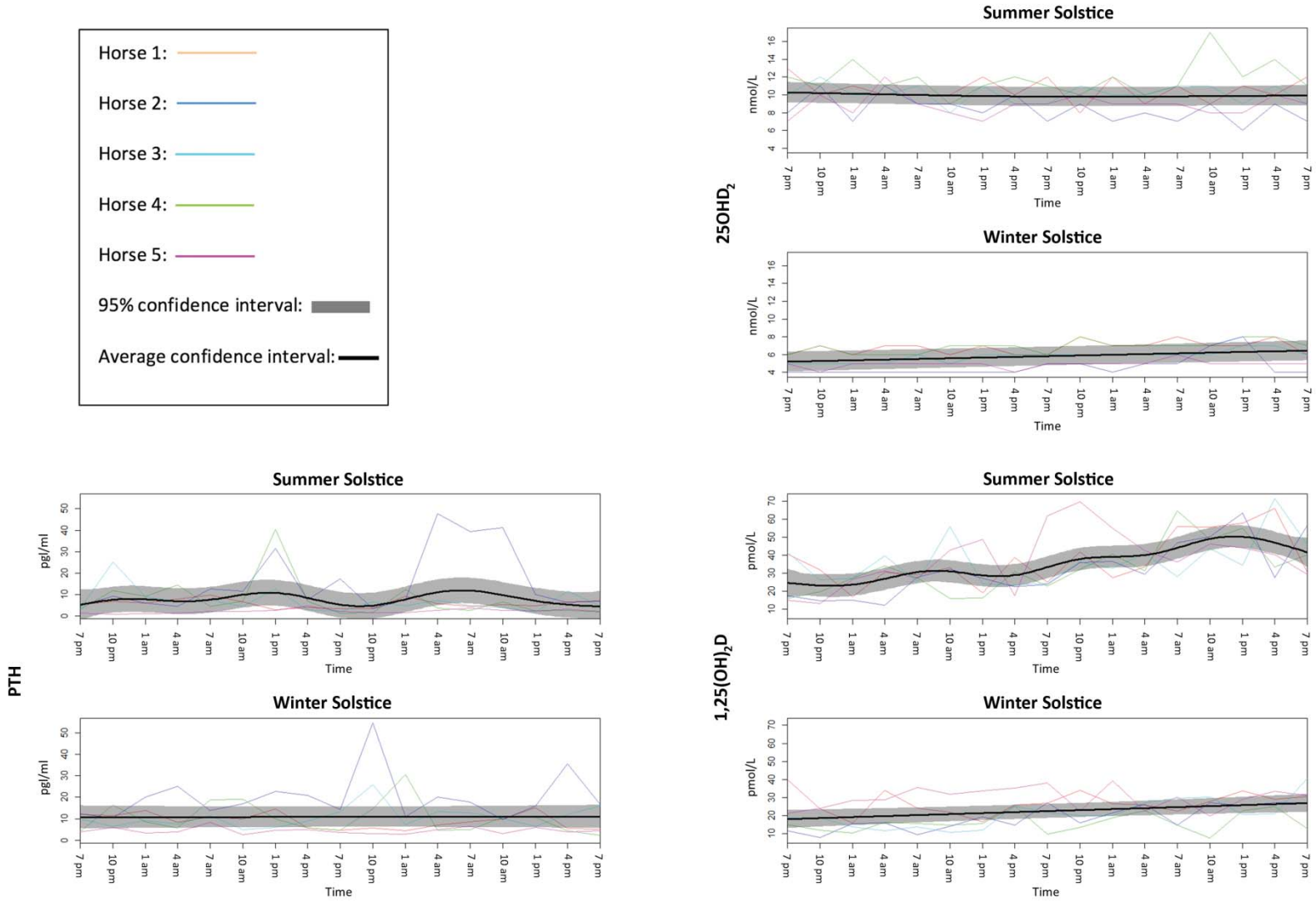


Figure 3.1 Trend of 25-hydroxyvitamin D_2, parathyroid hormone and 1,25-dihydroxyvitamin D. The trend of serum 25-hydroxyvitamin D_2 ($25OHD_2$), parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) concentration through the longest (December 2013) and shortest (June 2013) days of the year in 5 horses in New Zealand.

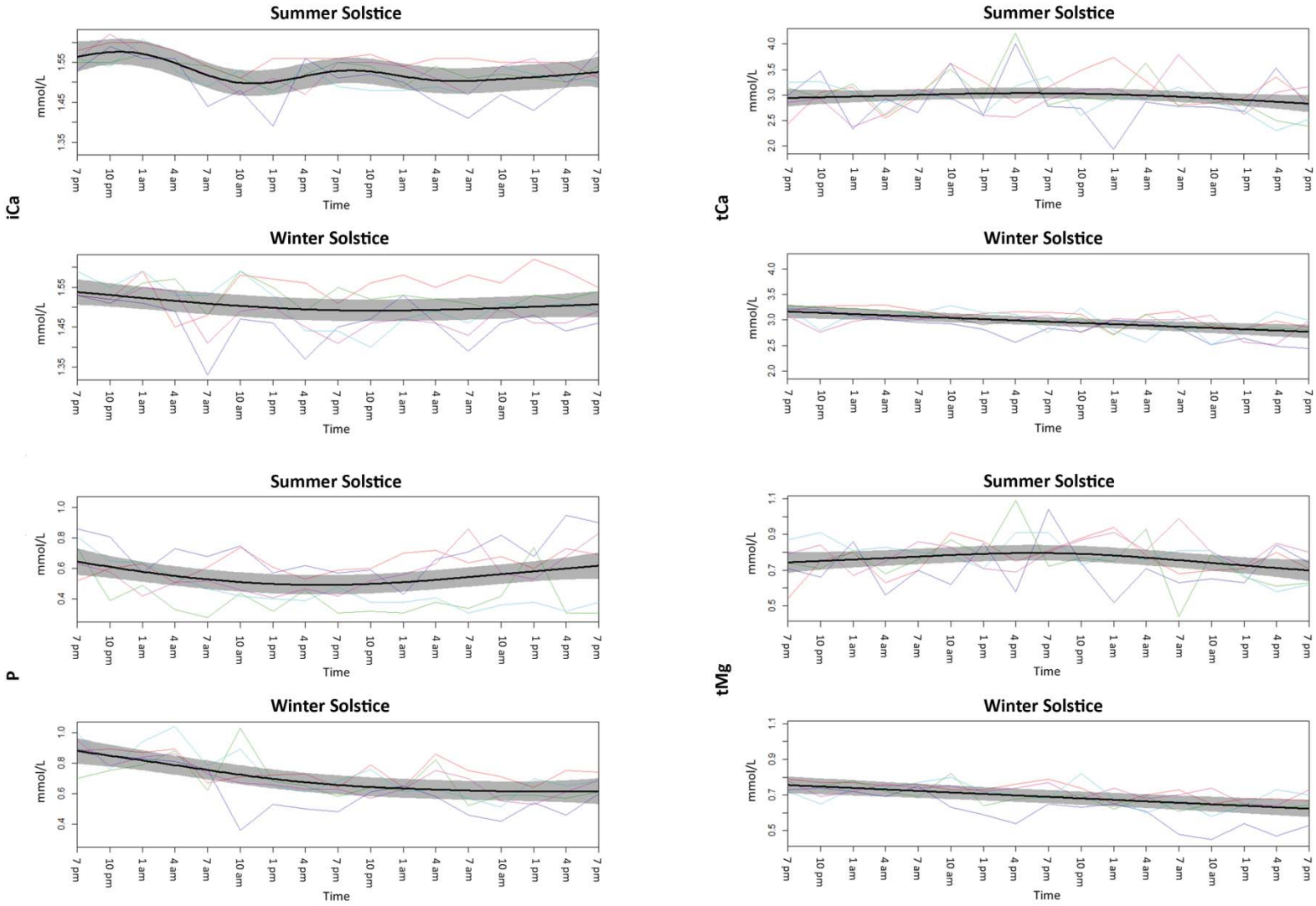


Figure 3.2 Trend of Ionised calcium, total calcium, phosphorus and total magnesium. The trend of serum ionised calcium (iCa), total calcium (tCa), phosphorus (P) and total magnesium (tMg) concentration through the longest (December 2013) and shortest (June 2013) days of the year in 5 horses in New Zealand.

Compared with the shortest days of the year, the serum concentration of $1,25(OH)_2D$ was higher (P<0.0001), while serum PTH was lower (P=0.006), during the longest days of the year (Table 3.3). For $1,25(OH)_2D$, there was a significant difference in trend through time between measurement periods (P<0.0001) with the longest days of the year showing an increasing periodic pattern. While the data showed a periodic trend for PTH during the longest days of the year, there was insufficient statistical evidence that this differed from the constant trend observed during the shortest days of the year (P=0.07) (Figure 3.1).

The serum iCa concentration was higher during the longest days of the year than the shortest days (P=0.0006) (Table 3.3). The trend through time differed between the longest and shortest days of the year (P=0.045), with a periodic pattern existing during the longest days of the year but not during the shortest days of the year (Figure 3.2).

Serum concentration of tCa did not differ significantly between the shortest and longest days of the year (P=0.90), whereas tMg was higher during the longest days of the year with a diurnal pattern and a maximum concentration in the middle of the sampling period (P<0.0001) (Table 3.3). During the shortest days of the year, a uniform constant downward pattern for serum concentrations of tCa and tMg was identified. The smooth trend for tCa and tMg in serum was significantly different between the shortest and longest days of the year (P=0.013 and P=0.003 respectively) (Figure 3.2).

Serum P concentration was lower during the longest days of the year with a diurnal pattern and a minimum concentration in the middle of measurement period, whereas P concentration was higher during the shortest days of the year with a downward constant pattern throughout the study (P<0.0001) (Table 3.3). The smooth trend for P

was significantly different between the shortest and longest days of the year (*P*=0.0001) (Figure 3.2).

3.3.1 Feed analysis

The concentrations of calcium (%), phosphorus (%), magnesium (%) and vitamin D (ng/g) were measured on a Dry Matter basis in the pasture and hay consumed by horses during the longest and shortest days of year (Table 3.4). The concentration of vitamin D was significantly higher in pasture on the longest days of the year than on the shortest days.

3.3.2 Fractional urinary clearance of calcium, phosphorus and magnesium

Urine and blood samples were collected simultaneously from the five horses that were involved in this study and fractional urinary clearance of calcium (FE_{Ca}), phosphorus (FE_P) and magnesium (FE_{Mg}) were calculated and are summarised in Table 3.6.

3.4 Discussion

There was a significant difference between the serum concentration of calcitropic hormones ($25OHD_2$, $1,25(OH)_2D$ and PTH), iCa, tCa, P and tMg between the shortest and longest days of the year in horses, although all analytes remained in the physiological range for horses (Estepa *et al,*. 2003; Berlin & Aroch, 2009; Toribio, 2011). Serum iCa, $1,25(OH)_2D$ and PTH concentrations clearly showed a circadian rhythm during the longest days of the year and serum tCa, P and tMg concentrations showed a diurnal pattern in the longest days of the year. None of the analytes demonstrated a circadian rhythm or diurial pattern during the shortest days of the year.

Fractional urinary clearance is mainly affected by physiological factors, including species, age, and the amount of each electrolyte present in the diet (Lefebvre *et al.*, 2008). The reported ranges of fractional urinary clearance for horses are different to other species (FE_{Ca}, FE_{P}, and FE_{Mg}), especially with regards to FE_{Ca} (Table 3.5). Fractional urinary clearance of calcium, phosphorus and magnesium significantly varied between individual horses in this study, but all were within the published reference range for horses (Lefebvre *et al.*, 2008).

For iCa, acrophases were recorded between 7 pm and 1 am on day one and day two of sampling in the longest days of the year and for PTH between 10 am and 4 pm on day one and between 4 am and 10 am on day two of the study in the longest days of the year. These results reflect the direct effect of these two analytes on each other, where increased serum calcium concentration suppresses PTH and *vice versa* (Lips, 2012). On day one of sampling during the longest days of the year, $1,25(OH)_2D$ showed peaks at the same time as iCa troughs; meanwhile PTH showed an opposite pattern to iCa. This pattern of serum $1,25(OH)_2D$ concentration on the longest days of the year supports the important regulatory role of this hormone on iCa and PTH where, due to the action of $1,25(OH)_2D$, active calcium absorption in the intestine is increased and release of PTH is suppressed (Lips, 2012).

Based on the statistical analysis on the raw data, diurnal rhythms were seen in serum concentrations of tMg, P and to some extent in tCa during the longest days of the year (Figure 3.2). However, tMg and tCa had opposite rhythms to P, supporting the concept that tMg has a similar relationship to P as tCa does (Toribio, 2011). This correlates with the finding in chapter 2 (Azarpeykan *et al.*, 2015) which showed similar

results and clearly revealed the direct effect and negative correlation of these analytes on each other in serum.

Previous work on the rhythmicity of serum concentrations of calcium, phosphorus and vitamin D metabolites in horses suggested that calcium, phosphorus and $25OHD_3$ had a strong and consistent circadian rhythm (Piccione *et al.*, 2008), which is not consistent with the findings of the current study. Light has a direct effect on circadian rhythms, although some non-photic parameters such as exercise patterns (Turek, 1989) and food availability (Stokkan *et al.*, 2001) may also influence these rhythms. It is suggested that the reason experimental horses and trained racehorses in other studies showed stable rhythms could be due to daily routine of the horses' environment with constant management regimens (e.g. feeding and exercise time). For example, a stronger rhythm of cortisol was seen in horses when they had habituated to a management routine including stabling, feeding and sometimes exercise (Irvine & Alexander, 1994). The fact that most horses in New Zealand are kept mainly in paddocks with limited stabling might reduce the management effects on horses, and therefore the rhythms of different analytes in their body. In addition, unlike previous studies, the horses in the current study had unlimited access to food (*ad libitum* forage), which was similar to horses' natural feeding habit where they spend most of their day eating at pasture (Broom & Fraser, 2015). Food intake and the time between ingestion of food and blood sample collection have a direct effect on the plasma concentration of many analytes (Young, 2012), suggesting that any rhythm in serum analytes measurements found in other studies might be the direct result of feeding and management practices, rather than changes in photoperiod.

Despite the fact that the concentration of the calcium, phosphorus, magnesium and vitamin D in pasture and hay consumed by the horses exceeded the minimum daily recommended intake requirements for horses (NRC, 2007), there was a significant variation in the concentration of most serum analytes between the shortest and longest days of the year. In the current study, the concentration of vitamin D metabolites ($25OHD_2$, $1,25(OH)_2D$) in serum was higher during the longest days of the year than during the shortest days. There was no $25OHD_3$ detected in the serum of horses, and $25OHD_2$ was the predominant vitamin D metabolite, and as discussed in Chapter 2 suggest that vitamin D_2 obtained from food is the principal source of vitamin D for horses. The amount of sunshine and UV radiation has a direct effect on the synthesis of vitamin D_2 in the pasture and hay that is consumed by horses (Wallis *et al.*, 1958; Jäpelt *et al.*, 2011). Perhaps the differences in vitamin D concentration, due to the greater availability of vitamin D in the pasture consumed by horses in the longest days of the year compared to the shortest days of the year, contributed to the variability in measurements of other analytes. It has been suggested that seasonal differences in UV light and its effect on vitamin D concentrations in humans may lead to seasonal changes in the activity of enzymes and transporters for drugs (Lindh *et al.*, 2011). This might also apply to serum mineral analyte concentrations in horses.

Another possible reason for differences in the concentration of serum analytes between the longest days of the year (summer) and the shortest days of the year (winter) is that all horses involved in this study were mares. Mares are seasonal polyoestrous long-day breeders and light is the main environmental factor influencing seasonal reproductive activity (Gastal *et al.*, 2004). Most mares are in anoestrus in winter and mares' cycle peaks are during summer (Kooistra & Ginther, 1975). Ovarian

activity has a direct effect on many hormone concentrations, and the degree of biological response. As the body needs to prepare itself for possible pregnancy (Evans & Irvine, 1975), it might need to establish hormonal and mineral ion homeostasis in a way that makes them responsive to the oestrous cycle and requirements of pregnancy. Inadequate nutrition or lower body condition may decrease pregnancy rate and fertility (Henneke *et al.*, 1984; Gastal *et al.*, 2004), therefore, diet has an influence on the mares' metabolism and consequently on reproduction which is mediated by body weight and/or condition. The physiological need of horses, which would be fulfilled through diet, may explain the higher concentrations of different analytes in the serum of mares during summer compared to winter.

This study could have significant relevance to horses in New Zealand and abroad, as there was a significant difference between serum concentrations of measured analytes between summer and winter. However, care should be taken in interpretation of serum analytes due to the seasonal differences and their applicability to the horses as the implications of the differences are not yet known.

Sections of this chapter have been published:

S. Azarpeykan, K. E. Dittmer, E. K. Gee, J. C. Marshall, P. Elder, E. Acke and K. G. Thompson (2016). Circadian rhythm of calciotropic hormones, serum calcium, phosphorus and magnesium during the shortest and longest days of the year in horses in New Zealand. *Journal of Animal Physiology and Animal Nutrition*. doi: 10.1111/jpn.12477

[Appendix 3]

Chapter 4

Inability of equine skin to synthesise vitamin D_3

4.1 Introduction

Vitamin D is known to play an essential role in the maintenance of calcium homeostasis and skeletal metabolism. In many animal species, the physiological requirement for vitamin D is mostly fulfilled by the conversion of 7-dehydrocholesterol (7-DHC) in the skin, through the influence of solar ultraviolet B radiation (UVB) (approximately 290-325 nm), to previtamin D_3, followed by thermal isomerisation at 37˚C to vitamin D_3 (DeLuca, 2004; Holick, 2007; Tavera-Mendoza & White, 2007). Other sources of vitamin D include: consumption from natural sources such as fatty fish (vitamin D_3), and irradiated plants (vitamin D_2) or from supplemented foods (vitamin D_3) (Lips, 2006; Zhu & DeLuca, 2012). Vitamin D enters the circulation where it binds to vitamin D-binding protein (DBP) and is transported to the liver and other organs, where it undergoes activation (DeLuca, 2004; Holick, 2004 a,b; Holick, 2006a).

The photoconversion of 7-DHC to vitamin D_3 has a direct correlation with the amount of ultraviolet B irradiation that reaches the skin (Holick, 2003; Chen *et al.*, 2007). It has been shown for instance, that skin from chickens covered by feathers is not able to synthesize vitamin D_3, and non-feathered skin has at least 10-fold higher concentration of 7-DHC (Tian *et al.*, 1994). In humans, the sum of exposure to ultraviolet B photons plus the dietary intake are considered important contributions to vitamin D status. These can be influenced by lifestyle, culture, traditional diets, behavioural parameters, geographic location (latitude and altitude), season, time of

day, cloud cover/pollution, amount of sun exposure, melanin quantity in the skin, age, the degree of protection from sunlight, and clothing traditions (Holick, 1994; Holick & Chen, 2008).

Melanin is a natural pigment responsible for determining skin and hair colour, and is present in the skin to varying degrees. Melanin has a photoprotective role; the amount of skin pigmentation and the incidence of skin cancers are inversely correlated (Clemens *et al.*, 1982). Fair-skinned individuals are approximately 70 times more likely to develop skin cancer than dark individuals (Halder & Bang, 1988; Gilchrest *et al.*, 1999; Halder & Ara, 2003). Melanin acts as a physical barrier to reduce the penetration of ultraviolet light through the epidermis; thus acting like a natural sunscreen (Kaidbey *et al.*, 1979; Kennedy *et al.*, 2003).

Studies in herbivores have shown that sheep (Chaudhary & Care, 1985; Dittmer *et al.*, 2011; Judson *et al.*, 2011; Kohler *et al.*, 2013; Kovacs *et al.*, 2015), goats (Kohler *et al.*, 2013; Kovacs *et al.*, 2015) and cows (Hymoller & Jensen, 2010) are likely able to produce vitamin D_3 in their skin. In contrast, experimental studies on carnivores, mainly cats and dogs, demonstrated they do not synthesise vitamin D_3 in their skin and rely on their nutrition and dietary supplements to fulfil their needs (How *et al.*, 1994; Morris, 1999). The prevailing assumption has been that horses can also synthesise vitamin D_3 in their skin through the action of sunlight (El Shorafa *et al.*, 1979), but, to the author's knowledge, this has not been demonstrated.

In Chapter 2 and 3 it was found that $25OHD_2$/ vitamin D_2 was the predominant form of vitamin D in equine serum, therefore, the main aim of this study was to investigate whether horses, like other herbivores, are able to synthesise vitamin D_3 in their skin in an experimental setting.

4.2 Materials and methods

4.2.1 Animals used and sample collection

Skin samples were collected from animals undergoing euthanasia at Massey University, Palmerston North for reasons unrelated to the experiment. Skin samples were excised within 30 min of euthanasia from the forehead, dorsum, and legs of five thoroughbred gelding horses (brown and bay), and the dorsum of two adult Romney cross female sheep (Table 4.1) . The skin samples were placed into sterile plastic storage tubes, wrapped in aluminium foil and stored at -80˚C until analysis.

Table 4.1 - Signalment of animals included in the study.

Animal	Age	Sex	Breed	Body score (out of 5)
Sheep 1	3	Ewe	Romney cross	2.5-3
Sheep 2	4	Ewe	Romney cross	3
Horse 1	1	Gelding	Thoroughbred	2.5-3
Horse 2	4	Gelding	Thoroughbred	3
Horse 3	5	Gelding	Thoroughbred	3
Horse 4	5	Gelding	Thoroughbred	3
Horse 5	5	Gelding	Thoroughbred	3

4.2.2 Sample irradiation and analysis

Prior to irradiation, the skin was thawed, and hair and subcutaneous tissue were removed in a dark room. Skin samples from each animal were pooled, measured and divided equally into three different groups, each 10 cm^2 in size. One skin pool from each animal was not irradiated and just wrapped in the aluminum foil to be used as control sample. The other skin pools from each animal were irradiated with an ultraviolet lamp (UV-Lampe für Reptilien und Vögel, Lucky Reptile, Waldkirch GmbH, Germany) output of between 290-305 nm. Each pool was irradiated for 8 h to obtain at least 5 J/cm^2 of accumulated UVB (Holick, 1981; Tian *et al.*, 1993; How *et al.*, 1994; Tian *et al.*, 1994; Holick, 2007). The ultraviolet lamp was used with an optimised

reflector box (size: width 28 cm^2, length 48 cm^2, depth 24 cm^2) UVB light was measured in $\mu W/cm^2$ by Solarmeter Model 6.2 UVB meter (Solartech Inc, Harrison Township MI, USA) in the 280-320 nm wavelength.

During irradiation, all samples were kept in a 37˚C room to allow for thermal isomerisation (Holick, 2007), and were moistened regularly with phosphate buffered saline. After irradiation, all samples were cut into small pieces (5 x 5 mm) in a dark room. The skin samples were weighed and freeze-dried using a freeze drier (Cuddon Freeze Dry model 0610, Blenheim, New Zealand) prior to saponification and lipid extraction.

The skin samples were processed as previously described (Morris, 1999; Hymoller & Jensen, 2011), and outlined in full below. The skin pools underwent ethanolic saponification with potassium hydroxide (KOH) as follows: the samples were combined with 0.1 mL of sodium ascorbate (0.2 g/mL), 8 mL of 1% ethanolic pyrogallol (1 g pyrogallol/100 mL ethanol) (Absolute Ethanol (200 Proof), Molecular Biology Grade, Fisher BioReagents™, Thermo Fisher Scientific, Massachusetts, USA) pyrogallol (MP Biomedicals™, Thermo Fisher Scientific, Massachusetts, USA) and 60% KOH (Analytical Reagent Grade, Thermo Fisher Scientific, Massachusetts, USA) in water in a round-bottom flask with a stirring bar and refluxed over a hot plate-stirrer. After cooling, the digest was filtered in a Buchner funnel with filter paper under partial vacuum. To perform lipid extraction, the residue on the filter paper was washed with ethyl acetate (HPLC Grade, Fisher Chemical, Thermo Fisher Scientific, Massachusetts, USA) (80:20) and then n-hexane (HPLC Grade, Fisher Chemical, Thermo Fisher Scientific, Massachusetts, USA), the combined filtrate was then transferred to a separating funnel, and water was added. The filtrate was extracted two more times with hexane,

and the combined hexane extracts were washed three or more times with water. Using a rotor vapor (Rotavapor® B-3, BÜCHI Labortechnik AG., Flawil, Switzerland) at 38˚C ± 2˚C, the samples were evaporated and the subsequent residue reconstituted with HPLC grade methanol (HPLC Grade, Fisher Chemical, Thermo Fisher Scientific, Massachusetts, USA), followed by sample clean-up using solid phase cartridges SPE C18 (EC 55 um, 70A) (Phenomenex, California, USA) in order to remove subcutaneous fat. The cartridge was activated with methanol (Fisher Chemical, Thermo Fisher Scientific, Massachusetts, USA) and the sample washed with 70% methanol, followed by elution with the mobile phase (95% methanol in water), which was collected and dried under nitrogen (Morris, 1999; Hymoller & Jensen, 2011).

Reverse phase high-performance liquid chromatography (HPLC) was performed using a Luna C-18 column (250 x 46 mm ID, 5 µm particle size) (Phenomenex, California, USA), with an isocratic mobile phase of methanol: H_2O (95:5), at a flow rate of 1.2 mL/min. Vitamin D_3 and 7-DHC were detected using an ultraviolet detector at 265 nm. Controls included vitamin D_3 (Sigma-Aldrich Corporation, St. Louis, USA) in concentrations ranging from 0.05 µg/ml up to 5 µg/ml, and 7-DHC (Santa Cruz Biotechnology, Inc., Texas, USA) from 0.05 µg/ml up to 3.5 µg/ml for the standard curves (Hymoller & Jensen, 2011).

4.3 Results

Reverse phase HPLC revealed the presence of 7-DHC in skin samples of both the sheep and horses included in this study. The concentration of 7-DHC was measured in one ovine and two equine samples.

Table 4.2 Skin results. The concentration of 7-dehydrocholesterol (7-DHC) and vitamin D_3 (Vit.D_3) in the equine and ovine skin samples before and after irradiation with 5 J/cm² (8 h) ultraviolet light.

Radiation	Sample	7-DHC			Vit.D_3		
		(µg/g)	(µg/cm²)	(nmol/cm²)	(µg/g)	(µg/cm²)	(nmol/cm²)
Control (0 h)	Sheep 1	1.5	1.23	3.2	< 0.03	< 0.02	< 0.05
	Sheep 2	-	-	-	< 0.03	< 0.02	< 0.05
	Horse 1	1.6	0.99	2.5	< 0.03	< 0.02	< 0.05
	Horse 2	8.6	5.95	15	< 0.03	< 0.02	< 0.05
	Horse 3	-	-	-	< 0.03	< 0.02	< 0.05
	Horse 4	-	-	-	< 0.03	< 0.02	< 0.05
	Horse 5	-	-	-	< 0.03	< 0.02	< 0.05
5 J/cm² (8 h)	Sheep 1	2.1	1.65	4.3	0.1	0.07	0.18
	Sheep 2	-	-	-	0.21	0.16	0.41
	Horse 1	1.9	1.05	2.7	< 0.03	< 0.02	< 0.05
	Horse 2	7.9	5.28	13	< 0.03	< 0.02	< 0.05
	Horse 3	-	-	-	< 0.03	< 0.02	< 0.05
	Horse 4	-	-	-	< 0.03	< 0.02	< 0.05
	Horse 5	-	-	-	< 0.03	< 0.02	< 0.05

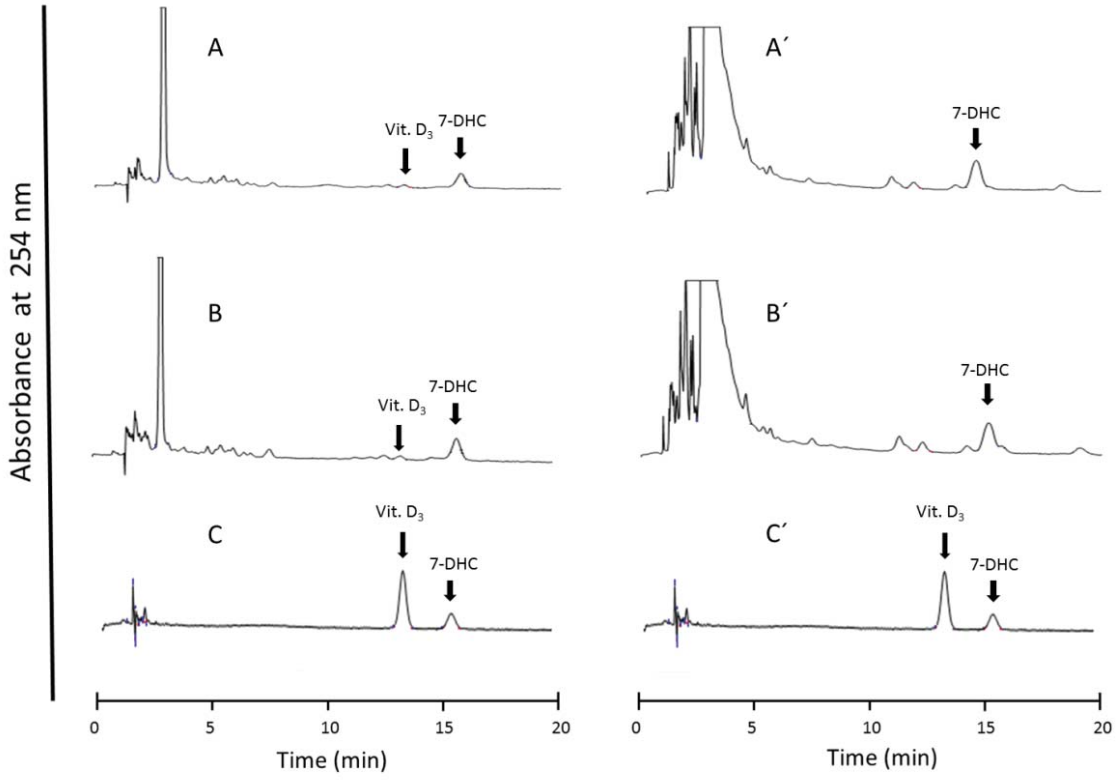


Figure 4.1 HPLC results. Reverse phase chromatography HPLC analysis of the skin of sheep and horse (7-DHC: 7-dehydrocholesterol and Vit.D_3: vitamin D_3). (A) Ovine skin irradiated with 2.5 J/cm² UVB light; (B) Ovine skin irradiated with 5 J/cm² UVB light; (C) the standard chromatograph of 7-DHC and Vit.D_3; (A´) Equine skin irradiated with 2.5 J/cm² UVB light; (B´) Equine skin irradiated with 5 J/cm² UVB light; (C´) the standard chromatograph of 7-DHC and Vit.D_3.

Irradiation of ovine skin resulted in production of vitamin D_3, whereas any vitamin D_3 produced in equine skin after 8 h irradiation with UV light was below the detection limit of the assay (<0.03 µg/g) (Figure 4.2).

4.4 Discussion

Ultraviolet radiation did not cause a detectable increase in the concentration of vitamin D_3 in the pooled skin samples from the forehead, dorsum, and legs of horses, but an increase in the amount of vitamin D_3 did occur in the pooled skin samples obtained from the dorsum of sheep.

It has been assumed that the ability of horses to photosynthesise vitamin D in the skin is similar to other herbivores (El Shorafa *et al.*, 1979). However, the results of this study on equine skin samples demonstrated that horses might be unable to synthesise appreciable amounts of vitamin D_3 in their skin, suggesting that they are reliant on their diet to fulfil their vitamin D requirements. This is a novel finding, which correlates with our recent study showing that the serum concentration of $25OHD_3$ in horses was negligible, and that $25OHD_2$ was the predominant form of vitamin D in their serum (Azarpeykan *et al.*, 2015).

The concentration of 7-DHC in the equine skin samples ranged from 0.99-5.95 ug/cm^2 (2.5-15 nmol/cm^2), which is similar to the concentrations detected in canine (n=5) (mean 1.86 µg/cm^2) (How *et al.*, 1994), feline (n=19) (mean 1.96 µg/cm^2) (How *et al.*, 1994; Morris, 1999) and human (n=5) (mean 1.28 nmol/cm^2) (Matsuoka *et al.*, 1992) skin, but higher than polar bear skin (n=5) (mean 0.11 nmol/cm^2) (Kenny *et al.*, 1998). From the results of the present study, and studies performed by others on cats and dogs, which showed similar concentrations of 7-DHC (How *et al.*, 1994; Morris,

1999), it can be concluded that the presence of 7-DHC in skin is no guarantee that it will be converted to vitamin D_3.

In humans, significant variations in the absolute concentration of epidermal 7-DHC have been reported, ranging from 2.41-11.65 μg/g (Maclaughlin & Holick, 1985). Variations in 7-DHC concentration may be associated with age and diet. Aging has a dramatic effect on the capacity of the skin to produce 7-DHC; an age-dependent decrease of greater than twofold was reported in the epidermal concentrations of 7-DHC in elderly individuals (Maclaughlin & Holick, 1985). Diet might also play an important role in the amount of 7-DHC in the body as 7-DHC is formed in the intestinal epithelium from the oxidation of cholesterol in food or bile (Glossmann, 2010). As a result, the amount of cholesterol in the diet, the ability of the bowel wall to form 7-DHC, and the amount of 7-DHC that is transported to the skin have a direct effect on the dermal concentration of 7-DHC (Glossmann, 2010). The variation in the concentration of 7-DHC in the skin samples of the horses in this study may have been due to a difference in age and/or diet. Horse 1, which was younger (1 year old), had a lower concentration of dermal 7-DHC than horse 2 (4 years old), both horses were young, and so this may just represent the individual variation in this species. Another possible explanation is that 7-DHC is a known precursor of cholesterol in the skin, and variations in cholesterol concentration in the body might be expected to reflect the dermal concentration of 7-DHC.

The four main phenotypes of horse coat colour are bay, black, brown and grey, which are controlled by epistatic interaction of genes (Sponenberg, 2009). Horses can vary in the amount of skin pigmentation, but even grey coated horses have pigmented skin (Toth *et al.*, 2006). In addition to its role in defining the skin and hair/coat colour,

melanin has a photoprotective responsibility and acts as a natural photon absorber mechanism to decrease the ultraviolet light penetration to skin (Springbett *et al.*, 2010). Studies in humans have shown that while all people produce vitamin D_3 in their skin, the concentrations of 25OHD in hyperpigmented (dark-skinned) individuals were less than half those of hypopigmented (fair-skinned) individuals (Harris & Dawson-Hughes, 1998; Harris, 2006). It has been assumed that horses, like humans, could produce more vitamin D in the lighter regions of their skin than in the dark regions. The equine skin samples used in this study were obtained from five horses, two bay and three brown. It was noted that one of the brown horses had a patch of white hair in its forehead; however the skin beneath this was highly pigmented. Therefore, the skin samples in this study were all highly pigmented and this may have interfered with their ability to produce vitamin D.

Different factors have a direct correlation with photoconversion of 7-DHC to vitamin D_3 in skin. The photo isomerisation and conversion of 7-DHC to previtamin D_3 in the skin occurs under the influence of solar ultraviolet B radiation with a wavelength of approximately 290-325 nm, followed by thermal isomerisation at 37˚C to vitamin D_3 (DeLuca, 2004; Holick, 2007).

In the study that was performed in cats (Morris 1999), samples from the ear of animals were used, however other similar studies did not mention from which part of the body samples were obtained. When designing the study, the possible synthesis of vitamin D in the face and limbs was taken into account, whereby samples from three different parts of body, including dorsum, legs and forehead were collected and pooled to account for possible variation in skin vitamin D production based on location of skin. All skin samples from the sheep and horses in this study were shaved prior to

irradiation with the UV light in order to exclude any interference of hair coverage towards vitamin D synthesis. Shorn sheep (Chaudhary & Care, 1985; Kohler *et al.*, 2013) and goats (Kohler *et al.*, 2013) have been shown to have higher serum vitamin D_3 concentrations due to increased exposure of skin to sunlight.

The results obtained from ovine skin samples in the present study showed that the irradiation and exposure time to UVB light made the conversion of 7-DHC to vitamin D_3 possible. As no detectable increase in the amount of vitamin D_3 in the skin samples of the horses occurred, it is likely that horses do not make measerable amounts of vitamin D_3 in the skin. However, it may also be that the concentration of vitamin D produced was below the detection limit of the assay. These findings demonstrating that horses may not be able to perform photoconversion of 7-DHC to vitamin D_3 in their skin (Table 4.1), contrast with previous findings and conclusions published for horses, in particular the report by Elshorafa *et al.* (1979), which is often cited in reference to vitamin D metabolism in horses. In that report, factors that could have influenced the findings, such as whether the animals had access to fresh pasture which contains vitamin D_2, were not described, and could have invalidated the results (El Shorafa *et al.*, 1979).

Voluntary feed intakes in horses depends on the forage quality, where a reduction in quality of forage results in increased feed intake to maintain rates of energy and nutrient absorption (Janis, 1976) and decreased forage digestibility (Duncan *et al.*, 1990). Individual variability, however, can play a significant role in voluntary feed intakes (Edouard *et al.*, 2008). There are a number of computer programs that can calculate the formulation and nutrient requirements of horses under different housing and exercise conditions, including the current recommendation of vitamin D in horses

(Austbø, 2014 and 2013). The estimated voluntary feed intakes of horses are approximately 1.5-2.5% of their bodyweight in Dry Matter/Day (Edouard et al., 2008). The current minimum requirements of vitamin D in horses according to National Research Council (NRC) is 7.5 µg (300 IU)/kg feed dry matter or 0.17 µg (6.6 IU)/kg body weight (BW) (NRC, 2007 and 1989). As it has been addressed in Chapter 2 (Table 2.2) and Chapter 3 (Table 3.6), the concentration of vitamin D in pasture/feed consumed by horses in our studies exceeded the NRC requirements (NRC, 2007 and 1989). It should be noted that no recent experimental data on the vitamin D requirements and physiology of horses exists and this recommendation is mainly based on older literature and extrapolation from other species (NRC, 2007 and 1989), and the limited literature that does exist on the subject suggests that horses may have a very different vitamin D physiology compared with species (Pozza *et al.*, 2014).

The finding that horses appear to rely on dietary vitamin D_2 to fulfil their vitamin D requirements suggest an adaptation in the evolution of the horse that might not have occurred in other grazing species. The possible evolutionary benefits of this adaption could include variation and change in diet with time, expansion of global range and habitat, domestication and any possible impacts that this may have had. The evolution of the domesticated horse (*Equus ferus caballus*), for example, saw a change in diet from jungle foliage in the early species to grasses as horses evolved in size (Weinstock *et al.*, 2005). Their habitat and range may have naturally expanded into the grass-filled steppe regions of Europe and Asia, as with the evolution of the wild Przewalskii horse (*Equus ferus przewalskii*) (Weinstock *et al.*, 2005). This may have also been a consequence of the increasing use, management and breeding of horses by humans

around the world. Either way, the reduced amount of sun in these areas may have encouraged an adaptation to obtain vitamin D from their diet that other herbivores elsewhere may not have required. Other species in the *Equidae* family in different geographical locations may not have needed such an adaption, or may have evolved differently to best adapt to the conditions around them. Future research and comparison of the vitamin D uptake in some of these species, such as the wild zebra in the sunnier African savannah, would test some of these evolutionary drivers.

In conclusion, the results of this study show that horses may not be able to convert 7-DHC to vitamin D_3 in their skin after exposure to UVB light. This finding suggests that horses rely mainly on their diet as a primary source of vitamin D and the requirements of vitamin D in the equine diet should be reassessed.

Chapter 5

Evaluation of housekeeping genes for quantitative gene expression analysis in the equine kidney

5.1 Introduction

One of the most accurate and widely used methods for detecting differences in gene expression is real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) (Kayis *et al.,* 2011; Feng *et al.,* 2012). RT-qPCR is a method with high sensitivity and specificity, low template requirements, and allows rapid quantification of gene expression (Kayis *et al.,* 2011; Lin & Lai, 2013).

While RT-qPCR is a sensitive and accurate method for measuring gene expression in cells and tissues, inaccurate pipetting, the quality of RNA, efficiency of cDNA synthesis by reverse transcriptase, and PCR amplification efficiencies can diminish RT-qPCR accuracy (Paris *et al.*, 2011). To reduce and eliminate these factors and prevent misinterpretation of the results, reference genes are used as internal controls for normalising and calculating the relative expression of target genes (Feng *et al.,* 2012; Brito *et al.,* 2013). It is assumed that reference genes, commonly known as housekeeping genes (HKGs), are stably expressed in the tissues/cells and that experimental parameters and pathological conditions do not alter their expression; therefore choosing a suitable internal reference gene is a vital factor to allow accurate interpretation of the results (Huggett *et al.,* 2005).

HKGs are generally used as internally expressed reference genes due to their stable expression levels. However, there is no unique universal HKG commonly used

for normalisation of gene expression levels and HKGs vary among organisms and tissues, and even according to environmental and experimental conditions (Kayis *et al.,* 2011; Brito *et al.,* 2013). It has been recommended that a combination of HKGs is used in order to acquire a much more stable and reliable reference (Beekman *et al.,* 2011). As a result, validating HKGs expression stability before using them in an experimental protocol is essential, as inaccurate normalisation results in inadequate quantification and false conclusions (Vandesompele *et al.,* 2002).

A number of studies on the appropriate internal controls for studying gene expression in equine tissues using quantitative RT-PCR have been published (Bogaert *et al.,* 2006; Cappelli *et al.,* 2008; Figueiredo *et al.,* 2009; Zhang *et al.,* 2009; Beekman *et al.*, 2011; Paris *et al.,* 2011), however no studies have been specifically performed to determine reliable HKGs for the equine kidney. The aim of this study therefore was to determine the most stably expressed HKGs in the equine kidney, in order to use these for normalisation of gene expression in subsequent RT-qPCR experiments examining vitamin D responsive genes in the equine kidney. The HKGs evaluated using RT-qPCR included 18S ribosomal RNA (18S), 28S ribosomal RNA (28S), ribosomal protein L32 (RPL32), ß-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase complex (SDHA), zeta polypeptide (YWHAZ), hypoxanthine phosphoribosyltransferase 1 (HPRT1).

5.2 Materials and methods

5.2.1 Tissue used and sample collection

Renal tissue samples were harvested post-mortem (Pathobiology post-mortem room, Massey University, Palmerston North) from nine adult horses (Thoroughbred

and Standardbred) that were euthanized for reasons unrelated to this study. Tissue samples for RNA isolation were collected within half an hour of euthanasia, cut into small pieces, snap frozen in liquid nitrogen and stored at -80˚C until processing.

5.2.2 RNA extraction and reverse transcription

Total RNA was extracted from renal tissue samples using the RNeasy Mini Kit (QIAGEN, USA) including the optional on-column DNAse digestion step (RNase-Free DNase Set, Qiagen) with the following protocol. Each kidney sample was thawed on ice and then cut into smaller portions using scalpel blade. A maximum of 30 mg of each sample was then placed into a 2 mL micro tube containing 2 x 5 mm stainless steel ball bearings and 600 µL of Buffer RLT reagent. The tubes were homogenized by agitation in a Mini-Beadbeater-16 (BioSpec, Oklahoma, USA) for 1 min and then centrifuged for 3 min at 16000 xg. Then 600 µL of 70% ethanol was added to the tubes and mixed well. Subsequently, 700 µL of the homogenate was transferred to an RNeasy spin column placed in a 2 mL collection tube. The spin column was centrifuged for 30 s at 8000 xg and the flow-through discarded. Subsequently, the remainder of the RNA extraction was performed according to the manufacturer's instructions. RNase-free water (50 µL) was directly added to the spin column membrane and centrifuged for 1 min at 8000 xg to elute the RNA. Samples were immediately stored at -80°C until required, except for a 4 µL aliquot which was tested for RNA and DNA concentration (to check for absence of DNA) using the Qubit® 2.0 Fluorometer and Qubit® RNA HS and DNA HS Assay kits (Invitrogen, Life Technologies Corporation, California, USA) as per manufacturer's instructions.

The TURBO DNA-free™ Kit (Ambion®, Life Technologies Corporation, California, USA) was used for treatment and removal of any contaminating genomic DNA from

the extracted RNA samples with the following protocol. The TURBO DNase Buffer reagent (5 µL) and TURBO DNase (1 µL) were added to the RNA sample, mixed gently and incubated at 37°C for 30 min. Then 5 µL of resuspended DNase Inactivation reagent was added to the RNA sample, mixed well and incubated at room temperature (22-25°C) for 5 min, with occasional mixing to redisperse the DNase Inactivation reagent. The sample was centrifuged at 10,000 xg for 1.5 min, then the clear (RNA-containing) supernatant was transferred to a fresh 2 mL tube and stored at -80˚C until later analysis.

5.2.3 Primer design

Primers were designed according to primer sequences previously published (Kayis *et al.,* 2011; Klein *et al.,* 2011) or using NCBI primer BLAST (http://www.ncbi.nlm.nih.gov/nucleotide) (Table 5.1). The primers were designed to have the following features: PCR product of less than 150 bp, span an exon-exon junction, show no complementarity to extraneous targets, have minimal primer-dimer and primer hairpin formation, and possess similar melting temperature and guanine-cytosine content (GC content %).

The best primer set was selected and the PCR amplicon sequence tested for secondary structures at 60˚C using the mFold program (http://mfold.rit.albany.edu/?q=mfold). If there were no hairpin loops in the primer binding area, then the primer pair was selected. If there was hairpin loop formation, the entire process was repeated until all conditions were satisfied.

5.2.4 Real-time PCR

Real-time PCR was performed using the StepOne Plus real-time PCR machine (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA). The primer concentrations determined to be optimal for each primer pair PCR are shown in Table 5.1. Real-time PCR reactions (10 µL) contained 5 µL Fast SYBR Green real-time PCR Master Mix (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA), the primer pair at concentrations given in Table 5.1, 10 ng of cDNA template and RNAase-DNAse free water. The PCR protocol consisted of a denaturation step at 95°C for 20 s; followed by 40 cycles at 95°C for 3 s and 60°C for 30 s; ending with a melt curve ranging from 60°C to 95°C with a heating rate of 0.3°C/15 s. Negative controls of water and reaction mix without reverse transcriptase were included in every PCR run and all samples were run in duplicate. Standard curves were produced for each target to determine the accuracy (R^2 ≥0.98) and efficiency (90-105%) of the real-time PCR reactions (Table 5.1). The real-time data were analysed using the StepOne plus software (Applied Biosystems, Life Technologies Corp., Calsbard CA, USA) and were exported into an Excel datasheet (Microsoft Excel 2010) for further analysis.

5.3 Data analysis

The HKGs expression stability data were analysed with two software packages; geNorm (qbase$^+$ 3.0, Biogazelle, Zwijnaarde, Belgium) (Vandesompele *et al.*, 2002; Hellemans *et al.*, 2007), and NormFinder (The Department of Molecular Medicine (MOMA), Aarhus University Hospital, Denmark http://moma.dk/normfinder-software) (Andersen *et al.*, 2004). Both methods generate a measure of HKGs stability, which can be used to rank the HKGs.

Table 5.1 Equine housekeeping genes. Housekeeping genes used in the real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) assay indicating gene name, function, GenBank accession code, primers, amplicon length, and their PCR parameters (efficiency and Regression coefficient (R^2)) from RT-qPCR analysis.

Gene	Full gene name	Function	GenBank (accession code)	Primer (5'-3')	Amplicon length (bp)	Primer concentration	PCR Efficiency	R^2
18S‡	18S ribosomal RNA	Ribosomal eukaryotic small subunit	AJ311673.1	F: ATGCGGCGGCGTTATTCC R: GCTATCAATCTGTCAATCCTGTCC	204	900:900 nM	91.17%	1
28S‡	28S ribosomal RNA	Ribosomal eukaryotic small subunit	EU554425.1	F: CGGGTAAACGGCGGGAGTAAC R:TAGGTAGGGACAGTGGGAATCTCG	109	350:300 nM	91.05%	0.995
RPL32	Ribosomal protein L32	Ribosomal eukaryotic large subunit	CX594263.1	F: TCGTGAAGCCCAAGATCGTC R: TTGAATCTTCTGCGCACCCT	130	350:150 nM	90.70%	0.998
B2M†	ß-2-microglobulin	Beta-chain of major histocompatibility complex class I molecules	NM_001082502.2	F: GTGTTCCGAAGGTTCAGGTT R: ATTTCAATCTCAGGCGGATG	102	300:300 nM	92.16%	0.998
GAPDH†	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	NM_001163856.1	F: AGAAGGAGAAAGGCCCTCAG R: GGAAACTGTGGAGGTCAGGA	87	900:500 nM	92.46%	0.994
SDHA†	Succinate dehydrogenase complex	Major catalytic subunit of the mitochondrial respiratory chain	XM_001490889.3	F: GCAGAAGAAGCCATTTGAGG R: CCTGTCGATTACGGGTCTGT	103	500:900 nM	93.51%	0.993
YWHAZ†	Zeta polypeptide	Mediate signal transduction	XM_001492988.3	F: TGTTGTAGGAGCCCGTAGGT R: ATTCTCGAGCCATCTGCTGT	95	300:300 nM	93.44%	0.994
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Protein Coding gene	AY372182	F: TTGCTGACCTGCTGGATTAT R: TTATGTCCCCTGTTGACTGGT	120	500:500 nM	93.80%	0.993

‡ Klein *et al.*, 2011

† Kayis *et al.*, 2011

5.4 Results

The geNorm program calculates the gene expression stability value M, which corresponds to the average pairwise variation (V) of a particular gene with all other control genes, to determine the benefit of adding extra reference genes for the normalisation process (Vandesompele *et al.*, 2002). The most stable HKG has the lowest M value, while the least stable has the highest M value. The geNorm program arbitrarily indicates genes with M values below the threshold of 1.5 as the most stable HKGs. To ensure the most stable genes will be selected, it is suggested that M values lower than 1.0 are considered as the most stable HKGs (Vandesompele *et al.*, 2002). When all nine samples were analysed together with geNorm, seven genes had an M value of less than 1.0 in the horse kidney (Figure 5.1).

To obtain reliable results from real-time PCR data, two or more reference genes should be used for data normalisation. The optimal number of reference genes can be determined by calculating the pairwise variation (V), and this was calculated for all the samples analysed. The proposed cut-off value for V is 0.15 (Vandesompele *et al.*, 2002), below which the inclusion of an additional control gene is not required. In this study, the paired variable coefficients indicated that two reference genes, HPRT1 and YWHAZ, would be optimal for normalising gene expression in the equine kidney (Figure 5.2).

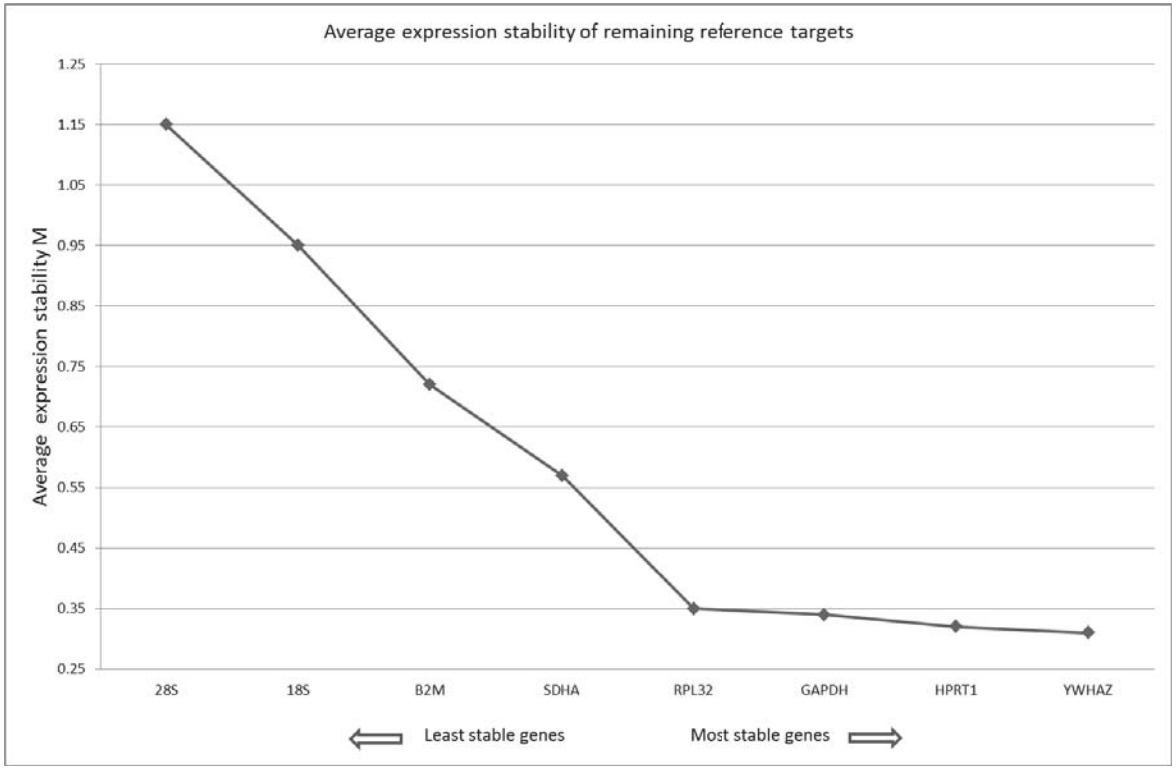


Figure 5.1 Gene stability results. Gene expression stability of the candidate housekeeping genes in equine renal tissue analysed by the geNorm program. Lower M values (M<1.0) correspond to the most stable and most suitable HKGs for normalisation.

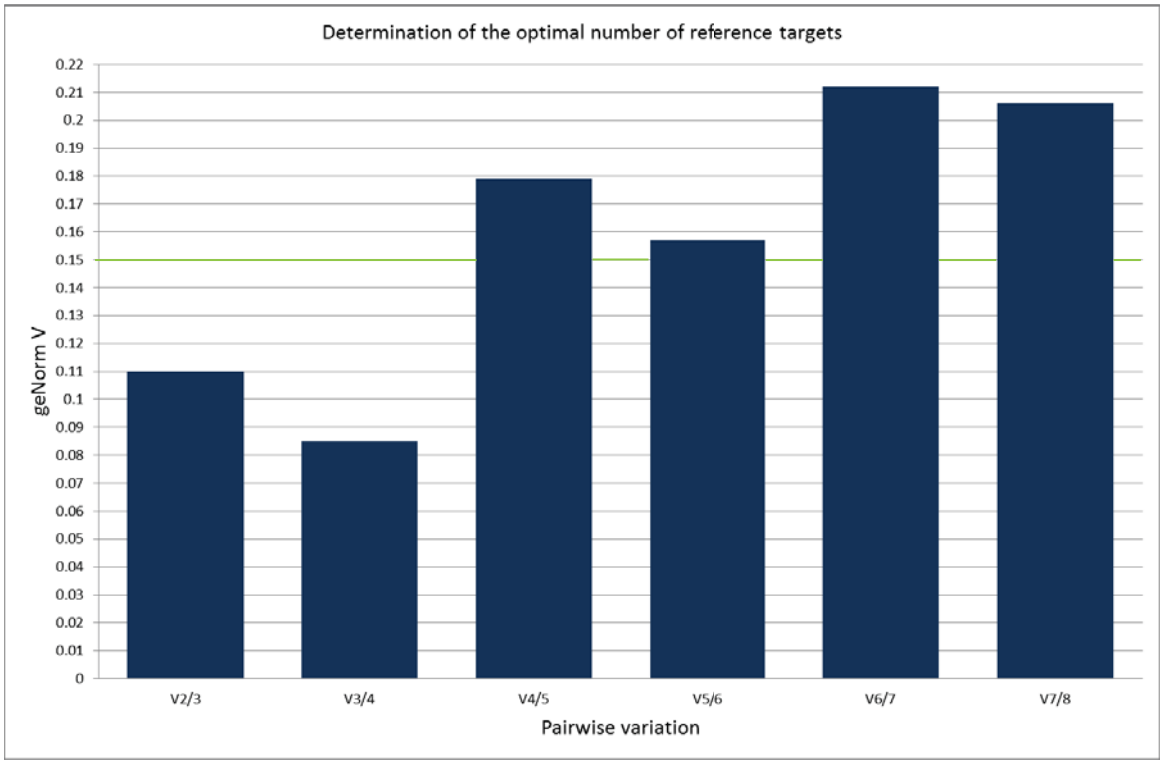


Figure 5.2 Pairwise variation. Evaluation of the optimum number of housekeeping genes according to the geNorm software. V≥0.15 are the sutable genes to be used for normalisation.

NormFinder is a Microsoft Excel-based Visual Basic application that estimates the stability values of a single HKG according to the similarity of their expression profiles by using a model-based approach (Andersen *et al.*, 2004). The results of the NormFinder analysis were similar to those of geNorm. Both methods ranked YWHAZ, HPRT1, RPL32, and GAPDH as among the four most stable reference genes, and 18S and 28S as the least stable genes (Table 5.2).

Table 5.2 Results comparison. Expression stability values of the candidate housekeeping genes calculated by the geNorm and Normfinder algorithms (ranking in parentheses).

Gene	18S	28S	RPL32	B2M	GAPDH	SDHA	YWHAZ	HPRT1
Stability Value	0.95	1.16	0.35	0.72	0.34	0.54	0.31	0.32
geNorm	(7)	(8)	(4)	(6)	(3)	(5)	(1)	(2)
Stability Value	0.90	1.12	0.10	0.86	0.14	0.49	0.24	0.16
Normfinder	(7)	(8)	(1)	(6)	(2)	(5)	(4)	(3)

5.5 Discussion

This study presents the validation and optimisation of eight potential HKGs for RT-qPCR in the equine kidney. Results analysed by geNorm and NormFinder algorithms showed that HPRT1, RPL32, GAPDH, and YWHAZ genes are the most suitable and most stable genes for normalising gene expression in further experiments on equine kidney.

The lack of stable HKGs during gene expression experiments will result in misinterpretation of the results (Huggett *et al.*, 2005). There are several different mathematical methods for determining the relative expression stability of genes including geNorm (Vandesompele *et al.*, 2002), Normfinder (Andersen *et al.*, 2004), Bestkeeper (Pfaffl *et al.*, 2004) and the comparative Delta Ct method (Silver *et al.*, 2006).

NormFinder and geNorm similarly ranked the top HKGs in this study, despite using different algorithms, where NormFinder ranked RPL32, GAPDH, HPRT1 and YWHAZ, and geNorm ranked YWHAZ, HPRT1, GAPDH and RPL32 as the top four most stably expressed genes, respectively. The small differences in gene ranking between two algorithms could be explained by the fact that during analysis of data by Normfinder the inter-tissue variation of gene expression is detected by the algorithm, whereas the geNorm algorithm does not take into account these inter-tissue variations during its calculations.

The geNorm algorithm determines the optimal number of HKGs required for accurate normalisation according to the stability measure (M) and the average pairwise variation of candidate HKGs by a stepwise exclusion method which excludes genes with the highest M value and ranks the candidate genes according to their expression stability. geNorm mostly detects the two optimal genes with the least variation in their expression ratio compared to other genes, whereas NormFinder is only able to identify the single best gene with the most stable expression in the tissue. It is reported that using a single HKG is often not suitable or accurate (de Jonge *et al.*, 2007) but using more than three HKGs is often unnecessary for gene expression studies (Erkens *et al.*, 2006).

The main limitation of geNorm is its sensitivity to coregulation (Andersen *et al.*, 2004). Coregulated genes have similar mRNA expression patterns by virtue of being in similar biological pathways (e.g. ribosomal proteins), therefore genes with similar functions are likely to be regulated via the same mechanisms and will have similar M values in the pairwise analysis (Allocco *et al.*, 2004). In contrast, NormFinder takes into account the inter- and intra-group variations and is not significantly affected

by coregulation of HKGs (Beekman *et al.*, 2011). Most HKGs that were chosen in this study, apart from 18S and 28S, belong to different functional classes thus avoiding coregulation as a problem in the analysis.

Nevertheless, results based on the two different algorithms, NormFinder and geNorm, were highly similar for normalisation of gene expression in equine kidneys. It was decided to use the geNorm ranking as it calculates V values for the proposed HKGs, which are useful for deciding the optimal number of HKGs to be used in gene expression studies (Vandesompele *et al.*, 2002). The geNorm algorithm reduces the pairwise variation between samples by inclusion of an additional HKG which indicates the number of HKGs required to achieve an arbitrarily selected threshold of HKG stability, a recommended cut-off value of 0.15. Therefore, it was decided that since YWHAZ and HPRT1 genes had good stability in both geNorm and NormFinder they would be appropriate to use as HKGs in further gene expression studies on kidney tissue from horses.

Studies have been reported where the most stable HKGs in kidneys of other species were determined. The most stable renal HKGs reported in the cat were RPL30 (ribosomal protein L30), HMBS (hydroxymethylbilane synthase), YWHAZ and B2M (Penning *et al.*, 2007), in dogs RPS19 (ribosomal protein S19), RPS5 (ribosomal protein S5), B2M, and HPRT (Brinkhof *et al.*, 2006) and RPL13A (ribosomal protein L13a) and RPL32 (Peters *et al.*, 2007), and in cattle GAPDH and YWHAZ (Lisowski *et al.*, 2008). This suggests that, to a certain degree, reference genes are species independent. However, YWHAZ and HPRT appear to be fairly stably expressed in the kidney of many species.

Two other studies have examined the stability of HKGs in equine renal tissue (Ahn *et al.*, 2011; Zhang *et al.,* 2009). However, in these studies, pan-tissue HKG which were not specific to the kidney, were examined. The proposed HKGs for normalisation in these studies were Ubiquitin B (UBB) (Ahn *et al.*, 2011), and 18S (Zhang *et al.,* 2009). UBB was not one of the HKGs that were used in the current study, and 18S ranked as the one of the least stable HKGs by both geNorm and NormFinder programs for equine. The previous studies examined different numbers of HKGs and validated them across a large number of tissues, and then chose the most stable HKG to be used in all of those tissues. The main focus of the current study was to determine the most stable HKGs in the kidney. Therefore, any comparison of different gene expression between individuals should consider that the expression of HKGs could vary according to the target tissue. Another limitation of previous studies is that only one HKG was suggested. Using a single HKG is often not suitable and not accurate for normalisation in gene expression studies. Hence, specific normalisation of potential HKGs for the respective tissue is an essential step for any gene expression study.

In summary, the current study is the first to specifically examine the stability of HKGs in the equine kidney and suggests that a combination of YWHAZ and HPRT1 genes can be used as HKGs for RT-qPCR studies.

Sections of this chapter have been published:

S. Azarpeykan and K. E. Dittmer (2016). Evaluation of housekeeping genes for quantitative gene expression analysis in the equine kidney. *Journal of Equine Science,* Vol. 27, No. 4.

Chapter 6

Evaluation and comparison of vitamin D responsive gene expression in ovine, canine and equine kidney

6.1 Introduction

In mammals, calcium is the most abundant cation in the body (Hoenderop *et al.*, 1999). Calcium participates in many different physiological processes, including neural transmission, muscle contraction, blood coagulation, and mineralisation of bone (Alexander *et al.*, 2014). The kidney, small intestine, and bone are the three organs that have key roles in calcium homeostasis (Hoenderop *et al.*, 2005).

The kidney plays an important role in calcium homeostasis through the filtration, reabsorption and excretion of calcium (Hoenderop *et al.*, 2005). The amount of calcium excreted in the urine is usually low as about 98%–99% of filtered calcium is reabsorbed by the renal tubules (Blaine *et al.*, 2015). However, increased intestinal calcium absorption or augmented calcium resorption from bone can result in increased plasma calcium concentration leading to increased excretion of calcium in the urine due to compensatory reductions in renal transport capacity (Pak *et al.*, 1975).

Vitamin D applies its endocrine activity on several calcium-transporting and calcium-sensing tissues to maintain plasma calcium homeostasis (Dittmer & Thompson 2011). However, vitamin D is biologically inactive and must undergo hydroxylation steps to become activated (Radlovic *et al.*, 2012). In blood, vitamin D binds to vitamin D binding protein (DBP) and is first transported to the liver, where it is hydroxylated to 25-hydroxyvitamin D (25OHD), followed by transport to the kidney where 25OHD

undergoes 1α-hydroxylation by cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1) resulting in the production of the bioactive form, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Dusso *et al.*, 2005). The kidney also expresses other genes important in vitamin D regulation and calcium homeostasis including the vitamin D receptor (VDR) and cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), the enzyme responsible for the breakdown of $1,25(OH)_2D$ (Kumar *et al.*, 2012). Vitamin D ($1,25(OH)_2D$) works as a high affinity ligand for VDR in target tissues and modulates the expression of vitamin D-dependent genes (Adams & Hewison, 2010).

Calcium is transported across the renal tubule through paracellular and transcellular pathways (Alexander *et al.*, 2014). Paracellular calcium transport is passive, whereas transcellular calcium transport is active and stimulated by $1,25(OH)_2D$ and parathyroid hormone (PTH) (Hoenderop *et al.*, 2005). Transcellular calcium transport is regulated by several vitamin D-dependent proteins, including apical influx of calcium by selective calcium channels called the transient receptor potential cation channel subfamily V member 5 (TRPV5) and TRPV6. Calcium diffuses through the cell to the basolateral membrane bound to calcium-binding proteins, such as calbindinD_{9k} ($calD_{9k}$) and calbindinD_{28k} ($calD_{28k}$), and is then secreted across the basolateral membrane via a plasma membrane calcium ATPase (PMCA) and/or sodium calcium exchanger 1 (NCX1) (van de Graaf *et al.*, 2007; Hoenderop *et al.*, 2005).

Species differ considerably in the physiology of their vitamin D and calcium metabolism and there are gaps in the current understanding of calcium transport mechanisms and calcium channels in veterinary science. The aims of the current study were to determine the relative abundance of calcium channels and vitamin D responsive genes (TRPV5, TRPV6, $calD_{9k}$, $calD_{28k}$, PMCA, NCX1, CYP27B1, CYP24A1 and,

VDR) in ovine, canine and equine kidney using real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR), and then to perform a comparison of the expression and correlation of these genes between sheep, dogs and horses in order to determine if the relationships between vitamin D responsive genes were similar between species. In addition, this study aimed to determine if differences in vitamin D responsive gene expression in kidney of horses contributed to the increase urinary excretion of calcium that is a feature of this species.

6.2 Materials and methods

6.2.1 Sample collection

Kidney samples were harvested post-mortem (Pathobiology Section post-mortem room, Massey University, Palmerston North) from 10 healthy adult horses (five Thoroughbreds, five Standardbreds) 4-14 years of age, 10 healthy adult Romney cross sheep 4-6 years of age, and five healthy adult mixed breed dogs 1-4 years of age, that were euthanised for reasons unrelated to this study. Tissue samples for RNA isolation were collected within half an hour of euthanasia, cut into small pieces, snap frozen in liquid nitrogen and stored at -80˚C until processing. Adjacent samples were collected into 10% neutral buffered formalin and processed for histological examination. Haematoxylin and eosin (H & E) stained sections were examined to confirm the absence of significant lesions.

6.2.2 RNA extraction and reverse transcription

Total RNA was extracted from renal tissue samples using the RNeasy Mini Kit (QIAGEN, USA) including the optional on-column DNAse digestion step (RNase-Free DNase Set, Qiagen) with the following protocol. Each kidney sample was thawed on ice

and then cut into smaller portions using scalpel blade. A maximum of 30 mg of each sample was then placed into a 2 mL micro tube containing 2 x 5 mm stainless steel ball bearings and 600 µL of Buffer RLT reagent. The tubes were homogenized by agitation in a Mini-Beadbeater-16 (BioSpec, Oklahoma, USA) for 1 min and then centrifuged for 3 min at 16000 xg. Then 600 µL of 70% ethanol was added to the tubes and mixed well. Subsequently, 700 µL of the homogenate was transferred to an RNeasy spin column placed in a 2 mL collection tube. The spin column was centrifuged for 30 s at 8000 xg and the flow-through discarded. Subsequently, the remainder of the RNA extraction was performed according to the manufacturer's instructions. RNase-free water (50 µL) was directly added to the spin column membrane and centrifuged for 1 min at 8000 xg to elute the RNA. Samples were immediately stored at -80°C until required, except for a 4 µL aliquot which was tested for RNA and DNA concentration (to check for absence of DNA) using the Qubit® 2.0 Fluorometer and Qubit® RNA HS and DNA HS Assay kits (Invitrogen, Life Technologies Corporation, California, USA) as per manufacturer's instructions.

The TURBO DNA-free™ Kit (Ambion®, Life Technologies Corporation, California, USA) was used for treatment and removal of any contaminating genomic DNA from the extracted RNA samples with the following protocol. The TURBO DNase Buffer reagent (5 µL) and TURBO DNase (1 µL) were added to the RNA sample, mixed gently and incubated at 37°C for 30 min. Then 5 µL of resuspended DNase Inactivation reagent was added to the RNA sample, mixed well and incubated at room temperature (22-25°C) for 5 min, with occasional mixing to redisperse the DNase Inactivation reagent. The sample was centrifuged at 10,000 xg for 1.5 min, then the clear (RNA-

containing) supernatant was transferred to a fresh 2 mL tube and stored at -80˚C until later analysis.

6.2.3 Primer design

All primers were designed using the National Centre for Biotechnology Information (NCBI), primer Basic Local Alignment Search Tool (BLAST) (Bethesda, Maryland, USA) (http://www.ncbi.nlm.nih.gov/nucleotide).

The primers were designed to have the following features: PCR product of less than 150 bp, primers must span an exon-exon junction, show no complementarity to extraneous targets, have minimal primer-dimer and primer hairpin formation, and similar melting temperature and guanine-cytosine content (GC content %). The best primer set was selected and the PCR amplicon sequence tested for secondary structures at 60˚C using the mFold program (http://mfold.rit.albany.edu/?q=mfold). If there were no hairpin loops in the primer binding area, then the primer pair was selected. If there was hairpin loop formation, the entire process was repeated until all conditions were satisfied. Primer sequences are listed in Tables 6.1 to 6.3.

6.2.4 Real-time PCR

Real-time PCR was performed using the StepOne Plus real-time PCR machine (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA). The primer concentrations determined to be optimal for each primer pair PCR are shown in Tables 6.1 to 6.3. Real-time PCR reactions (10 µL) contained 5 µL Fast SYBR Green real-time PCR Master Mix (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA), the primer pair at concentrations given in Tables 6.1 to 6.3, 10 ng of cDNA template, and RNAase-DNAse free water. The PCR protocol consisted of a denaturation step at 95°C

for 20 s, followed by 40 cycles at 95°C for 3 s and 60°C for 30 s; ending with a melt curve ranging from 60°C to 95°C with a heating rate of 0.3°C/15 s. Negative controls of water and reaction mix without reverse transcriptase were included in every PCR run and all samples were run in duplicate. Standard curves were produced for each target to determine the accuracy (R^2 ≥0.98) and efficiency (90-110%) of the real-time PCR reactions (Tables 1 to 3). The real-time data were analysed using the StepOne plus software (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA) and then exported into an Excel datasheet (Microsoft Excel 2010) for further analysis.

All samples were normalised relative to the expression of appropriate housekeeping genes (HKGs) as follow: zeta polypeptide (YWHAZ) (Kayis *et al.*, 2011) and hypoxanthine phosphoribosyltransferase 1 (HPRT1) in horses (Chapter 5), ribosomal protein L32 (RPL32) and beta-actin (ß-actin) in dogs (Peters *et al.*, 2007), and succinate dehydrogenase complex (SDHA) and phosphoglycerate kinase 1 (PGK1) in sheep (personal communication K.C. Perera *et al.*).

Table 6.1 Ovine kidney genes. Transient receptor potential cation channel subfamily V member 5 (TRPV5), transient receptor potential cation channel subfamily V member 6 (TRPV6), Calbindin D_9k ($calD_{9k}$), Calbindin $D_{28}k$ ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR) in ovine kidney.

Gene	Full gene name	GeneBank (Accession code)	Primer (5'-3')	Amplicon length (bp)	Primer concentration	PCR Efficiency	Regression coefficient (R^2)
TRPV5	Transient receptor potential cation channel subfamily V member 5	XM_004008320.1	F: CGGGTCAGCAATCATCCTAT R: ATTGTGATGACGTGGAATGG	108	250:250 nM	110.6%	0.96
TRPV6	Transient receptor potential cation channel subfamily V member 6	EU310242.1	F: TGATGCTGGAGAAGAAGCTG R: TGGTTGATGTCCTGTTTCTCTT	118	250:250 nM	93.9%	0.98
$calD_{9k}$	Calbindin D9k	NC_019484.1	F: TCACTGCTGAACGGCCAGGACA R: AGCTCCTCCTTGGACAGTTGGT	122	250:250 nM	99.3%	0.99
$calD_{28k}$	Calbindin D28k	NC_019466.1	F: GCTGGAAAAAGCAAACAAGACTGTTGA R: TTCTCCTGCACGGGTAGTAATCTGG	139	400:400 nM	90.8%	0.98
PMCA	Plasma membrane calcium ATPase	NC_019460.1	F: TGCAGCCATAGTATCATTGGGCCT R: TTGCCGCTCCTTCAATCCAACCA	128	250:250 nM	94.9%	0.99
NCX1	Sodium calcium exchanger 1	NC_019460.1	F: TGGCGAACATCAACCCGTGCT R: TGCAGATTGTAGCGTCGCATCTCG	93	300:300 nM	98.4%	0.99
CYP27B1	Cytochrome P450 family 27 subfamily B polypeptide 1	XM_004006519.1	F: GCAGAGCTTGAGTTGCACAT R: CTTCTCTCAGGCACCAGGAC	119	250:250 nM	102.2%	0.92
CYP24A1	Cytochrome P450 family 24 subfamily A polypeptide 1	NC_019470.1	F: CTGTGATGAGAGAGGCCGCATTGA R: AGCTTCCTCCCCTGCCTTCTT	128	600:600 nM	103.9%	0.99
VDR	Vitamin D receptor	NC_019460.1	F: TCATGCTGCGCTCCAACCAGT R: TGGAACTTGATGAGGGGCTCGAT	140	400:400 nM	93.5%	0.98
SDHA†	Succinate dehydrogenase complex	NC_019460.1	F: ACCTGATGCTTTGTGCTCTGC R: CCTGGATGGGCTTGGAGTAA	126	300:300 nM	97.05%	0.99
PGK1†	Phosphoglycerate kinase 1	NC_019460.1	F: ACTCCTTGCAGCCAGTTGCT R: AGCACAAGCCTTCTCCACTTCT	101	300:300 nM	94.5%	0.99

† Personal communication K.C. Perera *et al.*

Table 6.2 Canine kidney genes. Transient receptor potential cation channel subfamily V member 5 (TRPV5), transient receptor potential cation channel subfamily V member 6 (TRPV6), Calbindin D_{9k} ($calD_{9k}$), Calbindin D_{28k} ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR) in canine kidney.

Gene	Full gene name	GeneBank (Accession code)	Primer (5'-3')	Amplicon length (bp)	Primer concentration	PCR Efficiency	Regression coefficient (R^2)
TRPV5	Transient receptor potential cation channel subfamily V member 5	XM_003639556.2	F: CCACATGCTGCAACAGAAGA R: AAGTCACAGTTCCGGTCCAG	108	500:500 nM	92.7%	0.99
TRPV6	Transient receptor potential cation channel subfamily V member 6	XM_539861.5	F: AGAGCCGAGATGAGCAGAAC R: CTTGCTGAGAGCCTGGACAT	98	500:500 nM	102.3%	0.98
$calD_{9k}$	Calbindin D9k	XM_843973.2	F: TCTTCTAGCTGCCTTGCTG R: CTTCTTTGGCTGCGTATTTT	104	900:900 nM	101.7%	0.99
$calD_{28k}$	Calbindin D28k	XM_848991.4	F: CAGGGAATCAAAATGTGTGG R: TCCTTCAGTAAAGCATCCAGTTC	107	800:800 nM	90.1%	0.99
PMCA	Plasma membrane calcium ATPase	XM_005628823.1	F: TGAAAGCTCATTGACTGGTGA R: CATTCTTCCAGAGCCTTCCA	103	250:250 nM	91.7%	0.99
NCX1	Sodium calcium exchanger 1	XM_005630379.1	F: CACCATCGGCTTGAAAGATT R: GCGTCTGCATACTGATCCTG	114	350:350 nM	104.3%	0.98
CYP27B1	Cytochrome P450 family 27 subfamily B polypeptide 1	XM_538254.4	F: GAGCTGCAAATGGCTTTGGCTCAG R: CTGTAGGTTGATGCTCCTCTCGGG	123	300:300 nM	89.2%	0.99
CYP24A1	Cytochrome P450 family 24 subfamily A polypeptide 1	XM_543059.3	F: GAGGCCGCATTGAAGACTTA R: CATTCTTCCGAAGGAGTCCA	102	450:450 nM	94.7%	0.98
VDR	Vitamin D receptor	XM_005636920.1	F: AGCATCCAAAAGGTCATTGG R: GCACTTGATTTCAGCAGCAC	92	900:800 nM	95.2%	0.97
RPL32‡	Ribosomal protein L32	XM_848016.1	F: TGGTTACAGGAGCAACAAGAAA R: GCACATCAGCAGCACTTCA	100	900:900 nM	98.53%	0.99
ß-actin‡	Beta-actin	AF021873	F: CCAGCAAGGATGAAGATCAAG R: TCTGCTGGAAGGTGGACAG	100	900:900 nM	102.6%	0.98

‡ Peters *et al.*, 2007

Table 6.3 Equine kidney genes. Transient receptor potential cation channel subfamily V member 5 (TRPV5), transient receptor potential cation channel subfamily V member 6 (TRPV6), Calbindin D_{9k} ($calD_{9k}$), Calbindin D_{28k} ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR) in equine kidney.

Gene	Full gene name	GeneBank (accession code)	Primer (5'-3')	Amplicon length (bp)	Primer concentration	PCR Efficiency	Regression coefficient (R^2)
TRPV5	Transient receptor potential cation channel subfamily V member 5	AY944068	F: ACACTGTGATGTTCCAGCACCTGA R: AGGAGTCGATCTCTGTGAGGTCAT	102	75:75 nM	96.9%	0.99
TRPV6	Transient receptor potential cation channel subfamily V member 6	XM_001490905.2	F: CCTCAAGCCCATCACCAGTA R: GCCATCCTTAGGGGTCAAGT	97	250:250 nM	104.5%	0.97
$calD_{9k}$	Calbindin D9k	AY229893	F: GCGTGAAAAAGTCTCCTGAA R: TCACTAACACCTGGAATTCTTCAA	218	700:700 nM	97.08%	0.99
$calD_{28k}$	Calbindin D28k	NM_001163952	F: ACGGCTTGGTCTTCCTTGACAG R: TGCGTGTGTGAGTATGTGTGAGTG	101	75:100 nM	102.7%	0.99
PMCA	Plasma membrane calcium ATPase	DQ160196	F: AAACGCCGCCGATAGTGCAAATAC R: CCTTCTTGTGCATGTTGGCCTTCTTC	182	200:200 nM	102.7%	0.99
NCX1	Sodium calcium exchanger 1	DQ178640	F: TGGCCATAACTTTACTGCGGGAGA R: GGACCACATAAACACAAAGCGCGA	100	500:250 nM	92.4%	0.99
CYP27B1	Cytochrome P450 family 27 subfamily B polypeptide 1	NM_001163957.1	F: CAGAGACATTCATGTGGGTGA R: GCTGGACGAAAAGAATTTGG	117	300:300 nM	93.9%	0.98
CYP24A1	Cytochrome P450 family 24 subfamily A polypeptide 1	XM_003363957.2	F:GTGTGATGAAAGAGGCCACATTGA R: CGTTCTGCTGGAGGAGCCCG	113	350:350 nM	91.6%	0.99
VDR	Vitamin D receptor	XM_005611070.1	F: ACAGCATCCAAAAGGTGGTC R: TGACTTCAGCAGCACGATCT	89	500:500 nM	99.9%	0.99
YWHAZ[Ɛ]	Zeta polypeptide	XM_001492988.3	F: TGTTGTAGGAGCCCGTAGGT R: ATTCTCGAGCCATCTGCTGT	95	300:300 nM	93.4%	0.99
HPRT1	Hypoxanthine phosphoribosyltransferase 1	AY372182	F: TTGCTGACCTGCTGGATTAT R: TTATGTCCCCTGTTGACTGGT	120	500:500 nM	93.8%	0.99

[Ɛ] Kayis *et al.*, 2011

6.3 Statistical analysis of the RT-qPCR data

Expression of nine calcitropic genes (TRPV5, TRPV6, calD$_{9k}$, calD$_{28k}$, PMCA, NCX1, CYP27B1, CYP24A1 and VDR) was evaluated in 10 ovine, five canine and 10 equine kidney samples. The Ct (cycle threshold) values from RT-qPCR runs were exported into an Excel datasheet (Microsoft Excel 2010). The Ct values of each gene in each animal were normalised with the species related HKGs using:

$$\hat{\mathrm{C}}t_{ij} = Ct_{ij} - \overline{Rt_j} + \overline{Rt}$$

where Ct_{ij} is the level of expression of gene *i* for sample *j*, $\overline{Rt_j}$ is the mean expression of sample *j* on the HKGs, and $\overline{Rt}$ is the mean expression on the HKGs across all samples (Hellemans *et al.*, 2007). The expression per gene was estimated using a linear mixed effects model including a random effect accounting for the two replicates per sample. The model equation was:

$$\hat{\mathrm{C}}t_{ijk} = Gene_i + \mu_j + \epsilon_{ijk}$$

where $\hat{\mathrm{C}}t_{ijk}$ is the normalised level of expression of gene *i* for sample *j* and replicate *k*, $Gene_i$ is the average expression for gene *i*, μ_j is a random effect for sample, and ϵ_{ijk} are the residuals. Mean expression levels and 95% confidence intervals were computed by simulating from the fitted model, accounting for the variation due to the per-sample random effects. Spearman's pairwise correlation and scatterplots were used to study the correlation between normalised expression across different genes.

All statistical models and plots were produced in R (R Development Core Team, 2014). The mixed effects model was fit using the lme4 package for R (Bates *et al.*, 2015).

6.4 Results

Tissue-specific expression of TRPV5, TRPV6, calD$_{9k}$, calD$_{28k}$, PMCA, NCX1, CYP27B1, CYP24A1, and VDR in the sheep, dog and horse kidney was analysed by RT-qPCR, and all target genes were detected (Figures 6.1 to 6.6). Low Ct values equal high expression.

6.4.1 Comparison of the expression of calcium and vitamin D responsive genes in ovine, canine and equine kidney

Differences in the normalised expression levels of calcium channels and vitamin D responsive genes TRPV5, TRPV6, calD$_{9k}$, calD$_{28k}$, PMCA, NCX1, CYP27B1, CYP24A1 and VDR were identified between ovine, canine and equine kidney, presented in Figures 6.1 to 6.3.

TRPV5 and TRPV6 had similar expression patterns in sheep, dogs and horses, with TRPV6 having greater expression than TRPV5. CalD$_{9k}$ and calD$_{28k}$ were both expressed in the ovine, canine and equine kidney. The difference in expression between calD$_{28k}$ and calD$_{9k}$ appeared to be greater in horses than sheep but the pattern was similar, in that expression levels of calD$_{28k}$ were lower than calD$_{9k}$ in both sheep and horses. In dogs, calD$_{9k}$ had lower expression than calD$_{28k}$ (Figures 6.1 to 6.3).

Ovine, canine and equine kidney showed similar patterns of NCX1 and PMCA expression, where the expression levels of NCX1 were always lower than PMCA. Differences in expression between CYP27B1 and CYP24A1 were particularly obvious between species, where horses showed very similar levels of expression for CYP27B1 and CYP24A1, while in sheep and dogs CYP27B1 expression was lower than CYP24A1. VDR had a similar high level of expression in all species (Figures 6.1 to 6.3).

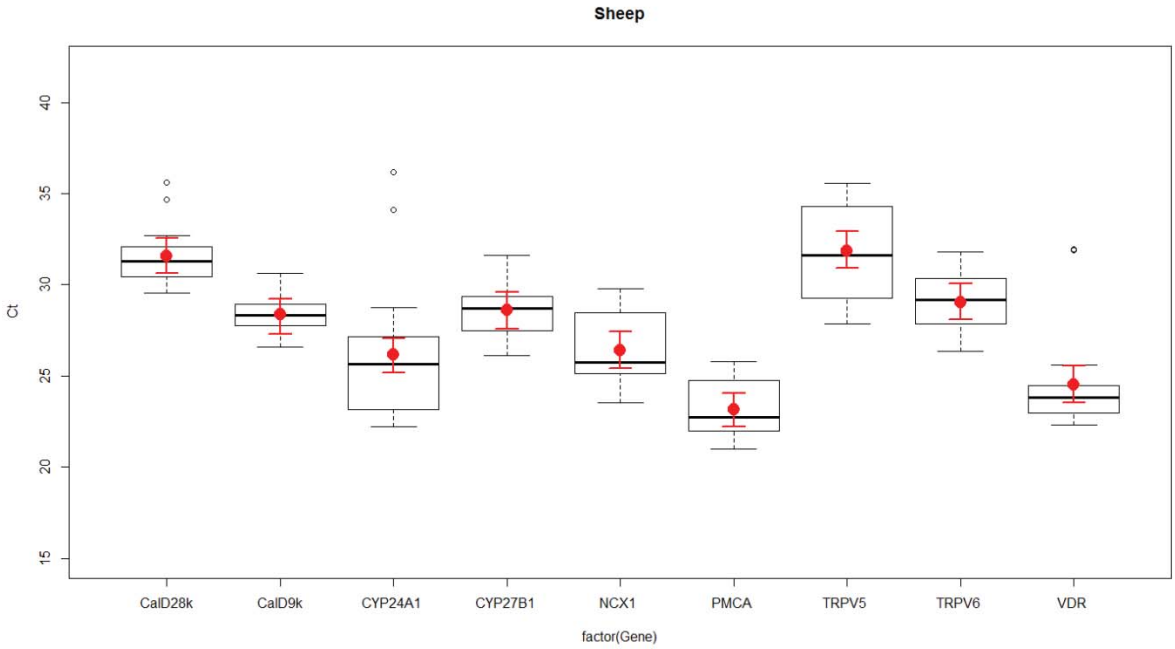


Figure 6.1 Sheep vitamin D responsive gene expression. Expression of calcium channels the transient receptor potential cation channel subfamily V member 5 (TRPV5), the transient receptor potential cation channel subfamily V member 6 (TRPV6), Calbindin D_{9k} ($calD_{9k}$), Calbindin D_{28k} ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR) in ovine kidney as determined by RT-qPCR. Boxplots represent normalised cycle threshold numbers (Ct values), where the median expression levels of genes are presented as black bars, the average expression of genes are presented as red dots, and red line bars represent 95% confidence interval (CI) of genes, accounting for replicates.

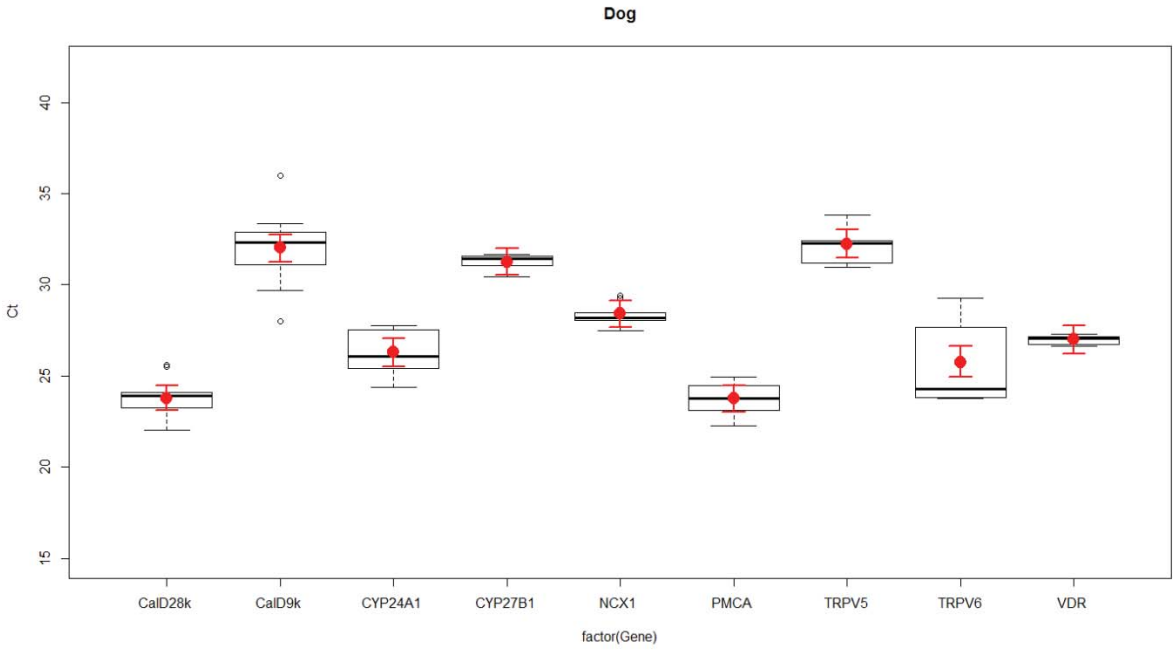


Figure 6.2 Dog vitamin D responsive gene expression. Expression of calcium channels the transient receptor potential cation channel subfamily V member 5 (TRPV5), the transient receptor potential cation channel subfamily V member 6 (TRPV6), Calbindin D_{9k} ($calD_{9k}$), Calbindin D_{28k} ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR) in dog kidney as determined by RT-qPCR. Boxplots represent normalised cycle threshold numbers (Ct values), where the median expression levels of genes are presented as black bars, the average expression of genes are presented as red dots, and red line bars represent 95% confidence interval (CI) of genes, accounting for replicates.

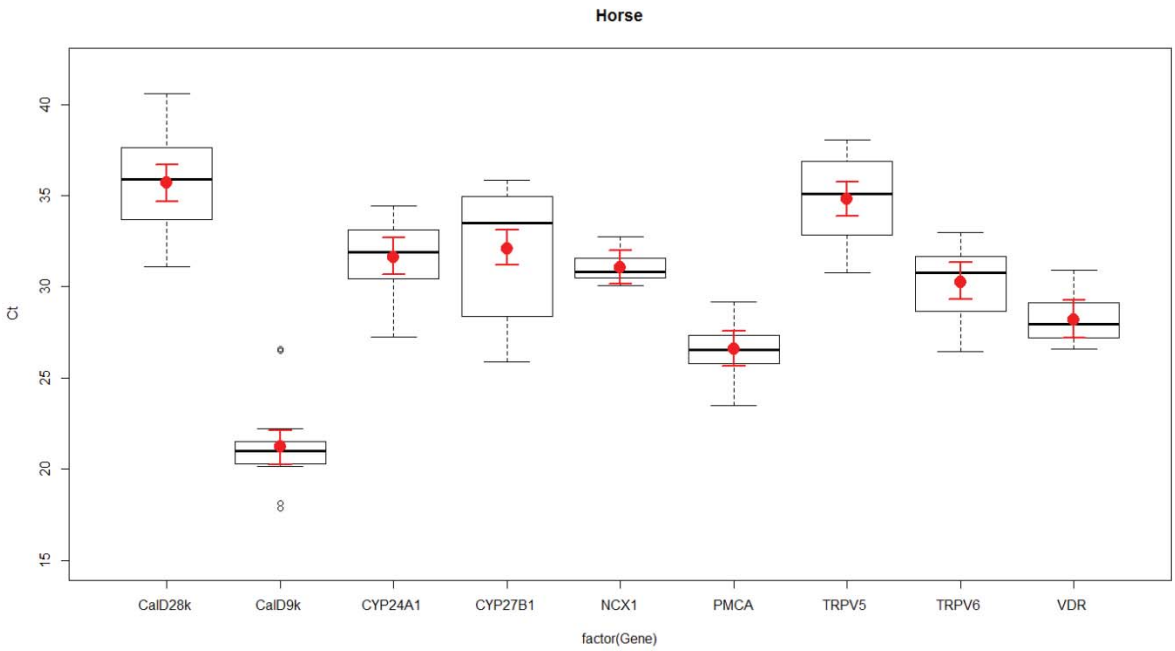


Figure 6.3 Horse vitamin D responsive gene expression. Expression of calcium channels the transient receptor potential cation channel subfamily V member 5 (TRPV5), the transient receptor potential cation channel subfamily V member 6 (TRPV6), Calbindin D_{9k} ($calD_{9k}$), Calbindin D_{28k} ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR)in equine kidney as determined by RT-qPCR. Boxplots represent normalised cycle threshold numbers (Ct values), where the median expression levels of genes are presented as black bars, the average expression of genes are presented as red dots, and red line bars represent 95% confidence interval (CI) of genes, accounting for replicates.

The RT-qPCR data were analysed and plotted to determine the pairwise correlation of vitamin D responsive genes with each other in the sheep, dog and horse kidney, presented in Figures 6.4 to 6.6.

$CalD_{28K}$ showed significant positive correlation with NCX1 and CYP24A1 in dogs, CYP27B1, TRPV5, and CYP24A1 in horses, and NCX1, CYP27B1, and TRPV5 in sheep (P<0.05). $CalD_{9k}$ showed significant positive correlation with PMCA in dogs and horses (P<0.05), whereas strong positive correlation with NCX1 and strong negative correlation with VDR was present in sheep. $CalD_{28k}$ and $calD_{9k}$ were positively correlated in sheep and horses (Figures 6.4 to 6.6).

TRPV6 and PMCA showed significant positive correlation in sheep, whereas significant negative correlation was seen in dogs and horses (P<0.05). PMCA showed

significant negative correlation with VDR in dogs, compared with significant positive correlation with VDR in horses (*P*<0.05). TRPV6 showed significant negative correlation with VDR in equine kidney (*P*<0.05). NCX1 showed significant positive correlation with TRPV5 in dogs, and significant negative correlation with VDR in horses (*P*<0.05) (Figures 6.4 to 6.6).

CYP27B1 showed significant positive correlation with NCX1 in sheep (*P*<0.05), but its expression was not significantly correlated with other genes in dogs. CYP24A1 and CYP27B1 were significantly positively correlated with TRPV5 in sheep, and TRPV5 and VDR in horses (*P*<0.05). CYP24A1 and CYP27B1 were significantly positively correlated in equine kidney (*P*<0.05) (Figures 6.4 to 6.6).

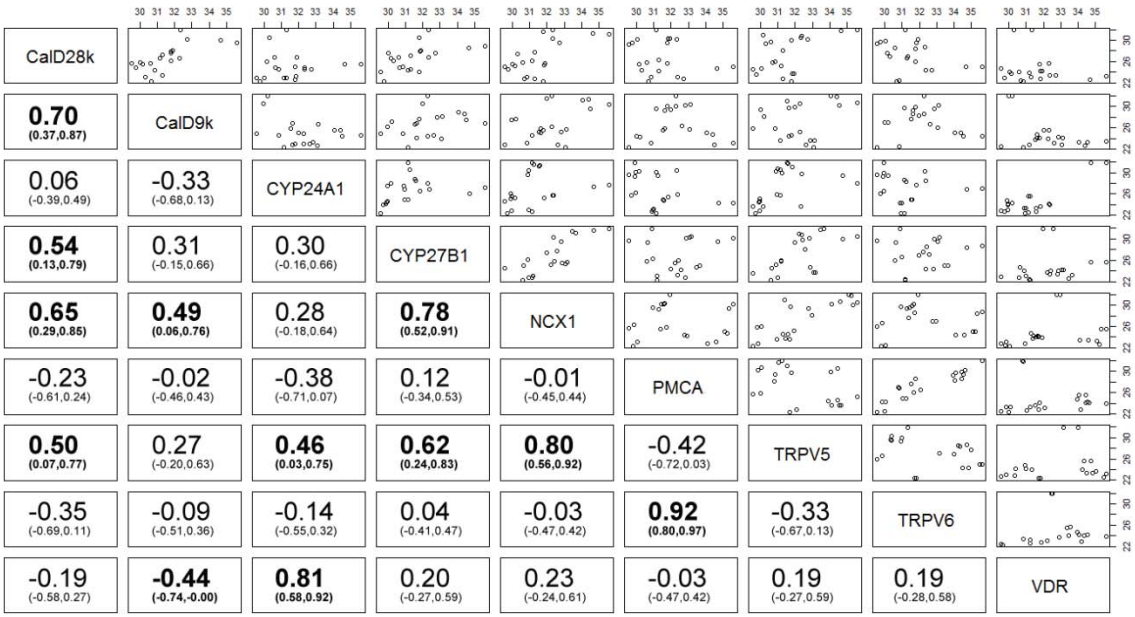


Figure 6.4 Ovine kidney vitamin D responsive genes correlation. Spearman's correlation (95% Confidence Intervals) between the transient receptor potential cation channel subfamily V member 5 (TRPV5), the transient receptor potential cation channel subfamily V member 6 (TRPV6), calbindin D_{9k} ($calD_{9k}$), calbindin D_{28k} ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR). Bolded numbers indicate statistically significant positive and/or negative correlation of different genes towards each other (P<0.05).

Figure 6.5 Canine kidney vitamin D responsive genes correlation. Spearman's correlation (95% Confidence Intervals) between the transient receptor potential cation channel subfamily V member 5 (TRPV5), the transient receptor potential cation channel subfamily V member 6 (TRPV6), calbindin D_{9k} ($calD_{9k}$), calbindin D_{28k} ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR). Bolded numbers indicate statistically significant positive and/or negative correlation of different genes towards each other (P<0.05).

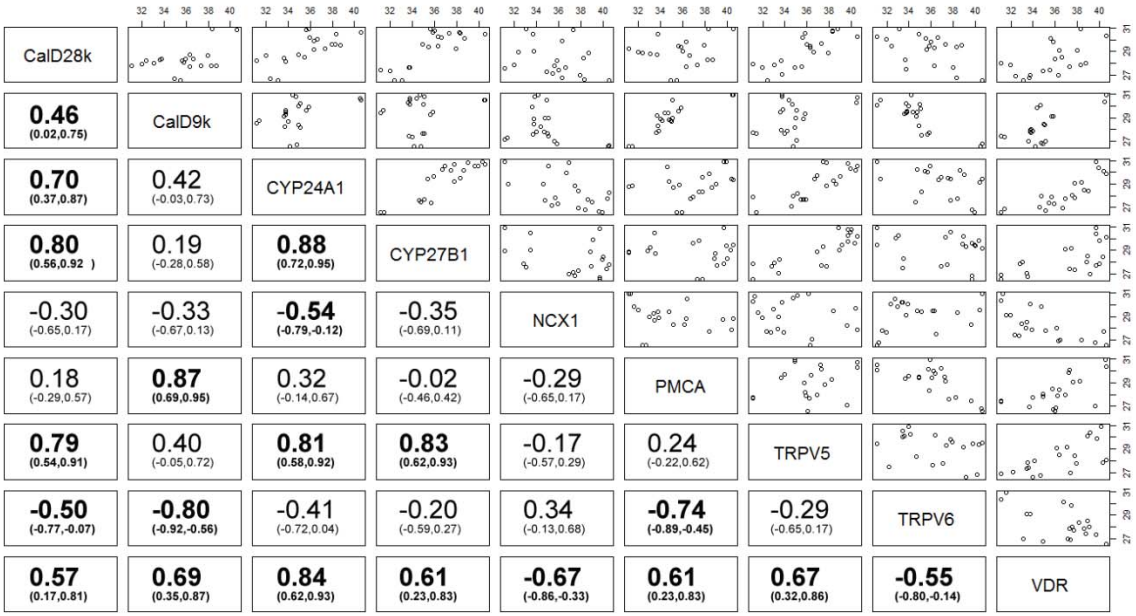


Figure 6.6 Equine kidney vitamin D responsive genes correlation. Spearman's correlation (95% Confidence Intervals) between the transient receptor potential cation channel subfamily V member 5 (TRPV5), the transient receptor potential cation channel subfamily V member 6 (TRPV6), calbindin D_{9k} ($calD_{9k}$), calbindin D_{28k} ($calD_{28k}$), plasma membrane calcium ATPase (PMCA), sodium calcium exchanger 1 (NCX1), cytochrome P450 family 27 subfamily B polypeptide 1 (CYP27B1), cytochrome P450 family 24 subfamily A polypeptide 1 (CYP24A1), and vitamin D receptor (VDR). Bolded numbers indicate statistically significant positive and/or negative correlation of different genes towards each other (P<0.05).

6.5 Discussion

To our knowledge this is the first investigation describing and comparing the expression of the vitamin D responsive genes involved in renal calcium transport in sheep, dogs and horses. Together these genes, TRPV5, TRPV6, $calD_{9k}$, $calD_{28k}$, PMCA, NCX1, CYP27B1, CYP24A1, and VDR regulate transcellular calcium transport in epithelial cells (Hoenderop *et al.*, 2005; van de Graaf *et al.*, 2007). Different studies published on calcium related genes in mice (Loffing *et al.*, 2001), humans (Boros *et al.*, 2009; Bonny & Edwards, 2013), sheep (Wilkens *et al.*, 2009; Wilkens *et al.*, 2012; Wilkens *et al.*, 2014; Herm *et al.*, 2015), rabbits (Hoenderop *et al.*, 2000), goats (Wilkens *et al.*, 2012; Wilkens *et al.*, 2014; Herm *et al.*, 2015), horses (Rourke *et al.*, 2010; Hwang *et al.*, 2011; Sprekeler *et al.*, 2011), and dogs (Palm *et al.*, 2010; Kim *et al.*, 2011), demonstrated a correlation between the expression of calcium transport genes and the capacity of cells for calcium absorption (Hoenderop *et al.*, 2000). Therefore, gene function is strongly related to their location and magnitude of expression.

The results of this study showed that TRPV5, TRPV6, $calD_{9k}$, $calD_{28k}$, PMCA, NCX1, CYP27B1, CYP24A1 and VDR were detectable in the kidney of sheep, dogs and horses although, the expression levels of each gene was different in each species. The nine genes investigated can be divided into five groups based on their function in calcium metabolism. The two selective calcium channels, TRPV5 and TRPV6, mediate apical influx of calcium into epithelial cells of the renal tubules (Hoenderop *et al.*, 1999). $CalD_{9k}$ and $calD_{28k}$, as vitamin D-dependent calcium-binding proteins, mediate the diffusion of calcium through the basolateral membrane (Hoenderop *et al.*, 2005), while PMCA and NCX1 mediate the secretion of calcium across the basolateral

membrane (Magyar *et al.*, 2002). These calcium channels and calcium binding proteins are vitamin D responsive genes, therefore 1,25$(OH)_2$D initiates its biological effect in gene expression via binding to VDR (Adams & Hewison, 2010). VDR interacts with the retinoid X receptor (RXR) to form a heterodimer receptor complex, VDR-RXR, which binds to vitamin D responsive elements in the region of genes directly controlled by 1,25$(OH)_2$D and alters gene expression (Haussler *et al.*, 2013). CYP27B1 and CYP24A1 play a critical role in governing plasma concentration of 1,25$(OH)_2$D through activation and degradation of vitamin D (Jones *et al.*,2012).

Mammalian transient receptor potential channels (TRPs) are cation-permeable channels involved in a variety of physiological processes and are categorised into six different subgroups, including TRPV channels (Hoenderop & Bindels, 2008). The most calcium selective channels within the TRPV subgroup are TRPV5 and TRPV6, which are closely related and considered the gatekeepers of transcellular calcium transport (Hoenderop *et al.*, 1999). Although TRPV5 and TRPV6 are very similar from an electrophysiological point of view and share many functional properties, including calcium dependent inactivation and regulation by 1,25$(OH)_2$D and calcium (Hoenderop *et al.*, 2003), some significant differences were revealed through detailed comparison of the N- and C-termini of these channels. For instance, TRPV5 channels are approximately 100 times more sensitive to the potent channel blocker ruthenium red than TRPV6 channels, the kinetic differences between calcium (Ca^{2+}) and barium (Ba^{2+}) currents are more pronounced for TRPV6 than for TRPV5, and TRPV6 has faster initial inactivation than TRPV5 (Hoenderop *et al.*, 2001). It is suggested that TRPV5 is involved in transcellular calcium reabsorption in the kidney and predominantly expressed in the distal convoluted (DCT) and connecting tubule (CNT), whereas TRPV6

regulates and increases calcium transport in the intestine (Hoenderop*et al.*, 2005). Our findings showed that both TRPV5 and TRPV6 are expressed in ovine, canine and equine kidney, which is in agreement with previous reports (Palm *et al.*, 2010; Rourke *et al.*, 2010; Wilkens *et al.*, 2009; Hwang *et al.*, 2011; Kim *et al.*, 2011). However, the high renal expression of TRPV6 compared to TRPV5 was an unexpected finding, since TRPV5 is thought to be the main renal and TRPV6 the main intestinal apical calcium channel in mammals (Mensenkamp *et al.*, 2007). TRPV6 has quicker calcium dependent inactivation and slower recovery from inactivation than TRPV5 (Hoenderop *et al.*, 2001), therefore greater expression of this gene may be needed in the tissue to compensate for this and regulate calcium transport. While TRPV5 and TRPV6 are the principal targets of calcitropic hormones, extracellular and intracellular signalling by plasma calcium concentration and associated proteins play important roles in their regulation (Schoeber *et al.*, 2007). There was limited information on the dietary regimen of the animals used in the present study, including the dietary content of calcium, phosphorus and other minerals, however, the results do indicate that TRPV6 acts cooperatively with TRPV5 in the kidney and plays a substantial role in renal calcium reabsorption.

Calbindin proteins are specialised calcium buffering proteins involved in facilitating different steps of epithelial calcium transport and control a continuous in-flow of intracellular calcium from the TRPV5 and TRPV6 channels (Hoenderop *et al.*, 1999; Hoenderop *et al.*, 2005; Schoeber *et al.*, 2007). The findings in this study indicate that $calD_{28K}$ and $calD_{9K}$ are co-expressed in the kidney, suggesting that $calD_{9K}$ acts cooperatively with $calD_{28K}$ and has an important role in calcium reabsorption in the kidney of sheep, horse and dog. The expression patterns of $calD_{28K}$ in ovine, canine

and equine kidney have been previously studied (Palviainen *et al.*, 2012; Palm *et al.*, 2010; Rourke *et al.*, 2010), although to the authors' knowledge there are no studies performed on $calD_{9K}$ in ovine and canine kidney. The only previously published research on $calD_{9K}$ in equine kidney reported that the expression of $calD_{28k}$ was much higher in the horse kidney than $calD_{9k}$ (Rourke *et al.*, 2010), in contrast with the present findings. The horses used in the current study were older (4-14 years of age) than horses used in the previous research (2-8 years of age), which may explain the differences as calcium demands are higher in younger horses. In the study by Rourke *et al.*, 2010, $calD_{28k}$ expression was detected primarily in the kidney and $calD_{9K}$ in the intestine, however, the results of the current study indicated that $calD_{9K}$ expression was significantly higher than $calD_{28K}$ in ovine and equine kidney but not in canine kidney. This may be associated with the nutritional alkali load in grazing animals (sheep and horses) compared with the acid load in carnivores (dogs), based on the nature of their diet. Urine composition is directly related to the animals diet, leading to alkaline urine (pH 7.0-8.0) in herbivores and more acidic urine in carnivores (pH 5.5-7.0) (Stockham & Scott, 2008). Different dietary sources, along with our finding, might suggest $calD_{9K}$ is the specialised calcium buffering protein for calcium transport in grazing animals, whereas $calD_{28K}$ is the specialised form in carnivores.

The extracellular concentration of calcium is much greater than the intracellular concentration and calcium cannot exit cells by diffusion. The extrusion of calcium across the basolateral membrane into the peritubular fluid is mediated by PMCA (Borke *et al.*, 1989) and/or NCX1 (Costanzo & Windhager, 1978). PMCA is powered by ATP, where one molecule of ATP is hydrolysed to export each calcium ion, while NCX1 removes a calcium ion in exchange for three sodium ions entering the cell

(Magyar *et al.*, 2002). PMCA has a high affinity and low capacity towards calcium, therefore it binds tightly to calcium even when the concentration of calcium in the cell is low, but the removal rate of calcium from the cell through the PMCA pump is slow (Strehler, 2015). In contrast, NCX1 has a low affinity and high capacity towards calcium, thus it does not bind tightly to calcium and is capable of transporting and removing large amounts of calcium rapidly (Magyar *et al.*, 2002). PMCA and NCX1 are expressed in many different tissues including the kidney, and their activities complement each other (Magyar *et al.*, 2002). Our findings showed high expression of PMCA and NCX1, suggesting the co-localisation of NCX1 and PMCA in ovine, canine and equine kidney, which is in agreement with the previous studies in dogs and horses (Palm *et al.*, 2010; Rourke *et al.*, 2010; Hwang *et al.*, 2011; Kim *et al.*, 2011). We found that the most dominant channel with relatively higher expression in the kidney was PMCA, which fits with the high expression of TRPV6 and suggests slow transport of calcium is predominantly used in the kidney of the animals studied. These findings suggest that TRPV6 and PMCA are expressed more for slow continuous transepithelial calcium transport in the kidney with TRPV5 and NCX1, as fast transepithelial calcium transport, perhaps being expressed as required to rapidly remove large amount of calcium from the cell. Another possible explanation could be the animals being in a state of normocalcemia at the time of sampling.

When the correlation of genes was examined it became clear that TRPV6, $calD_{9k}$ and/or $calD_{28k}$, and PMCA were significantly correlated ($P<0.05$) in ovine, canine and equine kidney. Therefore, it can be suggested that this group of genes are primarily in control of the rate of entry, transport and removal of calcium in renal epithelial cells.

Urine composition and the concentration of calcium are different in mammalian species, with a direct link to their diet. For instance, reported fractional urinary clearances for calcium (FE_{Ca}%) in sheep range from 0.010–8.90, in dogs from 0.04–5.6, and in horses from 1.3–33.0 (Lefebvre *et al.*, 2008). Horses have higher urinary excretion of calcium and lower serum 1,25$(OH)_2$D concentration compared with sheep and dogs (Maenpaa *et al.*, 1988; Breidenbach *et al.*, 1998). In equine kidney, CYP27B1 and CYP24A1 had very similar expression levels, while in sheep and dogs expression of CYP24A1 was higher than CYP27B1. This suggests that in horses the rate of activation and degradation of vitamin D (1,25$(OH)_2$D) occurs in a parallel manner and that 1,25$(OH)_2$D production is tightly controlled, while in sheep and dogs degradation of 1,25$(OH)_2$D outstrips production. However, in sheep and dogs there was no consistently significant correlation of the calcium transporting genes with the VDR, whereas in horses all the calcium transport genes were significantly correlated with VDR. This finding may suggest that even with the very low serum concentration of 1,25$(OH)_2$D in horses compared to other animals, it does closely and tightly regulate these vitamin D-responsive calcium transport genes in the kidney. It does however not explain the high excretion of calcium in equine urine.

The current study has some limitations and it is not possible to draw firm conclusions regarding transepithelial calcium transport in the kidney without information on serum biochemistry and the dietary history of the animals. In addition, RT-qPCR performed in this study is a snapshot in time of calcium homeostasis in the animal, and factors such as age, diet, and life history will alter gene expression.

The correlated expression patterns of TRPV6, $calD_{9k}$ /$calD_{28k}$, and PMCA detected in the ovine, canine and equine kidney emphasise the importance of these

genes in active transepithelial calcium transport in the kidney of these species. In equine kidney, all vitamin D-responsive and calcium transporting genes were highly correlated with VDR, whereas sheep and dogs did not show such high correlation, indicating the important regulatory function of VDR in the active renal transepithelial calcium transport in horses. The renal expression pattern and correlation of CYP27B1 and CYP24A1 in horses were particularly different to those in sheep and dogs suggesting that despite low serum vitamin D concentrations, vitamin D still plays a significant role in calcium metabolism in horses. The strong co-expression of renal vitamin D-responsive genes suggests that these genes work in harmony to facilitate the transepithelial calcium transport in ovine, canine and equine kidney.

Sections of this chapter have been published:

Azarpeykan S, Dittmer KE, Marshall JC, Perera KC, Gee EK, Acke E, et al. (2016). Evaluation and Comparison of Vitamin D Responsive Gene Expression in Ovine, Canine and Equine Kidney. PLoS ONE 11(9): e0162598. doi:10.1371/journal.pone.0162598

Chapter 7

General discussion

7.1 Introduction

This thesis set out to explore vitamin D metabolism in horses and has identified novel findings that help explain some of the mysteries concerning vitamin D physiology in horses and the role of vitamin D in calcium metabolism. The literature on this subject, specifically in the context of equine vitamin D, is lacking and this study sought to fill some of the gaps in our knowledge. The research questions included:

1. How do common management practices of blanketing affect the synthesis of vitamin D in horses?
2. What is the main source of vitamin D in horses? How do horses obtain their vitamin D requirements?
3. How does season affect serum vitamin D concentrations in horses, and does this impact other serum analytes?
4. Does the circadian rhythm in vitamin D change at different times of year?
5. Are the relationships between calcitropic hormones, Ca, P, and Mg in horses similar to those in other species?
6. Does assessing the expression of vitamin D responsive genes involved in active calcium transport in horse kidney compared with other species tell us why horses have high urinary excretion of calcium?

7.2 Summary of the results presented in this thesis

The main experimental findings are chapter specific and are summarised below:

- Blanketing does not have any impact on serum vitamin D metabolites ($25OHD_2$, $25OHD_3$ and $1,25(OH)_2D$), PTH, Ca, P, and Mg concentrations in horses
- The main vitamin D metabolite in serum is $25OHD_2$ and the concentration of $25OHD_3$ is undetectable and/or below the detection limit of the assay
- Season has a direct impact on serum concentration of vitamin D metabolites ($25OHD_2$, and $1,25(OH)_2D$), and its related serum analytes
- A circadian rhythm occurs for iCa, $1,25(OH)_2D$ and PTH in summer, but disappears in winter
- Equine skin appears to be unable to synthesise measurable amounts of vitamin D_3
- TRPV6, $calD_{28k}$, and PMCA appear to be the main pathway involved in active transepithelial calcium transport in equine kidney
- Vitamin D responsive and calcium transporting genes are highly correlated with VDR in equine kidney, in comparison to other species where the correlation between VDR and vitamin D responsive genes is less obvious.
- CYP27B1 and CYP24A1 have very similar levels of expression and significant positive correlation towards one another in the kidney of horses, whereas CYP24A1 expression is much greater than CYP27B1 in the kidney of dogs and sheep.

7.3 Experimental Findings

7.3.1 Vitamin D synthesis in horses

The main objective of Chapters 2 to 4 was to determine the effect of sunlight/UVB radiation on vitamin D production in horses. Studies in herbivores suggest that these animals are likely able to produce vitamin D_3 in their skin (Chaudhary & Care, 1985; Hymoller & Jensen, 2010; Dittmer *et al.*, 2011; Judson *et al.*, 2011; Kohler *et al.*, 2013, Kovacs *et al.*, 2015). Shorn sheep showed higher concentrations of vitamin D than unshorn ones (Smith & Wright, 1984; Chaudhary & Care, 1985) and skin coverage of Danish Holstein dairy cows resulted in decreased $25OHD_3$, suggesting skin coverage and serum vitamin D ($25(OH)D_3$) concentrations were strongly inversely correlated (Hymoller & Jensen, 2010). Blankets are commonly worn by horses during cold seasons to keep them warm and dry, and during hot seasons to keep them fly-free, and are a common practice among horse owners. Therefore, the question arises as what impact blanketing has on vitamin D synthesis in horse skin. For instance, do horse owners need to be concerned about vitamin D concentrations in their horses when they are wearing a blanket and, if so, how much vitamin D supplements should be added to their feed to fulfil their dietary requirements?

Our studies on the impact of skin coverage and its relation to the serum concentration of vitamin D metabolites ($25OHD_3$, $25OHD_2$, and $1,25(OH)_2D$) in horses showed that blanketing has no effect on serum vitamin D metabolite concentrations. In addition, the serum concentration of $25OHD_3$ in horses was either undetectable or below the detection limit of the assay; the main form of 25OHD in circulation being

$25OHD_2$. These finding were in contrast to most studies on mammalian species, which conclude that skin coverage does have a direct impact on the synthesis of $25OHD_3$ in mammalian skin, particularly in humans (Matsuoka *et al.*, 1992; Salih, 2004; Hymoller & Jensen, 2010).

This finding led us to question whether horses are able to produce vitamin D_3 in their skin. Studies in domestic herbivores have shown that sheep (Chaudhary & Care, 1985; Dittmer *et al.*, 2011; Judson *et al.*, 2011; Kohler *et al.*, 2013, Kovacs *et al.*, 2015), goats (Kohler *et al.*, 2013; Kovacs *et al.*, 2015), and cows (Hymoller & Jensen, 2010) are able to produce vitamin D_3 in their skin. However, our results from the irradiation of horse skin with UVB light suggested that horses may not be able to effectively synthesise vitamin D_3 in their skin, and differ in this regard from other domestic grazing animals.

The unexpected results obtained from these studies (Chapter 2 to 4) suggest that horses rely on their diet as the primary source of vitamin D. Therefore, we can conclude that $25OHD_2$ is probably the main metabolic precursor for $1,25(OH)_2D$, and should be considered as the best available index of vitamin D status in unsupplemented horses at pasture. Our findings suggest that vitamin D intake from food is mostly adequate and appears to fulfil the vitamin D requirement of horses. Any additional vitamin D supplementation in the equine diet should be reassessed to protect horses from vitamin D intoxication and its consequences (e.g. hypercalcaemia). It may be desirable for vitamin D concentrations in food consumed by horses to be estimated through seasonal feed analysis and the mean daily intake of vitamin D from supplemental sources adjusted accordingly. In addition, given $25OHD_2$ is the form present in the natural situation, perhaps supplementing with ergocalciferol (vitamin

D_2) rather than cholecalciferol (vitamin D_3) would be more appropriate and effective in horses, although further study needs to be done in this regard.

7.3.2 Vitamin D and its relation to PTH and serum ions in horses

The findings on the effect of season, and time of day on serum vitamin D ($25OHD_2$ and $1,25(OH)_2D$), PTH, iCa, tCa, P, and tMg in horses are presented in Chapter 2 and 3. Due to the lack of assays available for $25OHD_2$, $25OHD_3$, $1,25(OH)_2D$ and PTH measurement in horses, commercially available human tests for PTH, $25OHD_2$, $25OHD_3$ and, 1,25-dihydroxyvitamin D (Chapter 2 and Chapter 3) were validated for use in horses, making them available for equine veterinary practitioners in New Zealand. Although, sample preparation and cost may preclude and limit widespread use of these assays in practice, they may be useful for the investigation of cases where imbalances in vitamin D and/or calcium metabolism are suspected.

The findings indicated a peak in serum $25OHD_2$ and $1,25(OH)_2D$ concentrations during summer, presumably reflecting a direct effect of season on these analytes. During summer, when the amount of sunshine and UVB radiation was higher, equine serum $25OHD_2$ and $1,25(OH)_2D$ concentrations were higher, supporting the effect of sunshine and UVB radiation on the synthesis of $25OHD_2$ in the pasture/hay that was consumed by the horses. Finding on the correlation of serum analytes with each other emphasised the important roles of vitamin D metabolites ($25OHD_2$ and $1,25(OH)_2D$) and PTH in the regulation and metabolism of mineral ions in horses, in a similar manner to that in other species.

Previous studies on seasonal and circadian rhythms in horses were performed with the focus on nutrition and vitamin D supplementation (Maenpaa *et al.*, 1978, 1988a,b; Piccione *et al.*, 2008). Therefore management, as the main interference

factor, had a direct effect on serum calcitropic hormones and mineral analysis and did not reveal the true physiological patterns and rhythms of the horse. In our studies, it was tried to reduce the effect of management factors on horses by giving them unlimited access to food (*ad libitum* forage), similar to horses' natural feeding habit. As a consequence, the very strong circadian rhythms presented in other studies were weaker in our study and disappeared during winter. Obvious differences in seasonal trend and circadian rhythms of serum calcitropic hormones and mineral concentrations during summer compared to winter suggested that the time of the year (season) should be considered an important factor during blood sampling and any serum mineral and hormonal measurements in horses.

The concentration of vitamin D in grass was higher in summer, which resulted in higher serum $25OHD_2$ and $1,25(OH)_2D$ concentrations. The higher vitamin D consequently results in increased active calcium absorption in the intestine, which suppresses the release of PTH from parathyroid glands and *vice versa* (Lips, 2012). Measurement of calcium, phosphorus and magnesium concentrations in pasture/hay consumed by horses during the year suggested they were relatively stable in concentration, and their concentration substantially exceeded the minimum daily recommended intake requirements for horses (NRC, 2007), explaining the fairly stable trends of serum mineral concentrations in horses. Although small troughs in mineral concentrations were seen in winter, they remained within reference ranges but perhaps suggest changes in digestibility of pasture. Little is known about Mg metabolism in horses and in general our findings suggested that Ca and Mg had similar metabolic pathways while regulation of serum P likely involved additional pathways, e.g. the recently discovered phosphatonin system.

The findings on circadian rhythms of calcitropic and mineral ion concentration in horses revealed the variation in rhythms of these serum analytes between seasons and highlight the importance of considering time of the year when horses need to travel long distances. Jetlag in horses, especially in equine athletes travelling by air to international equestrian competitions across huge time and seasonal differences, may affect their health, ability to adjust their circadian rhythms to new time zones, and their performances during the competition events. It may be beneficial to provide facilities that place horses under similar daylight hours, via artificial lighting, according to the new time zone that horses will face or, as no circadian rhythms were seen during winter, put them in the similar light hours as winter days to reduce jetlag and increase performance.

7.3.3 Vitamin D responsive genes

Two PCR-based experiments were performed to first validate and optimise the stability of HKGs in the equine kidney, as detailed in Chapter 5, and then investigate which vitamin D responsive genes are responsible for transcellular calcium transport in the equine kidney and compare them to those in sheep and dogs, as detailed in Chapter 6.

The aim of the second study was to determine the relative abundance and relationship of vitamin D responsive genes (TRPV5, TRPV6, $calD_{9k}$, $calD_{28k}$, PMCA, NCX1, CYP27B1, CYP24A1, and VDR) in ovine, canine and, equine kidney using RT-qPCR and then perform a comparison between the three species. Due to their consistent correlation with each other in each species, TRPV6, $calD_{9k}$/$calD_{28k}$, and PMCA appeared to be the main pathways involved in active transepithelial calcium transport in the kidney of the examined species.

Our findings indicate that all of the studied genes were expressed in the renal tissue of sheep, dogs and horses, although the expression levels and correlation of genes with each other were different from species to species. All vitamin D responsive and calcium transporting genes were highly correlated with VDR in equine kidney, but not in sheep and dogs. The genes CYP27B1 and CYP24A1 showed different renal expression pattern and correlation in horses than in sheep and dogs. Interestingly, given the high urinary calcium concentrationand low serum 1,25$(OH)_2$D concentration in horses, it could be expected that CYP27B1 expression would be lower than CYP24A1 in the horse, and this did not appear to be the case. Therefore, the hypothesis on urine calcium excretion in horses was not supported and further research needs to be performed to reveal this mystery. The findings suggest that despite low serum vitamin D concentrations, vitamin D still plays a significant role in calcium metabolism in horses, especially given the strong correlations between VDR and vitamin D responsive genes in these animals.

7.4 Limitations of the study

The studies reported in this thesis have several limitations. One limitation was the relatively small sample size of horses available for blanketing in Chapter 2, hospitalisation in Chapter 3 and the skin radiation experiment in Chapter 4 due to budgeting constraints. The high cost of any measurements related to vitamin D metabolites also limited the number of animals and samples used in these studies. The availability of horses during our study was limited therefore the numbers of horses involved in some experiments were low (e.g. blanketing and collection of horse skin). In addition, the small size of the blanketed group was based on the necessity that the

horses were blanketed for an entire year, and horses were therefore selected by temperament rather than randomly.

At the time of the biochemical studies determining the concentration of serum analytes in horses (Chapter 2 and Chapter 3), it would have been ideal to measure vitamin D binding proteins (DBP) and ionised magnesium (iMg). Unfortunately the high cost of DBP and iMg measurements and budget constraints did not allow this. The iMg test is not currently available in New Zealand, and the cost of sending samples overseas to be measured would have been prohibitive.

There is a possibility that high concentration of 25-hydroxyvitamin D_2 might have interfered with the 1,25-hydroxyvitamin D measurements. As mentioned in the previous chapters, the Diasorin kit used to measure serum concentrations of 1,25-hydroxyvitamin D actually measures both 1,25-hydroxyvitamin D_2 and 1,25-hydroxyvitamin D_3. Measuring different vitamin D metabolites in the samples for future studies would enable stronger conclusions to be drawn.

The expression of genes studied by quantitative real time-PCR, as performed in Chapter 6, may be influenced by the age, diet, and life history of the animals used in the research. Therefore, to make strong conclusions regarding transepithelial calcium transport in the kidney, additional information including serum biochemistry, dietary regimen, and the health history of animals used would be required

7.5 Future studies

The results of the studies presented in this thesis suggest that vitamin D metabolism and its relationship to other analytes in the body is complex in horses, and deserves further investigation. Some of the research presented in here has raised additional questions and there are several lines of research which should be pursued.

Studying the expression of vitamin D responsive genes (TRPV5, TRPV6, $calD_{9k}$, $calD_{28k}$, PMCA, NCX1, CYP27B1, CYP24A1 and, VDR) in equine intestine would enable us to obtain a better overview of the regulation of calcium. Calcium transport and absorption in horses can be better understood when the results of gene expression in the equine kidney and intestine are assessed and correlated with serum analyte concentration in the same animal. In addition, it would be useful to expand our studies to include the phosphatonin system in horses, as this interacts with vitamin D and is involved in control of body P regulation.

Another line of research would be to further investigate equine magnesium metabolism. There is a significant knowledge gap in the understanding of magnesium haemostasis in horses which needs to be addressed, given that magnesium is an essential co-factor for more than 300 biochemical reactions in the body. There is limited literature on some of the most important areas, especially their magnesium requirements, assessment of magnesium status, required dietary intake of magnesium and the effects of magnesium on exercise performance. Magnesium has been referred to as “the forgotten cation” because its importance was recognised much later than that of calcium. It has important effects on bone and mineral metabolism, the neuromuscular system and the cardiovascular system. Imbalances of magnesium may result in unwanted neuromuscular, cardiac or nervous disorders. In humans, the essential role of magnesium on heart rhythm, vascular tone, nerve function, muscle contraction and relaxation is well studied. It has also been suggested that magnesium may promote weight loss and fight obesity, as serum magnesium concentrations are significantly lower in obese people than non-obese ones.

Magnesium is also believed to help reduce signs of nervousness, excitability, and muscle tremors, giving magnesium its reputation for having a calming influence on horses. Despite the widespread use of magnesium products, evidence for the effect of magnesium on nervousness and anxiety in horses is absent and a number of questions remain unanswered. If magnesium has an impact on equine behaviour through its calming effect on nervous system, how much magnesium is required to reach to the calming phase? And which form of magnesium formula is the best to administer? Therefore, further studies on Mg, which we have shown has a strong relationship with calcium and dietary vitamin D, may provide a clearer picture of the cause and effect of changes in these analytes and might answer some of these questions.

Future studies with a larger sample size are needed to confirm some of our results, for instance, the horse skin irradiation could be performed in a larger sample size with a comparison in skin colours (e.g. grey vs brown/black). Studies on cats and dogs showed low concentrations of 7-DHC and lack of vitamin D_3 synthesis in the skin of these species (How *et al.,* 1994; Morris 1999). Therefore, following from Chapter 4 results, it would be useful to measure 7-DHC reductase activity and non-toxic vitamin D metabolites such as lumisterol, tachysterol, suprasterol I and II, and 5,6-trans-cholecalciferol in equine skin, if they do indeed form there. The addition of internal standards to the assay should be included to determine the recovery of 7-dehydrocholesterol and Vitamin D_3 and confirm that they were extracted. With a larger sample size and study of the enzyme that is directly involved in preventing vitamin D synthesis in skin of cats and dogs, the reliability of results will be greater.

Normal concentrations of vitamin D and associated analytes for horses at pasture in New Zealand have been determined and so now it would be desirable to investigate different aspects of vitamin D in equine health and disease. For instance, the association of vitamin D as an inhibitory factor against respiratory infections and pathogens (e.g. pneumonia), vitamin D deficiency and its association with increased autoimmunity as well as an increased susceptibility to infection, and the association of low vitamin D concentrations with fractures, especially catastrophic fractures, in horses. Through such studies, the effects of vitamin D supplementation in various situations would be clarified and would help to determine the efficacy optimal dosage and vitamin D requirements in horses.

7.6 Summary

The findings from this thesis have increased current knowledge regarding vitamin D synthesis and metabolism in horses, and helped fill some gaps in the equine literature. The apparent inability of horses to synthesise vitamin D_3 in their skin is a novel and potentially important discovery that warrants further investigation. Likewise, the discovery that the main vitamin D metabolite in their circulation is $25OHD_2$ emphasizes the importance of nutrition and necessity of providing an appropriately balanced diet in horses.

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Appendices

Appendix 1

Mean (± SE) serum concentration of ionised calcium (iCa) (mmol/L), total calcium (tCa) (mmol/L), phosphorus (P)(mmol/L) , total magnesium (tMg)(mmol/L) , 25-hydroxyvitamin D_2 ($25OHD_2$) (nmol/L), 25-hydroxyvitamin D_3 ($25OHD_3$) (nmol/L), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (pmol/L) and, parathyroid hormone (PTH) (pg/mL) in blanketed and non-blanketed horses at pasture in New Zealand from January 2013 to January 2014. (C: blanketed horses, NC: non-blanketed horses).

		Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	P-value
iCa (mmol/L)	B	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)	0.99
	NB	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 (mmol/L)	B	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)	0.93
	NB	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)	
P (mmol/L)	B	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)	0.28
	NB	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 (mmol/L)	B	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)	0.73
	NB	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)	B	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)	0.10
	NB	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)	B	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)	N/A
	NB	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)	B	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)	0.30
	NB	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)	B	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)	0.26
	NB	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)	

Appendix 2

Molecular Development Protocol

Introduction

The aim of this research was to develop a protocol for reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) for equine housekeeping genes, and calcium channel and vitamin D responsive genes in equine, ovine and canine kidneys. This chapter describes the development of novel PCR primers for RT-qPCR, and the protocols used for RNA extraction, cDNA formation and RT-qPCR.

Materials and methods

Sample collection

For processes including tissue collection, RNA extraction, cDNA formation and real-time PCR, all work surfaces and equipment were cleaned with DNAZap (Ambion/Applied Biosystems, Austin, TX, USA) according to the manufacturer's instructions. Care was taken (e.g. use of protective gloves) to maintain an RNAse- and DNAse-free work environment during sample collection and handling of samples.

Renal tissue samples were harvested post-mortem from 10 adult horses, 10 sheep and five dogs. All animals were euthanized for reasons unrelated to this study. Tissue samples for RNA isolation were collected within half an hour of euthanasia. Samples were obtained from the kidney such that each sample included both cortex and medulla, and the samples were snap frozen in liquid nitrogen and stored at -80˚C until processing.

RNA extraction protocol

For the first part of the study total RNA was extracted from renal tissue samples using the RNeasy Mini Kit (QIAGEN, USA) including the optional on-column DNAse digestion step (RNase-Free DNase Set, Qiagen) with the following protocol. Each kidney sample was thawed on ice and then cut into smaller portions using scalpel blade. A maximum of 30 mg of each sample was then placed into a 2 mL micro tube containing 2 x 5 mm stainless steel ball bearings and 600 µL of Buffer RLT reagent. The tubes were homogenized by agitation in a Mini-Beadbeater-16 (BioSpec, Oklahoma, USA) for 1 min and then centrifuged for 3 min at 16000 xg. Then 600 µL of 70% ethanol was added to the tubes and mixed well. Subsequently, 700 µL of the homogenate was transferred to an RNeasy spin column placed in a 2 mL collection tube. The spin column was centrifuged for 30 s at 8000 xg and the flow-through discarded. Subsequently, the remainder of the RNA extraction was performed according to the manufacturer's instructions. RNase-free water (50 µL) was directly added to the spin column membrane and centrifuged for 1 min at 8000 xg to elute the RNA. Samples were immediately stored at -80°C until required, except for a 4 µL aliquot which was tested for RNA and DNA concentration (to check for absence of DNA) using the Qubit® 2.0 Fluorometer and Qubit® RNA HS and DNA HS Assay kits (Invitrogen, Life Technologies Corporation, California, USA) as per manufacturer's instructions.

The TURBO DNA-free™ Kit (Ambion®, Life Technologies Corporation, California, USA) was used for treatment and removal of any contaminating genomic DNA from the extracted RNA samples with the following protocol. The TURBO DNase Buffer reagent (5 µL) and TURBO DNase (1 µL) were added to the RNA sample, mixed gently and incubated at 37°C for 30 min. Then 5 µL of resuspended DNase Inactivation

reagent was added to the RNA sample, mixed well and incubated at room temperature (22-25°C) for 5 min, with occasional mixing to redisperse the DNase Inactivation reagent. The sample was centrifuged at 10,000 xg for 1.5 min, then the clear (RNA-containing) supernatant was transferred to a fresh 2 mL tube and stored at -80˚C until required.

Primer design

Primers were designed using the National Centre for Biotechnology Information (NCBI), primer Basic Local Alignment Search Tool (BLAST) (Bethesda, Maryland, USA) (http://www.ncbi.nlm.nih.gov/nucleotide) as described below (Table 5.1 to 5.4). A number of equine housekeeping gene (HKGs) primers have previously been published (Kayis *et al.,* 2011; Klein *et al.,* 2011).

The primers were designed to have the following features: a PCR product of less than 150 bp, span an exon-exon junction, show no complementarity to extraneous targets, have minimal primer-dimer and primer hairpin formation, and have similar melting temperatures and guanine-cytosine content (GC content %). The best primer set was selected and the PCR amplicon sequence tested for secondary structures at 60˚C using the mFold program (http://mfold.rit.albany.edu/?q=mfold). If there were no hairpin loops in the primer binding area, then the primer pair was selected. If there was hairpin loop formation, the entire process was repeated until all conditions were satisfied.

Reverse transcriptase quantitative PCR

Care was taken during all laboratory work (e.g. cleaned surfaces, use of gloves, separate PCR preparation and thermal cycling area workflow) to prevent

contamination of samples with extraneous RNA or DNA. DNAse free water (Gibco UltraPure distilled water, Gibco/Invitrogen, Grand Island, NY, USA) was used in all reactions.

The Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) was used to synthesise cDNA according to the manufacturer's instructions. Each reaction tube contained 600 ng RNA, 2.5 µM Oligo (dT), 60 µM random hexamers, 8 mM RT reaction buffer, 1 mM dNTP, 10 U reverse transcriptase, 20 U RNase inhibitor and 6 µL DNAse free water for a total volume of 20 µL. The reactions were performed with an initial stage of 25°C for 10 min, followed by 55°C for 30 min, 85°C for 5 min and finally a hold at 4°C using Applied Biosystems® Veriti® Thermal Cycler (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA). Samples were immediately frozen to -80°C and stored at that temperature until required.

RT-qPCR was performed using the StepOne Plus real-time PCR machine (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA). Different primer concentrations were tested to optimize the amplification reactions. A matrix of primer concentrations ranging from 50 to 900 nM was tested. Reactions contained 5 µL Fast SYBR Green real-time PCR Master Mix (Applied Biosystems, Life Technologies Corp., Carlsbad CA, USA), the primer pair and 10 ng of cDNA template and made up to a total volume of 10 µL with DNAse-free water. Negative controls consisting of water and reaction mix without reverse transcriptase were included in each PCR run, and all samples were run in triplicate. The PCR protocol consisted of a denaturation step at 95°C for 20 s; followed by 40 cycles at 95°C for 3 s and 60°C for 30 s; ending with a melt curve analysis ranging from 60°C to 95°C with a heating rate of 0.3°C/15 s. Five point

standard curves were produced for each target to determine the accuracy (R^2 ≥0.98) and efficiency (90-105 %) of the RT-qPCR reactions. The real-time data were analysed using the qbase$^+$ 3.0 (Biogazelle, Zwijnaarde, Belgium) to produce relative expression ratios.

RT-qPCR assays require additional controls and/or quantification calibrators, which enabled comparisons across different samples, RT-qPCR data must be normalized to correct variations in pipetting, RNA concentration, reverse-transcription, and efficiency of PCR amplification. All molecular studies were performed and results presented in compliance with the "minimum information for publication of quantitative real-time PCR experiments guidelines" (MIQE; http://www.rdml.org/miqe.php) (Bustin *et al.*, 2009).

Table.1 Equine housekeeping genes. Housekeeping genes used in the real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) assay indicating gene name, GenBank, primers, amplicon length, primer concentration, and their PCR parameters (efficiency and Regression coefficient (R^2)) from RT-qPCR analysis.

Target Gene	Gene Bank	Primer sequence (5'-3')	Amplicon length (bp)	Primer concentration	Best R^2	Best Efficiency	Problem with the primer
18S	**AJ311673**	F:ACGGAAGGGCACCACCAGGA R:CACCACCACCCACGGAATCG	127	400:400 nM	0.999	79.076%	Not able to obtain acceptable R^2 and efficiency
		F: ATGCGGCGGCGTTATTCC ‡ R: GCTATCAATCTGTCAATCCTGTCC	**204**	**900:900 nM**	**1**	**91.17%**	**A matrix of different primer concentrations was performed, and 900:900 nM was determined to be optimal**
28S	EU554425.1	F: CGGGTAAACGGCGGGAGTAAC ‡ R:TAGGTAGGGACAGTGGGAATCTCG	109	350:300 nM	0.995	91.052%	A matrix of different primer concentrations was performed, and 900:900 nM was determined to be optimal
RPL32	CX594263.1	F:TCGTGAAGCCCAAGATCGTC R:TTGAATCTTCTGCGCACCCT	130	300:150 nM	0.998	90.706%	A matrix of different primer concentrations was performed, and 900:900 nM was determined to be optimal
B2M	NM_001082502	F: GTGTTCCGAAGGTTCAGGTT † R: ATTTCAATCTCAGGCGGATG	102	100:250 nM	0.977	89.131%	Not able to obtain acceptable R^2 and efficiency
	NM_001082502.2	**F:TCTCAAAGACCCCCTGATAGTT R:GTCAAATCCAAATGAGGGAATC**	**89**	**300:300 nM**	**0.998**	**92.167%**	**No problems**
GAPDH	NM_001163856	F: AGAAGGAGAAAGGCCCTCAG † R: GGAAACTGTGGAGGTCAGGA	87	900:500 nM	0.994	92.464%	Different dilutions and concentrations of cDNA was tried to get optimal results.
SDHA	XM_001490889	F: GCAGAAGAAGCCATTTGAGG † R: CCTGTCGATTACGGGTCTGT	103	500:900 nM	0.993	93.511%	A matrix of different primer concentrations was performed, and 900:900 nM was determined to be optimal
YWHAZ	XM_001492988	F: TGTTGTAGGAGCCCGTAGGT † R: ATTCTCGAGCCATCTGCTGT	95	300:300 nM	0.994	93.449%	No problems
HPRT1	AY372182	F:TTGCTGACCTGCTGGATTAT R:TTATGTCCCCTGTTGACTGGT	120	500:500 nM	0.993	93.809%	No problems
ACTB (ß-actin)	NM_001081838	F:CCCTGGAGAAGAGCTACGAG R:CCACAGGATTCCATGCCCAG	115	250:250 nM	0.976	78.335	Primer dimers
TUBA1	AW260995.1	F: GCCCTACAACTCCATCCTGA ‡ R: ATGGCTTCATTGTCCACCA	78	900:900 nM	0.978	90.09%	Primer dimers
SLC36A2	XM_005599277.1	F:CTGGGCTTCTGCTGTGTGTA R:TCGGAATCACTGTCTCGTTG	112	None	N/P	N/P	This gene did not amplify with these primers

† Klein *et al.*, 2011

‡ Kayis *et al.*, 201

Table.2 Vitamin D responsive gene primers for equine kidney.

Target Gene	Gene Bank	Primer sequence (5'-3')	Amplicon Length (bp)	Primer concentration	Best R^2	Best Efficiency	Problem with the primer
$calD_9k$	AY229893	F:GCGTGAAAAAGTCTCCTGAA R:TCACTAACACCTGGAATTCTTCAA	219	**700:700 nM**	**0.998**	**97.086%**	No problems
$calD_{28}k$	NM_001163952	F:ACGGCTTGGTCTTCCTTGACAG R:TGCGTGTGTGAGTATGTGTGAGTG	**101**	**75:100 nM**	**0.99**	**102.752%**	**A matrix of different primer concentrations was performed, and 75:100 nM was determined to be optimal**
	AY665136.1	F:GGCCAGGTTACTACCAGTGC R:TCTATGTACCCATTGCCATCC	120	None	N/P	N/P	Primers did not work
	NM_001163952	F:GCTGGAAAAAGCAAACAAGACGGTTGA R:TTCTCCTGCACTGGTAGTAACCTGG	137	None	N/P	N/P	Very late amplification
	AY665136.1	F:CAGGGAGTCAAAATGTGTGG R:TCCTTCAGCAAAGCATCCAGTTC	107	None	N/P	N/P	Very late amplification
PMCA	DQ160196	F:AAACGCCGCCGATAGTGCAAATAC R:CCTTCTTGTGCATGTTGGCCTTCTTC	182	200:200 nM	0.991	102.748%	Different dilutions and concentrations of cDNA was tried to get optimal results.
NCX1	DQ178640	F:TGGCCATAACTTTACTGCGGGAGA R:GGACCACATAAACACAAAGCGCGA	101	500:250 nM	0.992	92.4%	A matrix of different primer concentrations was performed, and 500:250 nM was determined to be optimal
TRPV5	AY944068	F:ACACTGTGATGTTCCAGCACCTGA R:AGGAGTCGATCTCTGTGAGGTCAT	102	**75:75 nM**	**0.992**	**96.909%**	**A matrix of different primer concentrations was performed, and 75:75 nM was determined to be optimal**
	NM_001163956.1	F:GCACAAACTCCCGAGATAACA R:GACAGTCACCAGCTCCCCTA	104	None	N/P	N/P	Primers did not work
	NM_001163956.1	F:CGTAGTGTCAACCGCACAAA R:GACAGTCACCAGCTCCCCTA	117	None	N/P	N/P	Very late amplification
	NM_001163956.1	F:GGTCAAGGAGCCTGTCATGT R:TCACCAAGTTCACGTTCTGG	86	None	N/P	N/P	Primers did not work
TRPV6	XM_001490905.2	F:CCTCAAGCCCATCACCAGTA R: GCCATCCTTAGGGGTCAAGT	97	250:250 nM	0.974	104.5%	No problems

Continue from Table.2 Vitamin D responsive gene primers for equine kidney.

Target Gene	Gene Bank	Primer sequence (5'-3')	Amplicon Length (bp)	Primer concentration	Best R^2	Best Efficiency	Problem with the primer
VDR	AY944069	F:TGACCTGGTCAGTTACAGCATCCA R:TGTCGTCCATGGTGAATGACTGGT	158	250:250 nM	0.985	99.2%	Primer dimers
	XM_005611070.1	F:ACAGCATCCAAAAGGTGGTC R:TGACTTCAGCAGCACGATCT	**89**	**500:500 nM**	**0.998**	**99.976%**	**No problems**
CYP27B1	NM_001163957	F:GGACTAGAAGGCATAGCCGC R:CGTGGACACAAACACGGAAC	117	None	N/P	N/P	Primers did not work
	NM_001163957.1	F:CAGAGACATTCATGTGGGTGA R:GCTGGACGAAAAGAATTTGG	**117**	**300:300 nM**	**0.984**	**93.989%**	**A matrix of different primer concentrations was performed, and 300:300 nM was determined to be optimal**
	NM_001163957.1	F:TGGTCAAGGAAGTGCTGAGA R:CGAAAAGAATTTGGCTCTGG	169	None	N/P	N/P	Very late amplification
	NM_001163957.1	F:GAGATCACAGCTGCCCTGGGCCC R:TACAGTCTCAGCACTTCCTTGACCA	107	None	N/P	N/P	Very late amplification
CYP24A1	NW_001867393.1	F:GCGTGCCATTTACAACTCGG R:TGCGGATGATTCCTTTGGGT	78	None	N/P	N/P	Many primer dimers
	XM_003363957.2	F:TCTGCCTGGTGCTGTATGAG R:GACCATCATCCTCCCGAAC	119	None	N/P	N/P	Very late amplification
	XM_003363957.2	F:GTGTGATGAAAGAGGCCACATTGA R:CGTTCTGCTGGAGGAGCCCG	**113**	**350:350 nM**	**0.99**	**91.647%**	**A matrix of different primer concentrations was performed, and 350:350 nM was determined to be optimal**
	XM_003363957.2	F:GAGGCCACATTGAAGACTTG R:AGCGTCAGCCCCTGCGTTCTG	116	None	N/P	N/P	Very late amplification

Table.3 Vitamin D responsive gene primers for canine kidney.

Target Gene	Gene Bank	Primer sequence (5'-3')	Amplicon Length (bp)	Primer concentration	Best R^2	Best Efficiency	Problem with the primer
calD$_9$k	XM_843973.2	F:TCTTCTAGCTGCCTTGCTG R:CTTCTTTGGCTGCGTATTTT	104	900:900 nM	0.99	101.7%	PCR products used for standard curve
calD$_{28}$k	XM_848991.4	F:CAGGGAATCAAAATGTGTGG R:TCCTTCAGTAAAGCATCCAGTTC	107	800:800 nM	0.99	90.16	A matrix of different primer concentrations was performed, and 800:800 nM was determined to be optimal
PMCA	XM_005628823.1	F:TGAAAGCTCATTGACTGGTGA R: CATTCTTCCAGAGCCTTCCA	103	250:250 nM	0.993	91.7%	No problems
NCX1	XM_005630379.1	F:CACCATCGGCTTGAAAGATT R:GCGTCTGCATACTGATCCTG	114	350:350 nM	0.98	104.3%	Different dilutions and concentrations of cDNA and primers were tried to get optimal results.
TRPV5	XM_003639556.2	F:CCACATGCTGCAACAGAAGA R:AAGTCACAGTTCCGGTCCAG	**108**	**500:500 nM**	**0.992**	**92.74%**	**A matrix of different primer concentrations was performed, and 500:500 nM was determined to be optimal**
	XM_003639556.2	F:AATCCGTCTAGTGGGGGAAT R:CCCGAGGATAGTCTGTCCAA	118	250:250 nM	0.87	116%	Despite the perfect melt curve, not able to get acceptable R^2 and efficiency
	XM_003639556.2	F:TGCTCCTAGAGATCCCAGACA R:CACCATGCAGGCATAGATGA	110	900:900 nM	0.80	76.9%	Despite the perfect melt curve, not able to get acceptable R^2 and efficiency
TRPV6	XM_539861.5	F:TGGAGCTGTGATCATTCTGC R:AGGCGTAGGTGATGATGAGG	119	250:250 nM	0.93	136%	Despite the perfect melt curve, not able to get acceptable R^2 and efficiency
	XM_539861.5	F:AGAGCCGAGATGAGCAGAAC R:CTTGCTGAGAGCCTGGACAT	**98**	**500:500 nM**	**0.989**	**102.3%**	**Used PCR products**
	XM_539861.5	F:TGGTCATCCTGGGCTTTG R:GCCATGGGGTAGTCGTAGAA	91	350:350 nM	0.91	107.1%	Primers did not work
VDR	XM_005636920.1	F:AGCATCCAAAAGGTCATTGG R:GCACTTGATTTCAGCAGCAC	92	900:800 nM	0.971	95.2%	A matrix of different primer concentrations was performed, and 900:800 nM was determined to be optimal
CYP27B1	XM_538254.4	F:AAAGACATTCGTGTGGGTGA R:TCGAGCTGGACGAAAAGAAT	120	250:250 nM	0.76	159.7%	Late amplification
	XM_538254.4	F:GAGATCACAGCTGCCCTGGGCCC R:TACAGTCTCAGCACCTCCTTGACCA	107	500:500 nM	0.85	136%	Late amplification
	XM_543059.3	F:GAGCTGCAAATGGCTTTGGCTCAG R:CTGTAGGTTGATGCTCCTCTCGGG	**123**	**300:300 nM**	**0.999**	**89.2 %**	**A matrix of different primer concentrations was performed, and 300:300 nM was determined to be optimal**
CYP24A1	XM_543059.3	F:GAGGCCGCATTGAAGACTTA R:CATTCTTCCGAAGGAGTCCA	102	450:450 nM	0.988	94.74%	A matrix of different primer concentrations was performed, and 450:450 nM was determined to be optimal

Table.4 Vitamin D responsive gene primers for ovine kidney.

Target Gene	Gene Bank	Primer sequence (5′-3′)	Amplicon Length (bp)	Primer concentration	Best R^2	Best Efficiency	Problem with the primer
calD$_9$k	NC_019484.1	F:TCACTGCTGAACGGCCAGGACA R:AGCTCCTCCTTGGACAGTTGGT	122	250:250 nM	0.991	99.3%	No problems
calD$_{28}$k	NC_019466.1	F:GCTGGAAAAAGCAAACAAGACTGTTGA R:TTCTCCTGCACGGGTAGTAATCTGG	139	400:400 nM	0.98	90.8%	A matrix of different primer concentrations was performed, and 400:400 nM was determined to be optimal
PMCA	NC_019460.1	F:TGCAGCCATAGTATCATTGGGCCT R:TTGCCGCTCCTTCAATCCAACCA	128	250:250 nM	0.994	94.9%	No problems
NCX1	NC_019460.1	F:TGGCGAACATCAACCCGTGCT R:TGCAGATTGTAGCGTCGCATCTCG	93	300:300 nM	0.99	98.4%	A matrix of different primer concentrations was performed, and 300:300 nM was determined to be optimal
TRPV5	NC_019461.1	F:ATCCTCGTCCTCCAGCCCAACA R:ACTTCCGCTTCTGCATCAGGTG	133	250:250 nM	0.79	85%	Late amplification and primer dimers in higher concentrations of primers
	XM_004008320.1	F:CTCTCAAGGCCCGTAGTGAC R:CCGGATTTTATCCTGCTGAG	104	250:250 nM	0.70	137.9%	Late amplification and primer dimers in higher concentrations of primers
	XM_004008320.1	F:CGGGTCAGCAATCATCCTAT R:ATTGTGATGACGTGGAATGG	**108**	**250:250 nM**	**0.962**	**110.6%**	**Used PCR products**
TRPV6	EU310242.1	F:TGATGCTGGAGAAGAAGCTG R:TGGTTGATGTCCTGTTTCTCTT	118	250:250 nM	0.98	93.9%	No problems
VDR	NC_019460.1	F:TCATGCTGCGCTCCAACCAGT R:TGGAACTTGATGAGGGGCTCGAT	140	400:400 nM	0.986	93.5%	A matrix of different primer concentrations was performed, and 400:400 nM was determined to be optimal
CYP27B1	NC_019460.1	F:TTGCATTCGCCCAGCAGCAC R:TCTCTGCTCTACGTGCTGCTGG	81	None	N/P	N/P	Primers did not work
	XM_004006519.1	F:GGGACCAGATGTTTGCATTC R:CTTCCCAAAATGGTTCCTCA	80	None	N/P	N/P	Primers did not work
	XM_004006519.1	F:GCAGAGCTTGAGTTGCACAT R:CTTCTCTCAGGCACCAGGAC	**119**	**250:250 nM**	**0.92**	**102.2%**	**A matrix of different primer concentrations was performed, and 250:250 nM was determined to be optimal**
CYP24A1	NC_019470.1	F:CTGTGATGAGAGAGGCCGCATTGA R:AGCTTCCTCCCCTGCCTTCTT	128	600:600 nM	0.992	103.9%	A matrix of different primer concentrations was performed, and 600:600 nM was determined to be optimal

Appendix 3

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Sara Azarpeykan

Name/Title of Principal Supervisor: Dr Keren Dittmer

Name of Published Research Output and full reference:

Influence of blanketing and season on vitamin D and parathyroid hormone, calcium, phosphorus and magnesium concentrations in horses in New Zealand

Domestic Animal Endocrinology, Accepted 2016

In which Chapter is the Published Work: Chapter 2

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 75%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Sara designed the study, assisted with sampling, and sample processing, collected the data, contributed to the statistical analysis and wrote the manuscript.

Sara Azarpeykan Digitally signed by Sara Azarpeykan Date: 2016.03.17 09:14:05 +13'00'

Candidate's Signature

16/3/16

Date

Keren Dittmer Digitally signed by Keren Dittmer DN: cn=Keren Dittmer, o=Massey University, ou=IVABS, email=K.Dittmer@massey.ac.nz, c=NZ Date: 2016.03.16 12:02:13 +13'00'

Principal Supervisor's signature

16/3/16

Date

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Available online 19 March 2016

In Press, Accepted Manuscript — Note to users

Influence of blanketing and season on vitamin D and parathyroid hormone, calcium, phosphorus and magnesium concentrations in horses in New Zealand

S. Azarpeykan[a], K.E. Dittmer[a], E.K. Gee[a], J.C. Marshall[a], J. Wallace[b], P. Elder[b], E. Acke[a], K.G. Thompson[a]

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doi:10.1016/j.domaniend.2016.03.003

Highlights

- We examined the influence of covering and season on vitamin D and PTH, Ca, P and Mg homeostasis in horses.
- Serum concentration of $25OHD_3$ in horses was barely detectable or below the detection limits of the assay, and the main form of 25OHD in the serum was $25OHD_2$.
- Strong relationships were seen between iCa and PTH, iCa and tMg, dietary vitamin D and $25OHD_2$.
- A strong seasonal trend was seen in serum $25OHD_2$ concentrations with a trough occurring towards the end of winter, while PTH and $1,25(OH)_2D$ showed opposing trends through the year.
- The results suggest horses may rely on dietary vitamin D_2 to fulfil their vitamin D requirements.

Abstract

The aims of the study were to determine effect of season and blanketing on vitamin D synthesis in horses, and examine the interaction between vitamin D and other analytes involved in calcium homeostasis. Twenty-one healthy horses at pasture were included; 5 were covered with standard horse blankets including neck rugs. Blood samples were collected for 13 mo and analyzed for 25-hydroxyvitamin D_2 ($25OHD_2$), and 25-hydroxyvitamin D_3 ($25OHD_3$), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), ionized calcium (iCa), total calcium (tCa), phosphorus (P), total magnesium (tMg) and parathyroid hormone (PTH). Grass and hay samples were collected and analyzed for vitamin D, calcium, phosphorus and magnesium. Climate data were also collected. The serum concentration of $25OHD_3$ in horses either undetectable or below the detection limit of the assay, and the main form of 25OHD was $25OHD_2$. No differences in serum $25OHD_2$, $1,25(OH)_2D$, iCa, tCa, P, tMg and PTH ($P \geq 0.05$) concentrations were seen between the 2 groups. Associations were seen between iCa and PTH ($P < 0.05$), iCa and tMg ($P < 0.05$) and dietary vitamin D and $25OHD_2$ ($P < 0.05$). A strong seasonal trend was seen in serum $25OHD_2$ ($P < 0.0001$) which was higher during spring and summer when the amount of sunshine and UV radiation was higher. Parathyroid hormone and $1,25(OH)_2D$ showed opposing trends with PTH higher in winter while $1,25(OH)_2D$ was higher in summer. The results suggest that dietary vitamin D maybe necessary for horses to fulfill their vitamin D requirements, however further research is required to determine the contribution of vitamin D_3 synthesis in the skin to the vitamin D status of the horse.

Keywords

Horse; Vitamin D; Calcium; Phosphorus; Magnesium; PTH

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Sara Azarpeykan

Name/Title of Principal Supervisor: Dr Keren Dittmer

Name of Published Research Output and full reference:

Circadian rhythm of calciotropic hormones, serum calcium, phosphorus and magnesium during the shortest and longest days of the year in horses in New Zealand

Journal of Animal Physiology and Animal Nutrition, Accepted 2016

In which Chapter is the Published Work: Chapter 3

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 75%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Sara designed the study, assisted with sampling, and sample processing, collected the data, contributed to the statistical analysis and wrote the manuscript.

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Candidate's Signature

16/3/16

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Principal Supervisor's signature

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GRS Version 3– 16 September 2011

Journal of Animal Physiology and Animal Nutrition

DOI: 10.1111/jpn.12477

ORIGINAL ARTICLE

Circadian rhythm of calciotropic hormones, serum calcium, phosphorus and magnesium during the shortest and longest days of the year in horses in New Zealand

S. Azarpeykan[1], K. E. Dittmer[1], E. K. Gee[1], J. C. Marshall[2], P. Elder[3], E. Acke[1] and K. G. Thompson[1]

1 Institute of Veterinary, Animal and Biomedical Science (IVABS), Massey University, Palmerston North, New Zealand
2 Institute of Fundamental Sciences (IFS), Massey University, Palmerston North, New Zealand, and
3 Canterbury Health Laboratories, Christchurch, New Zealand

Summary

A study was conducted to determine the circadian rhythms and trends of vitamin D metabolites including 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, 1,25-dihydroxyvitamin D and parathyroid hormone, in addition to serum calcium, phosphorus and magnesium concentrations in horses over 48 h on the shortest and longest days of the year in 2013. Five healthy adult horses (*Equus caballus*) were on a constant pasture feeding regimen, and blood samples were collected from each horse every 3 h over a 48-h period, starting at 07:00 PM on day one and finishing at 07:00 PM on day three, for the measurement of calciotropic hormones and electrolytes. There was a significant difference between the serum concentration of calciotropic hormones, iCa, tCa, P and tMg between the shortest (winter) and longest (summer) days of the year in horses. Serum concentration of $25OHD_3$ was very low and mostly undetectable. Serum iCa, $1,25(OH)_2D$ and PTH concentrations clearly showed a circadian rhythm on the longest days of the year and serum tCa, P and tMg concentrations showed a diurnal pattern on the longest days (summer) of the year. None of the analytes showed any circadian rhythm on the shortest days (winter) of the year. The result of this study could have significant relevance to equine athletes travelling to international equestrian competitions and facing a huge time and seasonal differences that might affect their ability to adjust their circadian rhythms to new time zones.

Keywords horse, circadian rhythm, vitamin D, calcium, phosphorus, magnesium

Correspondence S. Azarpeykan, Institute of Veterinary, Animal and Biomedical Science (IVABS), Massey University, Tennent Drive, Palmerston North 4442, New Zealand. Tel: +64 6 9518259; Fax: +64 6 350 5714; Email: S.Azarpeykan@massey.ac.nz

Received: 27 September 2015; accepted: 18 December 2015

Introduction

Life is a cycle regulated by a variety of internal chemical processes and biological rhythms including annual, seasonal, weekly, circadian (24 h) and diurnal (day/night) rhythms. Chronobiology is the science of these biological rhythms (Smolensky and Peppas, 2007). The term circadian is derived from Latin and means 'around the day'. A circadian rhythm is a biological process that is driven by an 'internal clock' and this 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 algae through to mammals, depend on sunlight, and use sunlight exposure to regulate their activity in order to optimize 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 possess a biochemical system driven by sunlight and known as the internal, biological and/or circadian clock (Whitmore et al., 2000; Buijs et al., 2003). 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