

**Influence of 25-hydroxyvitamin D and anionic salts on the calcium status of  
dairy cattle**

**by**

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**Submitted in partial fulfilment of the requirements of the degree**

MSc (Agric) Animal Science: Nutrition Science

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## **DECLARATION :**

I declare that this dissertation for the degree MSc (Agric) Animal Science: Nutrition Science at the University of Pretoria has not been submitted for a degree at any other University.

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## ABBREVIATIONS:

1,25-(OH) <sub>2</sub> D <sub>3</sub>	1,25-dihydroxyvitamin D <sub>3</sub>
Ca	Calcium
CaSR	Calcium sensing receptor
DCAD	Dietary Cation-Anion Difference
Dry Matter	DM
DNMT 1	DNA methyl transferase 1
DNMT 3b	DNA methyl transferase 3b
EGFR	Epidermal growth receptor
FGF23	Fibroblasts growth factor receptor 23
HDAC2	Histone deacetylase 2
Mg	Magnesium
PTH	Parathyroid hormone
P	Phosphorus
K	Potassium
RXR	Retinoid X receptor
Na	Sodium
SEM	Standard error of the means
S	Sulphur
TMR	Total mixed ration
VDIR	VDR interacting repressor
DBP	Vitamin D binding protein
VDR	Vitamin D receptor

## SUMMARY

### **The influence of 25-hydroxyvitamin D and anionic salts on the calcium status of dairy cattle**

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Milk fever (parturient paresis / hypocalcaemia) is a metabolic disorder that usually occurs near parturition and at the onset of lactation in high producing multiparous dairy cows. Milk fever can indirectly contribute to an increased incidence of several diseases in early lactation. This study was conducted to compare two different feeding strategies to prevent milk fever, namely (i) the established concept of feeding a diet with a negative DCAD and (ii) a feeding strategy combining a negative DCAD supplement with 25-hydroxyvitamin D<sub>3</sub> (25-OH-D<sub>3</sub>). Thirty dairy cows were used in a randomized block design and were selected and blocked by parity (second parity and later), 305 day mature equivalent milk production in the previous lactation and expected calving date. Within each of the 15 blocks, the cows were allocated to two experimental groups named DCAD and DCAD + HyD. Fifteen animals in the DCAD + HyD group received a daily oral dosage of 3 mg of 25-OH-D<sub>3</sub>. Plasma samples were collected from day 21 prepartum to 10 days postpartum and were analysed for 25-OH-D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>, total and ionized calcium, phosphorus and magnesium. Samples were collected on day 21, 14, 10 prepartum and every second day to calving, 4 and 6 h postpartum and every second day up to day 10 after calving. Urinary samples for determination of macro minerals (calcium and phosphorus) were collected via manual stimulation on day 21, 14, 8 and 4 prepartum and day 4 postpartum. These samples were used to ensure that mild metabolic acidosis was achieved in both treatment groups. The metabolic acidosis was demonstrated by decreased urinary pH. Milk samples were collected on day 1, 4 and 10 postpartum and used for macro mineral (calcium and phosphorus) determination. This study did not achieve all of the expected results observed in similar experiments. No treatment differences could be detected for plasma Ca<sup>2+</sup> concentrations (P>0.05) and the mean plasma Ca<sup>2+</sup> concentrations were [1.086<sub>a</sub> mmol/L ± 0.010 (DCAD treatment) and 1.083<sub>a</sub> mmol/L ± 0.010 (DCAD + HyD treatment)] respectively.

Furthermore  $1,25\text{-(OH)}_2\text{D}_3$  plasma concentrations did not indicate any treatment differences ( $P>0.05$ ). These results could be due to the fact that the experimental animals were not sufficiently challenged and therefore the combination of a low DCAD diet and Rovimix HyD did not influence the calcium homeostatic mechanisms as expected. A clear correlation between plasma  $25\text{-OH-D}_3$  concentration and treatment duration was however demonstrated ( $P<0.001$ ), indicating effective absorption of orally supplemented  $25\text{-OH-D}_3$ . Several authors demonstrated that feeding massive doses of vitamin  $\text{D}_2$  (30 million units) for extended periods led to clinical evidence of vitamin D toxicity. When 10 million IU of vitamin  $\text{D}_3$  were however administered intramuscularly within 10 days of parturition, a reasonable measure of protection against toxicity could be provided. It can be concluded from this study that longer feeding periods ( $\pm 21$  days) than the proposed 10 days prior to calving can safely be implemented when feeding 3 mg  $25\text{-OH-D}_3$  per animal per day (=240 mg Rovimix HyD 1,25%).

## CHAPTER 1

### INTRODUCTION

#### Chapter 1: Introduction

The transition period, 3 to 4 weeks before calving until 4 weeks postpartum, is typified by an increased risk of disease (DeGaris and Lean, 2009). This period is dominated by a series of adaptations which may lead to homeostatic disorders which include hypocalcaemia, the downer cow syndrome, hypomagnesaemia, ketosis, udder oedema, abomasal displacement, metritis and poor fertility. In broad terms, the transition cow should be adapted to provide minimal risk of metabolic disorders of macro mineral metabolism including absolute or conditioned calcium (Ca), magnesium (Mg) or phosphorus (P) deficiencies; or excesses of sodium (Na) and potassium (K). Furthermore disorders of lipid metabolism arising from inadequate energy intake in the dry period and early lactation; disrupted rumen function associated with dietary change and impaired immune response can be reduced during a smooth transition (DeGaris and Lean, 2009).

Milk fever (parturient paresis / hypocalcaemia) is a metabolic disorder that usually occur close to calving and at the onset of lactation in high producing, multiparous dairy cows. A summary by DeGroot et al. (2010) indicated that in a US survey conducted during 2007, 83.5% of all dairies in the United States reported clinical milk fever as a health problem with an incidence rate of 4.9% which resulted in an average cost of \$334 per incidence.

Several authors reported that the incidence of clinical milk fever ranges between 5 – 7% (Goff 2008, DeGaris and Lean, 2009, Reinhardt et al., 2010). Subclinical milk fever in various degrees of severity appear in 47% of all cows in their second lactation and later, which in some cases may be severe enough to alter physiological and immune functions (Reinhardt et al., 2010).

Treatment can be effective, but costly and inconvenient and may reduce the productive life of dairy cattle. It is thus clear that feeding strategies aimed at preventing milk fever could have significant economic implications for dairy farmers.

In the past, a reduced dietary Ca:P ratio of 1:1, compared to 2:1 during lactation, was the preferred method to prevent hypocalcaemia. Since the ground breaking research by Block (1984) the DCAD (Dietary Cation-Anion Difference) concept was adopted and is widely used to prevent milk fever. Although the DCAD

concept is applied fairly successfully, it is possible that the supplementation of additional 25-OH-D<sub>3</sub> can further improve the calcium status of the transition cow. The aim of this study is to compare the effects of feeding a combination of anionic salts and 25-OH-D<sub>3</sub> (Rovimix HyD from DSM Nutritional Products Ltd, Switzerland), with the effects of feeding anionic salts on calcium homeostasis in peri-parturient dairy cows.

## **CHAPTER 2**

### **LITERATURE REVIEW**

## Chapter 2: Literature review

In this review various aspects relating to hypocalcaemia will be discussed. Firstly the pathophysiology and mechanisms involved in calcium metabolism will be discussed followed by the vitamin D regulatory system, predisposing factors for milk fever and milk fever control principles.

### 2.1 Pathophysiology:

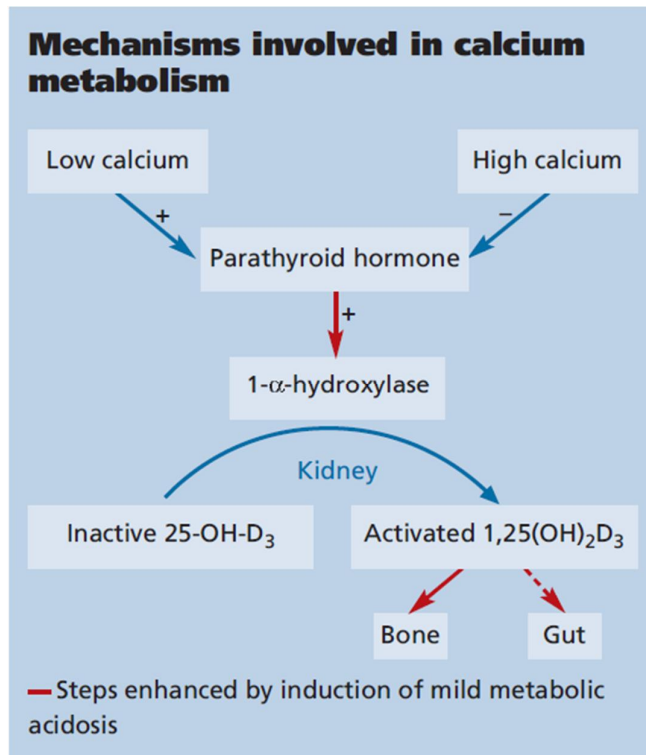
Calcium plays an important role in maintaining the integrity of the skeletal structure, whilst its presence in the intra- and extracellular fluids is linked to controlling a large number of biochemical processes. While intracellular calcium ions are needed in the activity of a large number of enzymes and are also involved in conveying information from the surface to the interior of the cell, extracellular calcium ions are required for neuromuscular excitability, blood clotting and hormonal secretion among many other functions (NRC, 2001; El-Samad et al., 2002). For all these biochemical roles to be achieved, the extra- and intracellular calcium concentrations are maintained within narrow ranges of 2.0 – 2.5 mmol/L. Cows however can afford to lose about 50% of their circulating calcium reserves before hypocalcaemia is induced (DeGaris and Lean, 2009). There is about 3 g calcium available in the entire plasma pool of a 600 kg cow, while the extracellular pool only contains about 8-9 g of calcium (Goff, 2000).

Approximately 45-50% of total calcium in plasma exists in the ionized form, which must be maintained at a relatively constant value of 1-1.25 mmol/L to ensure normal nerve membrane and muscle end plate electric potential and conductivity (NRC, 2001). In order to prevent the reduction of blood calcium levels after calving, the dairy cow needs to replace extracellular calcium lost to colostrum production (many cows are producing colostrum and milk that contains 20-30 g of calcium each day). This is achieved by withdrawing calcium from bone and increasing the efficiency of absorption of dietary calcium. By mobilizing bone calcium in order to maintain normal calcium levels in early lactation, the dairy cow can lose as much as 9-13% of her skeletal calcium sources during the first month after calving (Goff, 2008).

Calcium homeostasis is achieved through the interaction of 3 hormones (Figure 2.1):

1. Parathyroid hormone (PTH);
2. 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>);
3. Calcitonin.

(Husband, 2005; Horst, 1986)



**Figure 2.1** Mechanisms involved in calcium metabolism (Adopted from Husband, 2005)

Whenever the loss of calcium from the extracellular fluid exceeds the amount of calcium entering the extracellular fluid, there will be a reduction in the plasma calcium levels. The parathyroid glands monitor the calcium concentration in the blood and as soon as a decrease is detected, renal re-absorption mechanisms for calcium is stimulated, as well as the processes to enhance intestinal calcium absorption and bone resorption (NRC, 2001; Goff, 2008; DeGaris and Lean, 2009).



Bone is the major source of calcium during periods of low calcium intake and calcium exists mainly in two forms within bone. A small amount of readily available calcium exists in solution in the fluids surrounding the bone cells and within the canaliculi of the bone. The soluble calcium in the bone fluids is separated via a synctum of bone line cells from the extracellular fluids of the body. In reaction to PTH stimulation, the bone lining cells rapidly transfer the bone fluid calcium into the extracellular pool (Horst et al., 1997 a). Parathyroid hormone also induces the renal enzyme for the production of the vitamin D metabolite 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Horst et al., 1997 a). Both the PTH and 1,25-(OH)<sub>2</sub>D<sub>3</sub> is responsible for osteoclastic bone resorption and increasing renal tubular re-absorption of calcium. The principal function of the 1,25-(OH)<sub>2</sub>D<sub>3</sub> is to increase intestinal calcium absorption through active transport of dietary calcium across the intestinal epithelium (Horst et al., 1997 a; Goff, 2008; DeGaris and Lean, 2009).

It is important to note that as dietary calcium concentrations increase total dietary intake of calcium will also increase, resulting in reduced efficiency of intestinal calcium absorption as well as a decrease in calcium mobilization from bone and *vice versa* (Horst, 1986; NRC, 2001; DeGaris and Lean, 2009).

Though milk fever affects only a small percentage of dairy cows, nearly all animals experience some degree of hypocalcaemia during the first few days after calving, while the intestines and bones adapt to the calcium demands of lactation. The degree of hypocalcaemia may be related to the amount and rate prepartum colostrum production. For example, a cow producing 10 litres of colostrum, loses about 23 g of calcium during a single milking, which is 9 times more than the calcium available in the extracellular pool of the animals (Horst et al., 1997 a). Both the plasma and inorganic phosphorus concentrations decline at parturition, while magnesium remains unchanged or increases (Jorgenson, 1974). Normal plasma concentrations for calcium, inorganic phosphorus and magnesium are 8.5 – 11.4, 3.1 – 6.0 and 1.8 – 3.2 mg/100 ml respectively. A normal degree of hypocalcaemia may range from mild (7.5 – 8.5 mg/100 ml) to severe (5.0 – 6.0 mg/100 ml) (Jorgenson, 1974).

An understanding of how and why the calcium homeostatic mechanisms fail may arise from a thorough understanding of how these mechanisms work under normal circumstances and then exploring the possible sites for breakdown of homeostasis.

## **Hypomagnesaemia:**

The magnesium concentration of the dairy cow is normally maintained at 0.75 to 1.0 mmol/L.

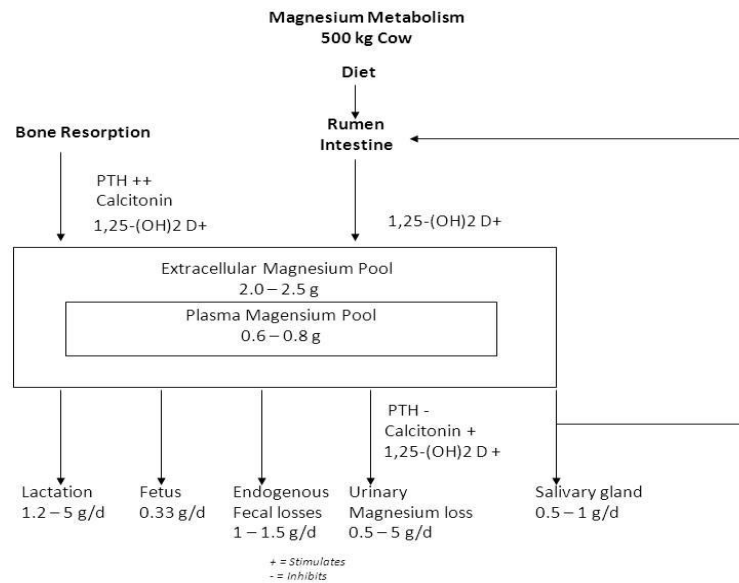
Hypomagnesaemia will affect calcium metabolism in one of two ways:

- i) Reducing PTH secretion in response to hypocalcaemia;
- ii) Reducing tissue sensitivity to PTH. (Goff, 2008; DeGaris and Lean, 2009).

Parathyroid hormone (PTH) has 3 main functions in the dairy cow:

- i) To mobilize calcium from bone;
- ii) To promote calcium absorption from the digestive tract through increased  $1,25\text{-(OH)}_2\text{D}_3$  concentration; and
- iii) To stimulate the kidneys to excrete excess phosphorus while retaining calcium for re-absorption.

Parathyroid hormone secretion is stimulated by minimal decreases in blood calcium concentrations. High PTH concentrations will promote the production of  $1\text{-}\alpha\text{-hydroxylase}$  in the kidney, thereby converting  $25\text{-OH-D}_3$  to  $1,25\text{-(OH)}_2\text{D}_3$  (Taylor et al., 2008). It was however found that hypomagnesaemia may inhibit this process (Goff, 2008). As PTH binds to its receptor on bone or kidney tissues, it initiates the activation of adenylate cyclase causing the production of the second messenger, cyclic AMP (Goff, 2006). Parathyroid hormone receptor interactions should cause the activation of phospholipase C in certain tissues, which will result in the production of the second messengers diacylglycerol and inositol 1,4,5-triphosphate. Both adenylate cyclase and phospholipase C have a magnesium binding site which must be occupied by a magnesium oxide for full activity (Goff, 2008).



**Figure 2.2 Hormonal control of magnesium metabolism (Adopted from Fontenot et al., 1989)**

Any dietary magnesium that is absorbed and not needed for maintenance, tissue growth, foetal development or lactation will be excreted via the kidneys. Most of the magnesium filtered across the renal glomeruli is re-absorbed by the renal tubular epithelium. At certain blood magnesium concentrations, the magnesium filtered across the glomerulus exceed the capacity of the renal tubules to re-absorb the filtered magnesium. This is known as the renal threshold for magnesium (Goff, 2006).

Calcium homeostasis is highly dependent on the integrity of the interaction between PTH and its receptor. Parathyroid hormone on the other hand can affect magnesium metabolism in two ways (Figure 2.2). Firstly, the renal threshold for magnesium excretion is partially under control of PTH and secondly, when cows are infused with PTH, urinary excretion of magnesium declines, resulting in increased plasma concentrations of magnesium and *vice versa* (Fontenot et al., 1989).

Parathyroid hormone increases renal tubular absorption of magnesium under normal conditions, which means that the kidneys are excreting less of the dietary magnesium absorbed. This in turn results in elevated blood magnesium levels as found in cows exhibiting milk fever (Goff, 2000). Should dietary magnesium be deficient or rumen absorption of magnesium be impaired, excess magnesium will be available to conserve and the plasma magnesium concentration will decline to levels below 1.85 mg/dl as a result of lactational drain of magnesium. If the serum magnesium concentration is not at least 0.8 mmol/L, it suggests inadequate dietary magnesium absorption and

that hypomagnesaemia may be limiting productivity and may contribute to hypocalcaemia in the herd (Goff, 2006).

Magnesium content of the close up dry cow ration should be between 0.35 and 0.4% to ensure adequate magnesium absorption during this critical period (Goff, 2000). Hypomagnesaemia can be prevented by increasing the dietary magnesium content and ensuring that it is supplied in a physiologically available form.

### **Low Calcium prepartal diets:**

Cows fed a diet with very low calcium concentrations (10 – 20 g of calcium/day) prior to calving cannot meet the calcium requirements for maintenance of foetal skeletal development (~30 g of calcium/day). These animals are in a negative calcium balance, causing a minor decline in blood calcium concentrations, which in turn stimulates PTH secretion and renal 1,25-(OH)<sub>2</sub>D<sub>3</sub> production prior to calving as well as osteoclastic bone resorption (Goff, 2008). Thus at the onset of lactation these homeostatic mechanisms for calcium are active, thereby preventing a severe decline in the concentration of plasma calcium (NRC, 2001; Goff, 2008). Preparing the dairy cow for a high calcium demand prior to calving avoids the 2-3 day required to activate the mechanisms in the fresh cow and thus assists the cow in avoiding prolonged hypocalcaemia.

It is a nutritional challenge to achieve truly low calcium diets (<20 g calcium/day) and as such a diet must supply significantly less absorbable calcium than is required, if it is to stimulate PTH secretion. For example, a 600 kg cow consuming 13 kg DM must be fed a diet that contains <1.5 g/kg (0.15%) absorbable calcium if it is to provide <20 g available Ca/day (Goff, 2008). The PTH secretion in turn leads to increased renal re-absorption of calcium, stimulating calcium resorption from bone and stimulates renal vitamin D metabolism towards production of 1,25-(OH)<sub>2</sub>D (Thilsing-Hansen et al, 2002 b). 1,25-(OH)<sub>2</sub>D stimulates the active transport of calcium across the epithelial cells (Horst et al., 1994). Due to the difficulty of keeping the calcium intake sufficiently low employing commercially available feeds, the low calcium-principle has more or less been abandoned in many countries.

Two methods to reduce the amount of dietary calcium absorbed have recently been developed. These include 1) Incorporation of Zeolite (an alumino-silicate such as clinoptilolite) into the ration which binds calcium and causes it to pass out in the faeces. Large amounts of zeolite must be ingested each day (0.25 to 1 kg/day for 2 weeks prior to calving) and may have a negative influence on phosphorus absorption (Thilsing-Hansen et al., 2002 b). 2) Administration of vegetable oils

which bind calcium to form an insoluble soap, thus preventing absorption of dietary calcium (Goff, 2008).

The low calcium-principle is a highly effective method in preventing milk fever, provided that the daily calcium intake is less than 20 g/day and the exposure period should be at least 2 weeks prior to calving.

### **Metabolic Alkalosis:**

Metabolic alkalosis is the result of a diet that supplies more cations (potassium, sodium, calcium and magnesium) than anions (chloride, sulphate and phosphate). This causes a disparity in the electrical charge of the body fluids as a larger number of positively charged cations enter the blood than negatively charged anions (Goff, 2008). In order to restore the electro-neutrality of the blood, a positive charge in the form of a hydrogen ion ( $H^+$ ) must be lost from the blood compartment. Reduction of the  $H^+$  concentration will be equivalent to an increase in the blood pH (Goff, 2008). Goff (2000) has clearly described the above with the following equation:

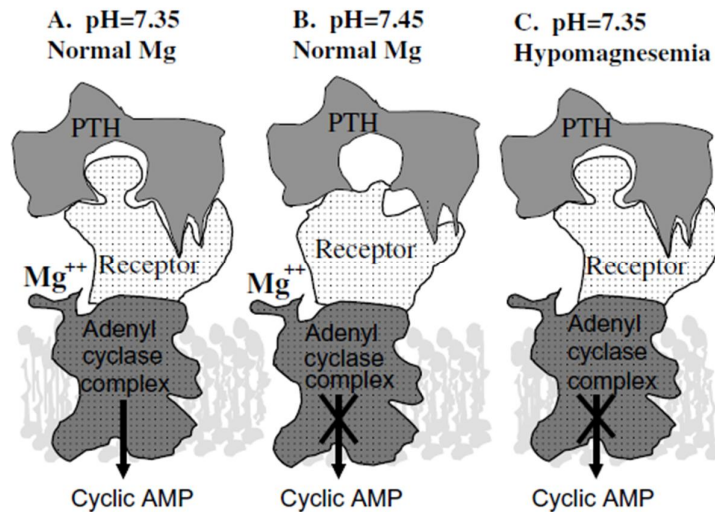
a. #Moles Cations = # Moles Anions

b.  $[H^+] \times [OH^-] = 1 \times 10^{-14}$

The number of moles of cations (positively charged particles) in any given solution (including body fluids) must be equal to the number of moles of anions (negatively charged particles). Secondly, the product of the concentration of hydrogen ions and hydroxyl ions is always equal to the dissociation constant of water (approximately  $1 \times 10^{-14}$ ). Both these equations must be satisfied simultaneously. As pH is the negative log of the concentration of hydrogen ions, this means that the pH of the solution will be dependent on the difference between the number anions and cations in the solution. If, for example, cations are added to a solution (e.g. plasma), the number hydrogen cations will decrease and the number of hydroxyl anions will increase to maintain the electro-neutrality of the solution. In other words the solution becomes more alkaline. Should the above be reversed and more anions are added, the pH will be reduced resulting in a more acidic solution (Goff, 2000).

It is clear that metabolic alkalosis may be the cause of subclinical hypocalcaemia and milk fever. Horst et al. (1994), indicated that should a cow be fed >100 g of calcium per day, the dairy cow will meet its daily calcium requirement almost entirely by passive absorption of dietary calcium. Active transport of calcium from the diet and bone calcium resorption mechanisms become homeostatically depressed. This may be due to a change in the conformation of the PTH receptor, rendering the tissues less sensitive to PTH (Figure 2.3). The lack of PTH responsiveness by bone tissue prevents the activation of osteoclastic bone resorption, while failure of the kidneys to respond to PTH reduces

renal re-absorption of calcium from the glomerular filtrate. The kidneys also fail to convert 25-OH-D<sub>3</sub> to the hormone 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Goff,2008). As a result, the cow will be unable to use bone calcium stores or intestinal calcium mechanisms at calving and is thus susceptible to hypocalcaemia until these mechanisms can be activated.



**Figure 2.3** Effect of PTH at the surface of target bone and kidney cells under various physiological circumstances (Adopted from Goff, 2008)

*Panel A: Under normal conditions, PTH released in response to hypocalcaemia interacts with its receptor, located on the surface of bone and kidney cells, in a lock-and-key fashion. This stimulates G-proteins and adenylate cyclase (adenylate cyclase complex) resulting in production of cyclic AMP, which acts as a second messenger within the cytosol of target cells. This initiates mechanisms such as bone calcium resorption and renal production of 1,25-dihydroxyvitamin D to restore blood calcium concentration to normal levels. Panel B: Alkalotic conditions induced by high potassium diets induce a change in the shape of the PTH receptor protein so that it is less able to recognize and bind PTH, resulting in failure to activate the cell by producing cyclic AMP. Panel C: Magnesium is required for full function of the adenylate cyclase complex. Hypomagnesaemia reduces the ability of PTH stimulated cells to produce cyclic AMP, resulting in failure to activate the cell.*

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## 2.2 The vitamin D regulatory system and its role in calcium homeostasis:

Our knowledge and understanding of vitamin D as an important regulator of calcium and phosphorus homeostasis has greatly improved during the latter part of the 20<sup>th</sup> century. The discovery of the vitamin D endocrine system resulted in the realization that calcium homeostasis in both mammals and birds involves a synchronized effort between the parathyroid hormone (PTH), calcitonin and the hormonally active form of vitamin D<sub>3</sub> – 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Spakauskas et al., 2006). A disruption of the calcium homeostasis at parturition may lead to clinical or subclinical hypocalcaemia. It has become apparent that the vitamin D sterols may play a significant role as a preventative measure for parturient hypocalcaemia.

The two major natural sources of vitamin D to ruminants are derived from photochemical conversion of 7-dehydrocholesterol to vitamin D<sub>3</sub> in the skin or from plants as a result of photochemical conversion of ergosterol to vitamin D<sub>2</sub> (Horst et al., 1994; Sahota and Hosking., 1999; NRC, 2001). Both vitamin D<sub>2</sub> and vitamin D<sub>3</sub> can either be derived from dietary sources or supplemented in the bovine diet via commercially available crystalline forms. Once vitamin D enters the blood, it circulates at relatively low concentrations (between 1 and 3 ng/ml) in dairy cows. According to Jones et al (1998), vitamin D does not circulate for an extended period in the bloodstream, but instead is immediately taken up by the adipose tissue for storage or liver for further metabolism.

Vitamin D<sub>3</sub> is activated by C<sub>25</sub> hydroxylation in the liver to form 25-OH-D<sub>3</sub> which is the major circulation form of vitamin D (Horst *et al.*, 1994). Jones et al (1998) indicated that the main reason for the stability of this particular metabolite is its strong affinity for the vitamin D-binding (globulin) protein of blood (DBP). He further states that the metabolic fate of 25-OH-D<sub>3</sub> depends on the calcium requirements of the animal i.e an urgent calcium requirement results in 1 $\alpha$ -hydroxylation, whereas an abundance of calcium results in 24-hydroxylation. The production of 25-OH-D<sub>3</sub> also depends on the vitamin D content of the diet. This suggests that plasma 25-OH-D<sub>3</sub> concentration is the best indicator of the vitamin D status of the animal (Littledike and Goff, 1987; NRC, 2001).

25-Hydroxyvitamin D<sub>3</sub> is transported to the kidney bound to vitamin D binding protein, where it is further hydroxylated by 1 $\alpha$ -hydroxylase to form the hormone 1,25-(OH)<sub>2</sub>D<sub>3</sub> (the major active vitamin D metabolite). This metabolite is responsible for most of the biological activity attributed to vitamin D. (Jones et al., 1998; Sahota and Hosking., 1999).

The vitamin D metabolites have several functions which include stimulation of calcium and phosphorus absorption in the intestines, mobilization of calcium and phosphorus from bone and increased renal absorption of calcium in the kidney. However, vitamin D also directly suppresses PTH and is a developmental hormone required for the recruitment of cells for osteoclast formation, female reproduction, skin development as well as treatment of certain malignant conditions (Jones et al., 1998; Spakauskas et al., 2006).

Sahota and Hosking (1999), stated that the  $1\text{-}\alpha$  hydroxylation is the rate limiting step in  $1,25\text{-(OH)}_2\text{D}_3$  synthesis and is mainly controlled by PTH and intracellular phosphate, but also by calcitonin, growth hormone, oestrogen, prolactin and  $1,25\text{-(OH)}_2\text{D}_3$ .  $1,25\text{-Dihydroxyvitamin D}$  along with PTH mediates blood calcium and phosphorus homeostasis as it is responsible for the adaptation to dietary calcium and phosphorus levels.

### **Parathyroid Hormone Function:**

The parathyroid gland can be seen as the calcium sensing organ of the body and will react to the slightest change in plasma calcium concentrations by secreting parathyroid hormone (PTH). Parathyroid hormone is responsible for the initiation of all events in order to maintain calcium homeostasis (Jones et al., 1998).

Should the plasma calcium levels decline to  $<10$  mg/dl, PTH will be produced by the parathyroid gland. In turn, PTH stimulates the activation of  $25\text{-OH-D}_3$  via up-regulation of  $1\text{-}\alpha$  hydroxylase in the kidney to produce  $1,25\text{-(OH)}_2\text{D}_3$ . If the plasma calcium concentration is  $>10$  mg/dl, PTH secretion as well as  $1,25\text{-(OH)}_2\text{D}_3$  synthesis is depressed (Horst et al., 1994, Taylor et al., 2008). Parathyroid hormone receptors can mainly be found throughout the length of the nephron of the kidney, but is not located in the intestines and is found in the osteoblasts not in the osteoclasts of the skeleton (Jones et al., 1998).

Parathyroid hormone stimulates the renal expression of the  $1\text{-}\alpha$  hydroxylase thereby favouring the formation of  $1,25\text{-(OH)}_2\text{D}_3$  which will in turn act on the gut to enhance calcium and phosphate absorption and secondly on the kidney to further increase tubular calcium re-absorption and will directly promote bone resorption as well (Goff, 2000; Bienaime et al., 2011). Parathyroid hormone also acts on the proximal tubule to inhibit phosphate re-absorption, thereby inducing phosphate diuresis, while activating the  $25\text{-hydroxyvitamin D-}1\alpha\text{-hydroxylase}$  in the proximal convoluted tubule cells (Jones et al., 1998; Bienaime et al., 2011).



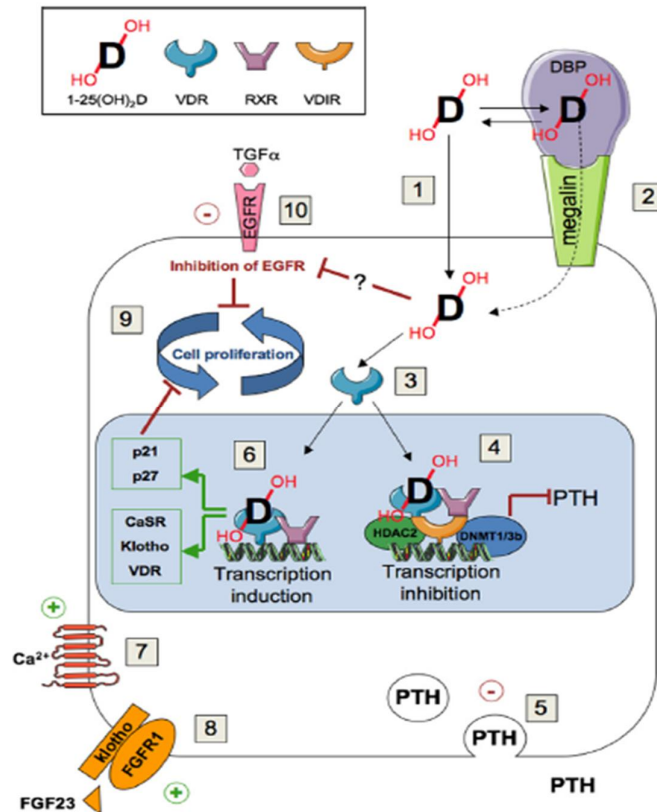
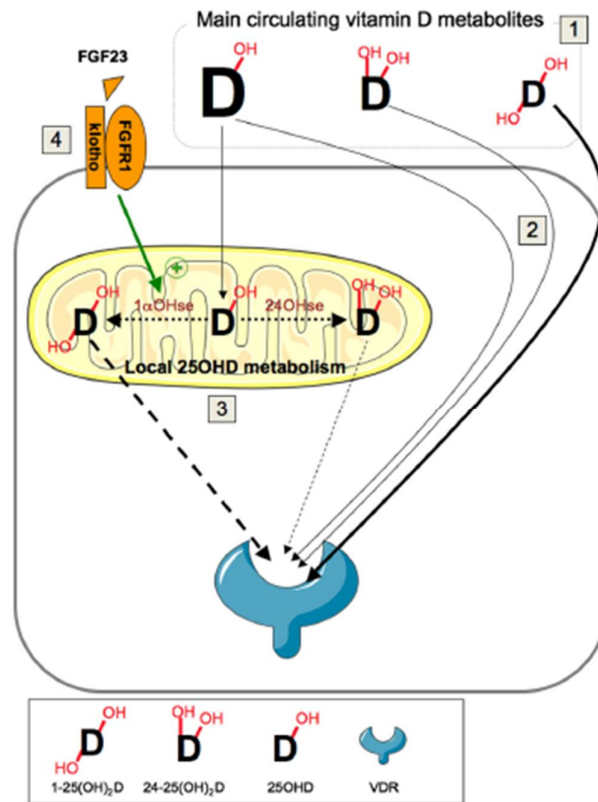


Figure 2.4 Overview of vitamin D function in parathyroid cells (Adopted from Bienaime et al., 2011)

Figure 2.4 gives an overview of vitamin D function in parathyroid cells and can be explained as follows:

- (1) Free vitamin D enters parathyroid cells by passive diffusion through the lipid bi-layer.
- (2) As parathyroid cells express megalin, the vitamin D binding protein (DBP) receptor, and active uptake of DBP bound to vitamin D is also possible.
- (3) Inside the cell vitamin D binds to VDR, which translocates to the nucleus.
- (4) Vitamin D bound VDR can form different complexes in different promoter regions. By associating with RXR and VDIR, it recruits the histone deacetylase (HDAC2) and the DNA methyl transferases DNMT1 and DNMT3b to repress PTH gene transcription.
- (5) Thereby reducing the amount of secreted PTH.
- (6) By associating with RXR directly on vitamin D responsive elements on DNA, vitamin D bound VDR induces the transcription of calcium sensing receptor (CaSR), Klotho, VDR, p21 and p27 genes
- (7) Increased CaSR transcription results in higher sensitivity to calcium of parathyroid cells,

- (8) Increased Klotho transcription results in a higher sensitivity to FGF23
- (9) P21 and P27 inhibits cell proliferation by inhibiting cell cycle progression
- (10) Vitamin D also inhibits parathyroid cells proliferation by reducing epidermal growth receptor (EGFR) signalling possibly in a VDR independent manner (Beinaime et al., 2011).



**Figure 2.5 Overview of vitamin D metabolism in parathyroid cells (Adopted from Bienaime et al., 2011)**

Figure 2.5 gives an overview of vitamin D metabolism in the parathyroid cells and can be explained as follows:

1. Total 25-OH-D<sub>3</sub> circulating concentration is 500-fold higher than 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration and
2. 5-fold higher than 24,25-dihydroxyvitamin D concentration.
3. All these metabolites enter the cell and bind the vitamin D receptor (VDR). However,
4. 1,25-(OH)<sub>2</sub>D<sub>3</sub> binds VDR with a 1000-fold higher affinity than either 25-OH-D<sub>3</sub> or 24,25-dihydroxyvitamin D.

5. Parathyroid cells express 1- $\alpha$ -hydroxylase and 24-hydroxylase and are therefore able to locally form 1,25-(OH)<sub>2</sub>D<sub>3</sub> and 24,25-dihydroxyvitamin D from 25-OH-D<sub>3</sub>.
6. FGF23 enhances 1- $\alpha$ -hydroxylase expression in parathyroid cells (Beinaime et al., 2011).

### **Role of 1,25-Dihydroxyvitamin D<sub>3</sub>:**

As mentioned earlier, 1,25-(OH)<sub>2</sub>D<sub>3</sub> is one of the most biologically potent vitamin D<sub>3</sub> metabolites and stimulates active transport of calcium as well as bone resorption. Its action requires it to bind to a low capacity, high affinity receptor in its target tissues before becoming active and therefore 1,25-(OH)<sub>2</sub>D<sub>3</sub> acts like a steroid hormone (Horst et al., 1983; Reinhardt et al., 2010).

1,25-Dihydroxyvitamin D<sub>3</sub> mainly circulates in blood bound to the vitamin D binding protein and there are usually less than 5% of this hormone circulating in the free state (Horst et al., 2003). The free form of 1,25-(OH)<sub>2</sub>D<sub>3</sub> will enter the target cells and associate with the 1,25-(OH)<sub>2</sub>D<sub>3</sub> receptor (VDR). This complex is phosphorylated and combines with a nuclear accessory factor, retinoic acid X receptor (RXR) creating a VDR/RXR heterodimer (Haussler et al., 1995). This VDR/RXR heterodimer has a high affinity for a number of vitamin D responsive elements located in the regulatory regions of 1,25-(OH)<sub>2</sub>D<sub>3</sub> controlled genes. A change in the expression of these genes results in target cell modification as well as the collection of biological effects of vitamin D which includes bone remodelling, intestinal calcium and phosphorus resorption, PTH suppression and catabolism of the 1,25-(OH)<sub>2</sub>D<sub>3</sub> hormone by 25-hydroxyvitamin D-24-hydroxylase (Haussler et al., 1995).

1,25-Dihydroxyvitamin D<sub>3</sub> can be seen as a major calcium mobilizing hormone. Jones et al. (1998) described the consequences of an increase in the concentration of this hormone as follows:

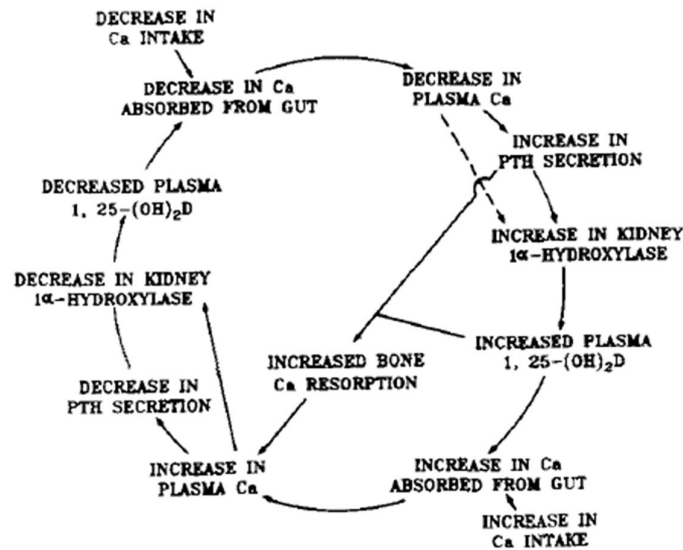
1,25-Dihydroxyvitamin D<sub>3</sub> acts by itself to initiate active intestinal calcium transport in the small intestine. This system has a relatively long lifetime (measured in days), whereas the other actions of 1,25-(OH)<sub>2</sub>D<sub>3</sub> are much shorter. Osteoblasts are activated by 1,25-(OH)<sub>2</sub>D<sub>3</sub> and the result of this activation is either to stimulate the osteoclast to resorb bone and/or the activation of the reverse transport of calcium from the bone fluid compartment to the plasma compartment. Finally the calcium is mobilized by the skeleton into the plasma compartment due to the action of the vitamin D hormone and PTH. Furthermore the action of these two hormones causes the re-absorption of the last 1% of filtered load of calcium into the plasma compartment, leading to an increase in serum calcium which clears the sensing point of the calcium receptor. The PTH secretion is then terminated (Jones et al., 1998).

### **Calcitonin Function:**

Hypercalcaemia may lead to the calcification of soft tissues such as kidney, heart, aorta and intestine causing organ failure and death. Calcitonin is released by the thyroid C-cells in response to hypercalcaemia and will reduce calcium removal from bone as well as increase urinary calcium excretion (Goff, 2000). In order to prevent hypercalcaemia it is thus important that PTH secretion is stopped and that calcitonin be released (Jones et al., 1998). This is a 34-amino acid peptide hormone that is responsible for lowering the serum calcium concentrations via its action on the skeleton. It has a direct action on osteoclasts and osteocytes by reducing the calcium mobilizing activity and eliminating calcium coming from the skeleton (Jones et al., 1998)

### **Calcium absorption:**

The amount of calcium that must be fed to meet the requirement for absorbed calcium is dependent on the availability of calcium from the feedstuffs and inorganic calcium sources in the diet, as well as the efficiency of intestinal absorption in the animal being fed. The amount of calcium absorbed will generally be equal to the requirement of the body for calcium, provided the diet contains enough available calcium (NRC, 2001). Horst et al. (1994) stated that the amount of dietary calcium absorbed will decrease as the dietary calcium increases above the requirement of the tissues for absorbed calcium (Figure 2.6). To determine the efficiency of dietary calcium absorption, animals should be fed less calcium than the amount of absorbed calcium needed, to meet their requirements. This will ensure that the intestinal calcium absorption mechanisms are fully active and the animal will absorb all the calcium from the feedstuff to its biological upper limit (NRC, 2001).



The dashed lines represent a response that occurs in rats but not in ruminants ( $(OH)_2$  – dihydroxyvitamin PTH – Parathyroid Hormone

**Figure 2.6** Mechanism of adaptation to alterations in dietary calcium (Adopted from Horst, 1986)

Calcium, magnesium and phosphorus are absorbed both actively in the duodenum and jejunum and passively in the colon. There is however only little information available on respective proportions of active and passive transport routes under different conditions i.e. amount of dietary calcium supply, source of calcium, species, age, sex and reproduction as well as production state (Schröder and Breves, 2007).

The quantitative proportions of individual segments of the gastro-intestinal tract in overall net calcium absorption have been studied in various balance experiments using single- or multi fistulated sheep (Rayssiguier and Poncet, 1980; Greene et al., 1983; Wylie et al., 1985). The majority of these experiments indicated that net calcium absorption occurs before the duodenum in addition to the upper small intestines (Schröder and Breves, 2007).

From the data obtained a mean daily calcium intake of 5.4 g and a mean daily faecal excretion of 4.3 g can be calculated resulting in a net absorption of 1.1 g/day. On average, about 50, 35 and 15% of this amount has been absorbed from the duodenum, small intestines and hindgut respectively. The pre-duodenal absorption should however be corrected for salivary calcium secretion as well as the potential role of the abomasum, which has not been studied in any detail with respect to its absorptive function for calcium (Schröder and Breves, 2007).

Similarly to the above mentioned studies in fistulated sheep, efforts have been made to illustrate the quantitative proportions of the individual segments of the gastro-intestinal tract involved in calcium absorption in cattle. It appears that, as in sheep, the pre-duodenal proportion becomes more important if the daily dietary calcium intake exceeds a certain amount (>120 g of dietary calcium per day) (Schröder and Breves, 2007). At present it is unknown to what extent passive and active pathways may contribute to overall calcium net absorption from the pre-duodenal compartments in the bovine rumen.

Proximal absorption is controlled by  $1,25\text{-(OH)}_2\text{D}_3$  and involves luminal permeability, intracellular calcium transport and active extrusion at the basolateral membrane (Sahota and Hosking, 1999). At the distant bowel, calcium is absorbed both by a  $1,25$ -dihydroxyvitamin-modulated carrier process and by passive paracellular diffusion down a concentration gradient, partially dependent on the luminal calcium concentration and via a high affinity calcium activated ATP or sodium/potassium exchange (Horst, 1986; Sahota and Hosking, 1999).

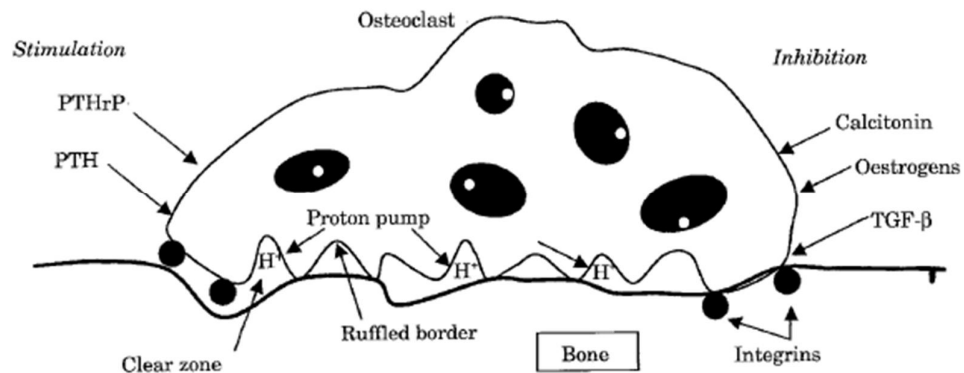
$1,25$ -dihydroxyvitamin  $\text{D}_3$  mediates these responses, by entering the enterocytes through diffusion and binding to its receptor in the cytosol. The subsequent complex formed, translocates the chromatin fraction of the nucleus. This nuclear receptor-hormone complex results in increased mRNA synthesis as well as synthesis of specific proteins that control calcium transport (Horst, 1986; Goff et al., 1991 b).  $1,25$ -dihydroxyvitamin  $\text{D}_3$  may facilitate the diffusion, but this facilitation does not appear to be the rate limiting step to calcium absorption. Calcium must then cross the cell to the basal lateral side of the cell and this is facilitated mainly by the calcium binding protein, which is cytosolic and dependent on vitamin D (Horst et al., 1994). The rate of transcellular calcium transport is directly correlated to the amount of calcium binding proteins in the cells (Goff et al., 1991 b).

### **Bone Resorption:**

Bone resorption (Figure 2.7) usually begins by the proliferation and differentiation of the stem cells of monocyte macrophage lineage and the formation of mature osteoclasts (Sahota and Hosking, 1999).

Calcium exists within bone in two states:

- i) The majority is tightly bound to the organic bone collagen matrix as calcium hydrogen phosphate ( $\text{CaHPO}_4$ ) deposits;
- ii) A small amount of calcium exists in solution in the fluids surrounding the bone cells within the canaliculi of the bone. (Goff et al., 1991 b)



Abbreviations: PTH = parathyroid hormone; PTHrP = parathyroid hormone-related peptide; TGF- $\beta$  = transforming growth factor -  $\beta$ .

**Figure 2.7 Osteoclastic bone resorption (Adopted from Sahota and Hosking, 1999)**

As indicated before, calcium concentrations are maintained under normal conditions by calcitonin, vitamin D and PTH. Bone resorption and intestinal calcium absorption are regulated by vitamin D and PTH (Liesegang et al., 2007). During conditions of acute calcium stress, e.g. onset of lactation, plasma calcium levels decrease, resulting in increased plasma PTH and  $1,25\text{-(OH)}_2\text{D}_3$  (Horst, 1986). Upon increased PTH concentrations, osteocytic osteolysis occurs, where PTH causes the removal of bone salts from the bone matrix by lacunar osteocytes. This occurs within minutes and proceeds without actual resorption of the bone matrix (El-Samad et al., 2002).

Osteocytic osteolysis only meets the more short term needs. If high PTH concentrations persists, a delayed response (hours to days) takes place due to activation of the bone osteoclasts. This is known as osteoclastic bone resorption. This process involves resorbing the bone matrix itself and allows the response to PTH to continue beyond what can be handled by osteoclastic osteolysis (El-Samad et al., 2002). This causes increased bone resorption rates, decreased calcium excretion via the kidney and increased Calcium absorption (Liesegang et al., 2007).

Should hypocalcaemic conditions persist, the animal's continued PTH secretion causes osteoblasts to secrete substances such as prostaglandins and interleukin-6. These substances stimulate the activity of existing osteoclasts prompting them to form new osteoclasts. Continued PTH secretion increases 1,25-(OH)<sub>2</sub>D<sub>3</sub> production which enhance osteoclastic activity (Goff et al., 1991 a). Furthermore, the presence of 1,25-dihydroxyvitamin D will enhance maturation of monocytes to macrophages which are the direct precursors to osteoclasts (Horst, 1986).

Osteoclasts do not contain receptors for PTH and 1,25-(OH)<sub>2</sub>D<sub>3</sub> as osteoblasts do. This implies that osteoclast activity is under the control of osteoblasts, causing osteoblasts to release acids and lysosomal enzymes onto the bone surface. The osteoclasts will release acids and lysosomal enzymes on the bone surface, causing digestion of the organic matrix and solubilisation of calcium salt deposits. These deposits are in turn transported across the osteoclast and released into the extracellular pool. Should hypercalcaemia develop, calcitonin will decrease osteoclast activity (Goff et al., 1991 a).

### **2.3 Predisposing Factors for Milk Fever**

Milk fever does not occur randomly and without bias. Several factors can predispose a cow to be affected by milk fever including age, breed and diet. These factors can act alone or in conjunction with one another to increase the severity of milk fever of the affected animal.

#### **Age:**

Heifers rarely develop milk fever. The risk of a cow developing milk fever will increase with age (Horst et al., 1997 a; NRC, 2001; Rezac, 2010). From the third lactation onwards, dairy cows produce more milk, resulting in a higher calcium demand. In addition to increased milk production, ageing also results in a diminished ability to mobilize calcium from bone stores and a decline in the active transport of calcium in the intestine, as well as impaired production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Horst et al., 1997 a; Rezac, 2010). The skeletal bones of heifers are still in a growth phase and therefore have a large number of osteoclasts present, which can respond to PTH more readily than the bones of mature cows (NRC, 2001). Increased age also causes a decrease in the number of 1,25-(OH)<sub>2</sub>D<sub>3</sub> receptors (Rezac, 2010).



**Breed:**

Certain breeds of dairy cows have been shown to be more susceptible to milk fever than others. It was shown that Jerseys had lower numbers of intestinal receptors for 1,25-(OH)<sub>2</sub>D<sub>3</sub> than same-aged Holsteins (Horst et al., 1997 a). Lower receptors would result in a loss of target tissue responsiveness and sensitivity to 1,25-(OH)<sub>2</sub>D<sub>3</sub>. At calving the plasma 1,25-(OH)<sub>2</sub>D<sub>3</sub> levels are increased due to the hypocalcaemic state of the dairy cow. Normally, the elevated levels would result in enhanced bone calcium resorption and intestinal calcium absorption, but with the reduced number of receptors available in an older animal, the activation of genomic events by 1,25-dihydroxyvitamin D is less sufficient, resulting in increased tendency to become hypocalcaemic (Horst et al, 1997 a).

**Diet:**

It is known that the manipulation of dietary calcium and phosphorus has dramatic effects on the incidence of milk fever (Horst et al., 1997 a). Jorgensen (1974) stated that cows with high calcium intake were at an increased risk of hypocalcaemia and milk fever compared to cows on a lower calcium diet. He also indicated that the total amount of dietary calcium was more important than the dietary ratio of calcium and phosphorus. As stated earlier, it is very difficult to achieve truly low calcium diets (<20 g calcium/day) as such a diet must supply significantly less absorbable calcium than required by the cow. For example, a 600 kg cow consuming 13 kg DM must be fed a diet that contains <1.5 g/kg (0.15%) absorbable calcium if it is to provide <20 g available Ca/day (Goff, 2008).

**2.4 Milk Fever Control Principles:**

Several milk fever control principles and preventative measures have been proposed, but for a number of reasons the most widely used measures include:

1. Oral drenching around calving with a supplement of easily absorbed calcium;
2. Feeding of acidifying rations by anionic salt supplementation during the last few weeks before calving;
3. Prepartum administration of vitamin D, vitamin D metabolites and analogues. (Thilising-Hansen et al., 2002 a).

## Oral Calcium drenching around calving:

There are a number of formulations available for oral calcium drenching and the majority of studies have been done with preparations containing easily absorbable calcium salts such as calcium chloride, providing 40-50 g of calcium per dose – either as a bolus, gel, paste or a liquid. This method was developed for therapeutic use in cows with milk fever, instead of intravenous calcium infusions (Thilsing-Hansen et al., 2002 a).

Normally cows will absorb calcium by 1 of 2 mechanisms:

- i) Active transport across intestinal epithelial cells;
- ii) Passive transport between intestinal epithelial cells. (Goff and Horst, 1993).

Passive transport depends on diffusion down a concentration gradient. Passive diffusion of calcium from the lumen of the gut to the extracellular fluids occur when the luminal ionized calcium concentration exceeds 1 mM (Thilsing-Hansen et al., 2002 a). Goff and Horst (1993), found that oral calcium treatment increased luminal calcium concentrations above 1 mM, favouring passive transport of calcium into the extracellular fluids. The capacity of passive transport of calcium is unlimited and independent of stimulation by  $1,25\text{-(OH)}_2\text{D}_3$ . This means that the net absorption of free calcium increases linearly with increasing luminal calcium concentrations (Thilsing-Hansen et al., 2002 a). In a study reported by Goff and Horst (1993), it was found that calcium chloride proved to be readily absorbable, thereby increasing the plasma calcium concentrations. Calcium chloride may also stimulate the oesophageal groove reflex due to the osmotic effect and thereby allowing rumen bypass. This means that the calcium solution avoids dilution in the rumen, causing a high concentration gradient in the abomasum, thus favouring passive transport (Goff and Horst, 1993).

Best results are usually obtained with doses between 50 and 125 g of calcium per dose (Goff, 2008). Fifty gram of soluble calcium results in the entry of about 4 g of calcium into the blood. It is suggested that the increased blood calcium levels may last between 4 and 6 hours, however the calcium chloride can be caustic and may have a strong irritating effect on the mucosal lining of the gastro-intestinal tract (Goff and Horst., 1994; Thilsing-Hansen et al., 2002 a).

Plasma pH is highly dependent on the dietary balance between the cations potassium (K) and sodium (Na) and the anions chlorine (Cl) and Sulphur (S). Treatment with calcium chloride will upset this balance due to the chlorine being readily absorbed from the diet, while the calcium is poorly absorbed. The enhanced, negatively charged anions will cause a reduction in the plasma pH, leading to metabolic acidosis (Goff and Horst., 1994).

It is unlikely that oral calcium treatment could successfully prevent all milk fever cases although these treatments may help to prevent relapses.

### **Acidifying Rations (Dietary Cation-Anion Difference, DCAD):**

Several experiments have shown that the adjustment of the Dietary Cation-Anion Difference (DCAD) is effective in reducing the incidence of clinical hypocalcaemia (Block, 1984; Oetzel et al., 1988; Goff et al., 1991 a). According to Overton & Waldron (2004), several points should however be noted that should affect decision making for feeding of low DCAD diets. These include the fact that much of the research conducted has utilized animals at a higher risk for milk fever e.g. multiparous cows and Jersey cows. They also pointed out that an important factor in DCAD research has been the use of highly cationic diets as controls, which means that forages with high cationic loads were used, that exaggerated the difference between the control and treatment groups in the cited experiments.

Several equations have been published for the calculation of DCAD in dairy cattle diets. The first published equation was  $(Na + K) - (Cl + S)$  (Charbonneau et al., 2006). Using this equation the prepartum dietary cation-anion difference (DCAD) could be altered. As shown by a number of authors, the alteration of the DCAD had dramatic effects on the incidence of milk fever (Moore et al., 2000; Thilsing-Hansen et al., 2002 (a); Goff and Horst, 2003; Roche et al., 2003). They determined that by lowering the DCAD of the diet before calving, the risk for clinical and sub-clinical milk fever would be reduced.

Since the first DCAD equation (as mentioned before) was published, other DCAD equations have been suggested to account for the contributions of other macro mineral ions that may affect the acid-base status of the animal, but are not completely bio-available.

These equations included:

- $(Na + K + 0.38 Ca + 0.30 Mg) - (Cl + 0.6 S + 0.5 P)$  (Charbonneau et al., 2006)
- $(Na + K + 0.15 Ca + 0.15 Mg) - (Cl + 0.2 S + 0.3 P)$  (Charbonneau et al., 2006)
- $(Na + K + 0.15 Ca + 0.15 Mg) - (Cl + 0.6 S + 0.5 P)$  (NRC, 2001)

A fifth equation,  $(Na + K) - (Cl + 0.6 S)$ , was proposed by Goff et al (2004) and discounts the acidifying effects of S by 40% compared to the original 4- mineral equation  $[(Na + K) - (Cl + S)]$ . Two recent meta-analyses conducted by Charbonneau et al. (2006) and Lean et al. (2006) concluded

that both the equations  $(Na + K) - (Cl + S)$  and  $(Na + K) - (Cl + 0.6 S)$ , predicted animal responses with similar accuracy.

Ramberg et al. (1996) noted that, with the exception of sulphur, the DCAD equation concerns only monovalent dietary electrolytes, not all cations or anions. Organic and inorganic ions with higher valence (Ca, Mg, P etc.) are ignored. The supposed explanation for this is that binding of ionized substances with valence higher than 1 (both in the diet and animal) and variable and incomplete intestinal absorption, would complicate the interpretation of the effects on acid-base balance. The efficiency of intestinal absorption of inorganic monovalent ions is generally high and homeostasis is maintained primarily via urinary excretion. This is in contrast with divalent and trivalent cations whose homeostasis involves regulation of intestinal absorptive efficiency (Ramberg et al., 1996).

### Calculating DCAD:

Husband (2005) used the following table to demonstrate the properties of the four cations and anions commonly used to calculate the DCAD of a ration or feedstuff:

**Table 2.1** Properties of the four cations and anions used to calculate the DCAD of a ration or feedstuff

Element	Molecular weight (g)	Valence (charge)	Equivalent weight (g)	Conversion factor (% to mEq/kg diet)
Sodium	23.0	+1	23.0	435.0
Potassium	39.1	+1	39.1	255.7
Chlorine	35.5	-1	35.5	-282.1
Sulphur	32.1	-2	16.0	-623.8

To calculate the DCAD, mineral concentrations must first be converted to *milliequivalents (meq)* as follows:  $meq/100g = (milligrams)(valence)(g \text{ atomic weight})$ .

Block (2011) used the following example in order to demonstrate the calculation of DCAD for a diet:

The mEq  $(Na + K) - (Cl + S)$  value of a diet with 0.1% Na, 0.65% K, 0.2% Cl and 0.16% S will be calculated. In 100 g of this diet there are 100 mg Na (0.10% = .10 g/100 g or 100 mg/100 g), 650 mg K (0.65% K), 200 mg Cl (0.2% Cl), and 160 mg S (0.16 % S) per 100 g diet DM.

Therefore, this diet contains:

$$\text{mEq Na} = (100 \text{ mg})(1 \text{ valence}) = 4.3 \text{ mEq Na (23 g atomic weight)}$$

$$\text{mEq K} = (650 \text{ mg})(1 \text{ valence}) = 16.7 \text{ mEq K (39 g atomic weight)}$$

$$\text{mEq Cl} = (200 \text{ mg})(1 \text{ valence}) = 5.6 \text{ mEq Cl (35.5 g atomic weight)}$$

$$\text{mEq S} = (160 \text{ mg})(2 \text{ valence}) = 10.0 \text{ mEq S (32 g atomic weight)}$$

The next step is to sum the mEq from the cations and subtract the mEq from the anions:

$$\text{DCAD} = \text{mEq (Na + K)} - (\text{Cl} + \text{S}) = 4.3 + 16.7 - 5.6 - 10.0 = + \mathbf{5.4 \text{ mEq/100 g diet DM.}}$$

Another way to calculate DCAD directly from the percentages of minerals present is to use:

$$\text{DCAD} = [(\% \text{Na in DM}/0.023) + (\% \text{K in DM}/0.039)] - [(\% \text{Cl in DM}/0.0355) + (\% \text{S in DM}/0.016)].$$

For example, using the same numbers as above:

$$\text{DCAD equals } (0.10\% \text{ Na}/0.023) + (0.65\% \text{ K}/0.039) - (0.2\% \text{ Cl}/0.0355) - (0.16\% \text{ S}/0.016) = + \mathbf{5.4 \text{ mEq/100 g diet DM.}}$$

There are 2 basic principles to the DCAD theory:

1. The number of moles of cations in any given solution must be equal to the number of moles of anions in the solution;
2. The product of the concentration of hydrogen ions and hydroxyl ions is always equal to the dissociation constant of water, which is approximately  $1 \times 10^{-14}$

It is important that both these equations are satisfied simultaneously (Goff and Horst, 2003).

This theory suggests that a net influx of any mineral cation or anion will have an influence on the acid-base status of the dairy cow. The extent of the influence will depend on the amount of ions that enters the system. This means that the difference in the number of cation and anion equivalents in a diet, available for absorption will determine the acid-base status of the animal (Horst et al., 1997 a) and therefore the pH of the blood. The animal will be acidotic should absorbable anions dominate or alkalotic when absorbable cations dominate (Horst et al., 1997 a).

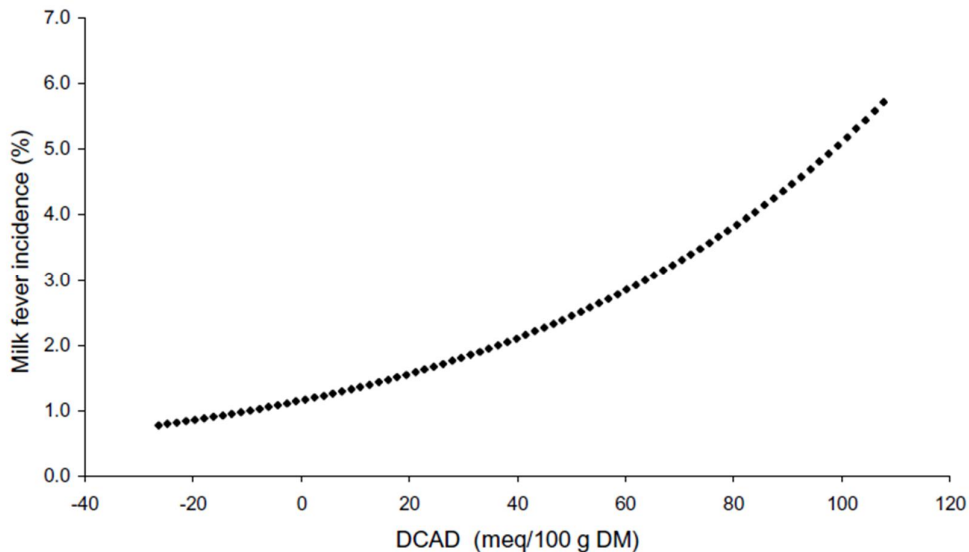
Ramberg et al. (1996) stated that the DCAD concept is an empirical hypothesis, not a physiological mechanism. It is thus based on the hypothesis that calcium homeostasis can be improved by feeding a diet with a lower DCAD, thus preventing parturient hypocalcaemia at calving. He further noted that should this hypothesis be accepted, application of the concept involves finding a combination of

dietary substances able to reduce the DCAD without causing toxicity, reduced palatability or expense beyond tolerable limits.

It is well known that the onset of lactation will cause a severe and rapid drain on blood calcium required for milk production. Dietary Cation-Anion Difference is one of the solutions to prevent the rapid decline in plasma calcium levels at calving. Ramberg et al. (1996) defined calcium turnover as the velocity of calcium movement (loss and replacement) through the exchangeable calcium pool.

This means that a cow with a higher calcium turnover would be more resistant to severe hypocalcaemia, than a cow with a lower calcium turnover, even though pre-partal plasma calcium concentration might be the same for both. Ramberg et al. (1996) noted that any changes in dietary calcium intake will not alter the steady state calcium turnover. Plasma calcium concentration can be defined as the sum of calcium inflows into the blood from gut and bone, divided by the total clearance of calcium from plasma. The inflow of calcium is subject to negative feedback regulation by PTH. Calcium clearances are disturbing signals and calcium inflow is a controlling signal for plasma calcium homeostasis. Increased urinary calcium clearance would tend to lower plasma calcium concentration and cite endocrine mechanisms to enhance calcium inflow. This metabolic exercise would prepare the calcium homeostatic system to respond more rapidly to the increased calcium clearance at calving (Ramberg et al., 1996). LeClerc and Block (1989), conducted a trial on peri-parturient cows, showing that there exists a significant negative correlation between DCAD and the plasma calcium concentration, which was the strongest from 12 hours before to 12 hours after calving. This shows that as DCAD is reduced prepartum, blood calcium concentration will be maintained at a higher level around parturition.

DeGaris and Lean (2009), showed that a linear relationship exists between DCAD and milk fever risk and thus predicts that any reduction in DCAD will reduce the risk of milk fever. This is clearly illustrated in Figure 2.8.



**Figure 2.8 Linear relationship between DCAD [(Na + K) - (Cl - S)] and risk of milk fever (Adopted from DeGaris and Lean, 2009)**

Lowered DCAD is associated with compensated metabolic acidosis, which can be seen by reduced plasma bicarbonate, lower urinary pH and higher urinary net acid excretion. Blood pH is compensated due to bone accepting hydrogen ions in exchange for calcium, while calcium excreted in urine due to the acidotic state, may be retained when calcium demand decreases (Charbonneau et al., 2006). Negative DCAD thus may impact on the plasma calcium concentration via 3 major ways:

1. Intestinal calcium absorption;
2. Bone resorption (mobilization);
3. Kidney reabsorption.

(Roche et.al., 2003; Hu et.al., 2007, Block, 2011, Grünberg et al., 2011).

It has been shown that the kidneys play a role but not by reabsorbing calcium and putting it into the blood. The effect of chronic acidosis on the kidney is rather to increase excretion of calcium (Goulding and Campbell, 1984; Block, 2011). Reducing the DCAD will cause metabolic acidosis, thereby increasing urinary calcium excretion, leading to increased calcium retention and causing the vitamin D parathyroid hormone axis to increase the signals for bone mobilization of calcium.

Furthermore, metabolic acidosis directly increases bone mobilization of calcium by:

1. creating the necessary acidic environment for lysosomal and mitochondrial enzymes in the osteoclasts (bone mobilization cells) to operate;
2. allowing the rapid production of other lysosomal and cytoplasmic acids in these cells, such as lactic and hyaluronic acids; and
3. allow for a localized reduction in pH around the bone cells to allow for bone mineral dissolution (Block, 2011).

Goff et al., (1991) found that milk alkalosis reduces the ability of the peri-parturient cow to maintain calcium homeostasis at or near calving by reducing the tissue responsiveness to PTH.

Block (2011) mentioned three classes of feedstuffs that can be used to reduce DCAD:

1. Forages (Purchased or produced on farm):

Forages alone will not likely be able to reduce DCAD to acceptable negative values. Careful selection of forages low in dietary potassium (K) can be used to reduce DCAD such that a minimal amount of purchased, speciality supplements, such as anionic salts would be needed.

2. Anionic Mineral Supplements:

The choices in this category range from purchasing specific mineral salts to reduce DCAD (e.g., the chloride and sulphate salts of calcium, magnesium and/or ammonium) to specifically formulated mineral packs containing these and other salts that may or may not be mixed with flavour enhancers (distillers grains, molasses, brewers grains etc.). Field experience with mineral salt products has been mixed from a palatability standpoint. They tend to be less palatable especially if more than 20 mEq/100 g DM of anions is added to the diet.



3. Manufactured supplements (not based on anionic salts designed to deliver a negative DCAD):

These supplements tend to be value-added in that they bring additional benefits to the diet other than negative DCAD. Field experience shows that these tend to be more palatable even when more than 20 mEq/100g DM of anions has to be added to the diet. The two major products available with published scientific literature are BIO-CHLOR<sup>®</sup> (Church & Dwight Co., Inc., Princeton, NJ) and SoyChlor<sup>®</sup> (West Central, Ralston, IA).

The DCAD recommendation may be influenced by a number of factors such as feed intake, acid-producing potential of the diet and concentrations of other fixed ions (Hu et al., 2007). DCAD equations only provide a theoretical basis for dietary manipulation of the acid-base status.

Goff (2008) and Goff and Horst (2003) describes simple guidelines for cation-anion supplementation and these include:

1. Sodium:

The sodium requirement of a late gestation cow is around 1.2 g/kg (0.12%). Only a small amount of salt is included in the diet to prevent pica. Unlimited access to salt during late gestation may lead to udder oedema.

2. Calcium:

A study by Goff and Horst (1997), indicated that the calcium concentration in the close-up diet should be between 8.5 and 10 g/kg (0.85 to 1.0%) calcium.

3. Magnesium:

To ensure adequate amounts of magnesium in the blood of the dry cow, the dietary magnesium concentration should be between 3.5 – 4.0 g/kg (0.35 – 4.0%).

4. Phosphorus:

The dietary phosphorus levels should be fed to meet the requirements of the late gestation cow (according to the NRC). This level is generally about 4.0 g/kg (0.4%). A diet supplying 80 g P/day will block renal production of 1,25-dihydroxyvitamin D<sub>3</sub> and cause milk fever.

5. Sulphur:

Dietary sulphur should be maintained above 0.22% to ensure enough available substrate for rumen microbial amino acid synthesis.

6. Potassium:

Dietary potassium levels should be kept as close as possible to NRC requirements for dry cows – about 10 g/kg (1.0%).

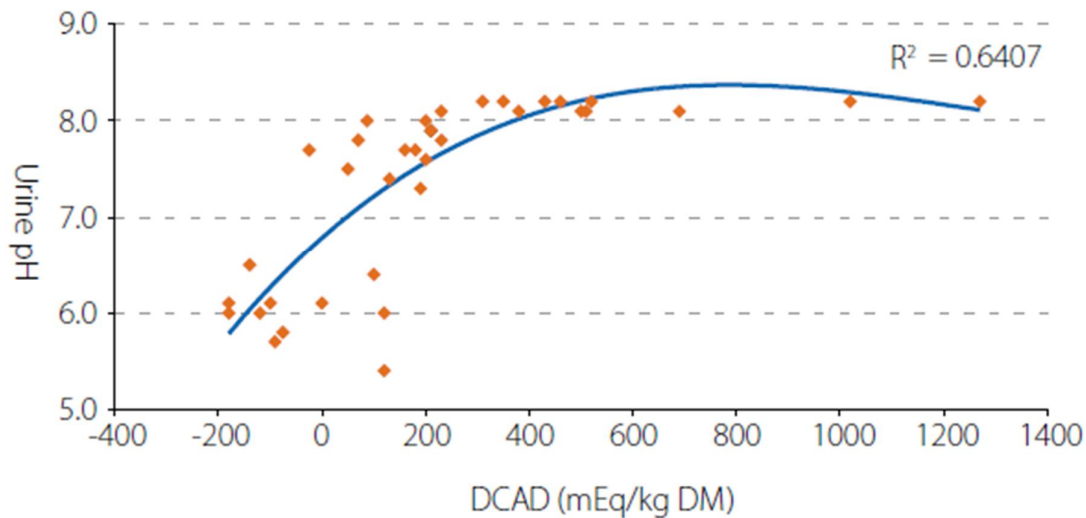
7. To be able to reduce subclinical milk fever, chloride must be added to the diet to counteract the effect of low potassium levels in the diet. The chloride level should thus be 5 g/kg (0.5%) lower than the amount of potassium in the diet. It is important that the chloride level in the diet is not too high as it is likely to decrease the dry matter intake and it may cause over-acidification.

A useful measure to determine if the animal is responding to the added dietary anions is to measure the urinary pH. Spanghero (2004), developed a model enabling practitioners to predict urinary (and blood) pH by knowing the DCAD intake of cows. By limiting the amount of dietary cations the urine pH will be reduced to 7.8 (Goff and Horst, 2003; Goff, 2008). Urinary pH between 5.5 and 6.2 is associated with effective administration of anions in the diet (Horst et al., 1997 a) although Charbonneau et al. (2006) concluded that a urinary pH of 7.0, regardless of breed, may be more appropriate for transition cattle.

It is however important to take care when interpreting results. Because, low DCAD diets will decrease urinary pH, a clinical finding of higher pH values indicate that the cows are not consuming the formulated DCAD; it does not indicate that negative DCAD does not work (Block, 2011). Block (2011) indicated that there are several possibilities to explore if a negative DCAD is offered and cows are showing high urinary pH values. These include:

1. Cows are not consuming as much DM as expected;
2. Total ration mix was not adjusted for additional cows entering the pen;
3. Other supplements have not been accounted for (i.e. free choice minerals);
4. Forage mineral contents are changing and have not been evaluated for current DCAD values.

According to Lean and DeGaris (2010), there is a curvilinear relationship between DCAD and urine pH with DCAD having little impact on urine pH until it reaches approximately 200 mEq/kg DM (Figure 2.9). They concluded that this relationship reflects renal buffering systems that maintain an alkaline urinary pH until overwhelmed. Due to this finding, Lean and DeGaris (2010) do not recommend urine pH as a tool to monitor efficacy of dietary acidification any longer.



**Figure 2.9 Relationship between DCAD and urine pH (Adopted from DeGaris and Lean, 2010)**

## **Prepartum administration of vitamin D, vitamin D metabolites and analogues:**

There has recently been renewed interest in the active metabolite of vitamin D<sub>3</sub>, 25-OH-D<sub>3</sub> as preventive measure or treatment for parturient paresis. 25-Hydroxyvitamin D<sub>3</sub> has several advantages over vitamin D<sub>3</sub>, which includes:

- It does not undergo 25-hydroxylation and is thus not subject to product inhibition;
- It acts more rapidly;
- It is rapidly metabolized;
- Only small amounts are needed (1 – 8 mg daily (1 mg = 50 000 iu Vitamin D<sub>3</sub>)). (Jorgensen, 1974).

As previously stated, vitamin D must first be metabolically activated to be able to produce its characteristic physiologic effects. The physiological controls over calcium homeostasis includes calcitonin secretion, which is stimulated in response to increased blood calcium levels, whereas PTH is released due to reduced blood calcium concentrations (DeGaris and Lean, 2009). Vitamin D is converted to 25-OH-D<sub>3</sub> in the liver via product inhibition. Parathyroid hormone stimulates the activation of 25-OH-D<sub>3</sub> (Horst et al., 1994), which in turn is converted to 1,25-(OH)<sub>2</sub>D<sub>3</sub> in the kidney and this process is regulated by a feedback mechanism (Jorgensen, 1974). The dairy cow will respond to a decline in plasma calcium concentrations by increasing the amount of PTH secreted and subsequently the amount of 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations (Sahota and Hosking, 1999; DeGaris and Lean, 2009). This causes higher bone resorption rates, decreased calcium excretion via the kidney and increased calcium absorption from the intestines in an animal that has a limited ability to respond to these increased metabolic demands (Liesegang et al., 2007).

Several researches examined various forms of vitamin D and their derivatives in relation to calcium mobilisation and possible prevention of hypocalcaemia (Olson et al., 1973; Horst et al., 1983; Hodnett et al., 1992; Okura et al., 2004). Administration of 20-30 million international units of vitamin D per day for a minimum of 3 days prepartum, reduced the milk fever incidence in parturient cows, but administration had to be discontinued after 7 days to avoid toxicity (Olson et al., 1973; Gast et al. 1977).

Hibbs and Conrad (1960), indicated that if vitamin D supplementation was discontinued for more than one day prepartum, the preventative effects disappeared and the milk fever incidence actually increased in some cases. Reasonable protection could be achieved by supplementing 10 million IU of vitamin D intramuscularly 10 days before parturition, provided that dietary calcium, phosphorus and magnesium are in balance (Olson et al., 1973; Gast et al., 1977). Lower doses may actually induce hypocalcaemia depending on the time of administration. This is due to high doses of 25-OH-D<sub>3</sub> and 1,25-(OH)<sub>2</sub>D<sub>3</sub> suppressing renal synthesis of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and thus inhibiting PTH release (Littledike and Horst, 1982).

Jorgensen (1974) conducted several studies and found that the administration of 25-OH-D<sub>3</sub> to third lactation and older cows can be considered. The major difficulty of using the vitamin D metabolites is to determine the accurate calving date, as treatment is generally most effective between 1 and 4 days prior to calving (Gast et al., 1979). It was found that when the 25-OH-D<sub>3</sub> was administered at recommended rates, parturient paresis was reduced, appetite was stimulated and no toxicity was caused. Vitamin D is typically supplemented in the diet or injected; however, variations in DM intake may lead to less than optimal doses of the vitamin being consumed and concerns regarding injection site lesions merit examination of alternative delivery methods (Rivera et al., 2005).

Okura et al (2004) confirmed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> can effectively be absorbed by Holstein heifers, after vaginal administration, while Rivera et. al. (2005) proved that buccal administration of 100 and 1000 mg 25-OH-D<sub>3</sub> increased vitamin D metabolites in serum and tissues. This should thus be an effective method to deliver the vitamin.

Several studies have been done to confirm the effectiveness of administering 25-OH-D<sub>3</sub> and anionic salts during the dry cow period. These include a study done by Elliot et al. (2006) which showed that a combination of MgCl<sub>2</sub> and 25-OH-D<sub>3</sub> resulted in amplification in calcium and phosphorus mobilization from the bone mass. The results of this experiment showed that creating metabolic acidosis in non-lactating cattle is essential to obtain a response to 25-OH-D<sub>3</sub> supplementation. A study completed by Wilkens et al. 2012 indicated that by lowering the DCAD plus the supplementation of 25-OH-D<sub>3</sub>, maintained higher calcium levels in the cows at the time of calving in comparison to all other treatments. This study also showed that 25-OH-D<sub>3</sub> supplementation caused higher phosphate levels after calving.

In view of the above observations of Elliot et al. (2006) and Wilkens et al. (2012) it is clear that a reduction in plasma  $1,25\text{-(OH)}_2\text{D}_3$  in cows suffering from hypocalcaemia and milk fever due to the feeding of a highly cationic diet prior to calving, the interaction between dietary supplements of anionic salts,  $\text{MgCl}_2$  and  $25\text{-OH-D}_3$  and their effect on the macro mineral (Ca) urinary excretion and blood concentrations prior to calving, justifies investigation.

### **Hypothesis:**

Milk fever (peri-parturient paresis) is a metabolic disorder that occurs due to the failure to maintain blood calcium concentrations at calving. This is caused by the failure of high producing dairy cows to mobilize enough calcium from their tissues or diet to meet the high calcium demand at the onset of calving. It was established that there is a reduction in plasma  $1,25\text{-(OH)}_2\text{D}_3$  in dairy cows suffering from hypocalcaemia during the feeding of highly cationic diets before calving (Goff et al., 1991 a; Goff., 2006; Goff., 2008). It is hypothesized that a combination of  $25\text{-OH-D}_3$  (Rovimix Hy-D) and anionic salts may be more effective than anionic salts alone to improve the calcium status of a group of close-up dairy cattle.

The materials and methods conducted during this study will be discussed in Chapter 3.

## **CHAPTER 3**

### **MATERIALS AND METHODS**

## Chapter 3: Materials and methods:

### 3.1 Experimental design and treatments:

The trial was conducted from January to May 2011 on a commercial dairy farm in the Gauteng area (Kaalfontein, Rayton). Thirty multiparous, pregnant Holstein type cows were used in a randomized complete block design. The cows were selected and blocked by parity (second parity and later), 305 day mature equivalent milk production in the previous lactation and expected calving date. Within each of the 15 blocks, the cows were randomly allocated to two experimental groups namely DCAD and DCAD + HyD. The experiment commenced 21 days prior to the expected calving date and continued up to day 10 postpartum. All animals received a commercial total mixed ration (TMR) for dry cows up to 39 days prepartum, which consisted of the following:

- 1 kg Economeal 17% (commercial concentrate supplied by AFGRI Animal Feeds, a division of AFGRI Operations Ltd, 12 Byls Bridge Boulevard, Highveld Ext 73, Centurion)<sup>1</sup>
- 2 kg dry brewer's yeast
- 7.5 kg maize silage (wet)
- 7-8 kg grass silage (dry)

Both experimental groups received the commercial steam-up ration fed as per standard practice to all steam-up animals on farm. The steam-up ration was fed from day 21 prepartum up to calving. All fresh cows were moved to the lactation groups postpartum. The steam-up ration consisted of the following:

- 4 kg dry cow concentration + anionic salts (R1001) (commercial concentrate supplied by AFGRI Animal Feeds, a division of AFGRI Operations Ltd, 12 Byls Bridge Boulevard, Highveld Ext 73, Centurion) (Table 3.1) Concentrate contained ammonium chloride, ammonium sulphate and magnesium sulphate as anionic salts, to obtain a negative DCAD of -160 mEq/kg DM.
- 1 kg brewer's grain
- 6 kg maize silage
- 3 kg grass silage
- 1.8 kg grass hay

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<sup>1</sup> The composition of the AFGRI Economeal 17% is confidential and is thus not displayed in this dissertation.

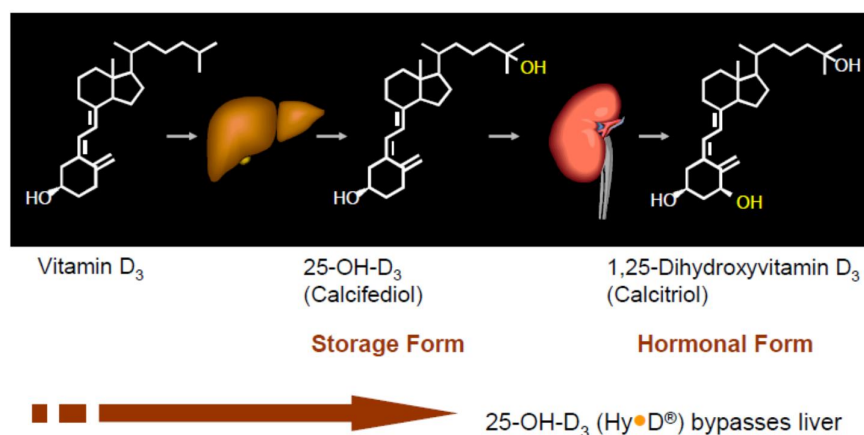


**Table 3.1** Nutrient composition of AFGRI R1001 dry cow concentrate and anionic salts

Nutrient	% DM
Protein (%)	18.0
Energy (MJ/kg)	11.0
NDF (%)	19.0
Ca (%)	0.60
P (%)	0.50
Na (%)	0.025
K (%)	0.77
Cl (%)	1.82
S (%)	0.52
Mg (%)	0.62

Nutrient composition as supplied by AFGRI Animal Feeds a division of AFGRI Operations Ltd, 12 Byls Bridge Boulevard, Highveld Ext 73, Centurion)

The commercial steam-up ration fed to the DCAD + HyD group was supplemented with 3 mg 25-OH-D<sub>3</sub> per animal per day (=240 mg Rovimix HyD 1.25%). Rovimix HyD 1.25% is a commercial product marketed by DSM Nutritional Products, Basel, Switzerland and consists of 25-OH-D<sub>3</sub>, the natural metabolite of vitamin D<sub>3</sub> that can easily be transported in the animal body via the bloodstream. 25-Hydroxyvitamin D<sub>3</sub> is absorbed differently than vitamin D<sub>3</sub> and bypasses a metabolic transformation step in the liver, leading to optimal 25-OH-D<sub>3</sub> plasma levels (Figure 3.1).



**Figure 3.2** Vitamin D metabolism and the mode of action of 25-OH-D<sub>3</sub>

The DCAD + HyD group received the aforementioned commercial steam-up concentrate (R1001) and Rovimix HyD 1.25% supplement as a hand addition at 4 kg per animal per day instead of a

complete TMR (Figure 3.2). The roughage was supplied separately via a feeding car. The DCAD group received a complete TMR.



**Figure 3.3 DCAD + HyD group receiving steam-up concentrate separately**

Due to on farm constraints a complete TMR could not be provided to the DCAD + HyD group. The computerized feeding system did not allow accurate weighing of the TMR for small numbers of animals. To ensure that each animal in the DCAD + HyD group received the required 4 kg concentrate per animal per day, it was decided that the steam-up concentrate should be supplied by hand.

Individual feed intake could not be determined due to constraints in the facilities and management practices. However the daily feed requirement for all groups of animals on farm was determined by visual observation of the feeding troughs every morning. Adjustments were calculated to ensure that all animal groups achieved their required daily intake. Feeding troughs were only emptied once every second day, causing difficulty in accurately determining the feed intake for all experimental animals.

The following section will focus on the sampling procedures and analyses conducted for all feed, blood, urine and milk samples collected during the experimental period.

## **3.2 Sampling Procedures:**

### **3.2.1 Feed Sampling and Analyses:**

DSM Nutritional Products SA (Pty) Ltd blended the Rovimix Hy-D 10% premix. The premix was supplied to AFGRI Animal Feeds and was mixed with the R1001 Steam-Up concentrate to produce a complete concentrate. The concentrate was delivered on farm and was used as part of the ration supplied to the DCAD + HyD Group.

The premix was analysed by DSM Kaiseraugst, Switzerland according to the internal DSM standard to verify the 25-OH-D<sub>3</sub> in premixes (Schüpfer et al., 2011). Total mixed ration (TMR) samples were taken on farm on alternate days and were analysed by UP Nutrilab (Department of Animal and Wildlife Sciences, University of Pretoria, Pretoria) for crude protein (CP) (nitrogen was determined using a Leco N analyser, model FP-428, Leco Corporation, St Joseph, MI, USA and CP (calculated as N x 6.25 (AOAC, 2000, procedure 968.06)), NDF (filter bag technique with the Ankom<sup>2000</sup> fibre analyser) (Robertson and Van Soest, 1981), calcium (AOAC, 2000, procedure 965.09), phosphorus (AOAC, 2000, procedure 965.17), sodium (AOAC, 2000, procedure 935.13), potassium (AOAC, 2000, procedure 935.13), chloride (AOAC, 2000, procedure 969.10), sulphur (AOAC, 2000, procedure 935.13) and magnesium (AOAC, 2000, 935.13). The TMR containing Rovimix HyD 1.25% was analysed by DSM Kaiseraugst, Switzerland according to the internal DSM standard for determination of 25-OH-D<sub>3</sub> in feed (Hofmann et al., 2010).

### **3.2.2 Blood Sampling and Analyses:**

Three prepartum blood samples from each animal were collected from the subcutaneous abdominal artery/vein on day 21, 14, 10 and every second day until parturition, as well as 4 and 6 hours postpartum. Sampling thereafter continued every second day up to 10 days postpartum. All blood samples were collected in Serum Gold (Gel Vacutainer) tubes (8.5 ml) and were immediately centrifuged for 10 minutes at 4000 rpm using a mobile centrifuge unit. Two millilitres of plasma from each sample were stored in Eppendorf vials and frozen at -80<sup>0</sup> Celsius until such time that analyses could be done. Ionized calcium was determined using a blood gas analyser, Rapidlab 348 (Rapidlab 348 System Operator's Manual, 2007). Total calcium, phosphorus and magnesium were determined by using standard diagnostic methods on the Cobas Integra 400 Plus Chemistry Analyser (Cobas Integra 400/700/800 Method Manual Edition V4, 2008). All of these analyses were conducted at the Clinical Pathology Laboratory at Onderstepoort, South Africa.

Furthermore, the plasma samples were analysed for 25-OH-D<sub>3</sub> by the Analytical Research Centre of DSM Nutritional Products in Kaiseraugst (Switzerland) and for 1,25-(OH)<sub>2</sub>D<sub>3</sub> by MLM Medical Labs located in Mönchengladbach in Germany. The method of Lauridsen et al. (2010) was used for determination of 25-OH-D<sub>3</sub> in plasma samples. This method was adapted by using a 1290 Infinity HPLC System from Agilent and no column switching system. For detection a triple quadruple mass spectrometer from AB Sciex was used (API 4000) with an atmospheric pressure photo ionization (APPI) source. The specific ion transition of the internal standard (m/z 389.50 → 211.27) and the analyte transition (m/z 383.42 → 211.27) were detected. For determination of 1,25-(OH)<sub>2</sub>D<sub>3</sub> MLM Medical Labs used a RIA (Radio Immuno Assay) method as described, using the IDS Gamma-B 1,25-dihydroxyvitamin D Kit (AA-54).

### **3.2.3 Urine Sampling and Analyses:**

Urine samples were collected on day 21, 14, 8 and 4 prior to calving and on day 4 after calving. Care was taken not to collect the first few dribbles of urine as this may have a higher urinary pH value than urine samples collected from a full stream. Samples were collected via spontaneous urination after gentle massaging of the perineum. The pH of these samples was determined immediately after collection, using a LaMotte pH Plus Direct pH Meter. The pH meter was calibrated each test day before determination using a 2-point calibration with pH = 7.0 and 4.0. The pH-meter was rinsed with clean water between measurements. All samples were centrifuged for 10 minutes at 2500 rpm and transferred to 2 ml Eppendorf vials and stored at -80<sup>0</sup> Celsius. These samples were analysed for calcium, phosphorus and creatinine according to a standard diagnostic method using the Cobas Integra 400 Plus Chemistry Analyser (Cobas Integra 400/700/800 Method Manual Edition 4, 2008) conducted by the Clinical Pathology Laboratories at Onderstepoort.

### **3.2.4 Milk Sampling and Analyses:**

Individual milk samples were collected in the parlour and stored in a plastic container with a bronopol tablet for preservation of the sample. Samples were collected on day 1, 4 and 10 postpartum and were analysed for calcium, milk fat, milk protein, milk urea nitrogen (MUN), and milk lactose using the System 4000 Infrared Analyzes (Foss Electric, Hillard, Denmark) at Lactolab (Pretoria).

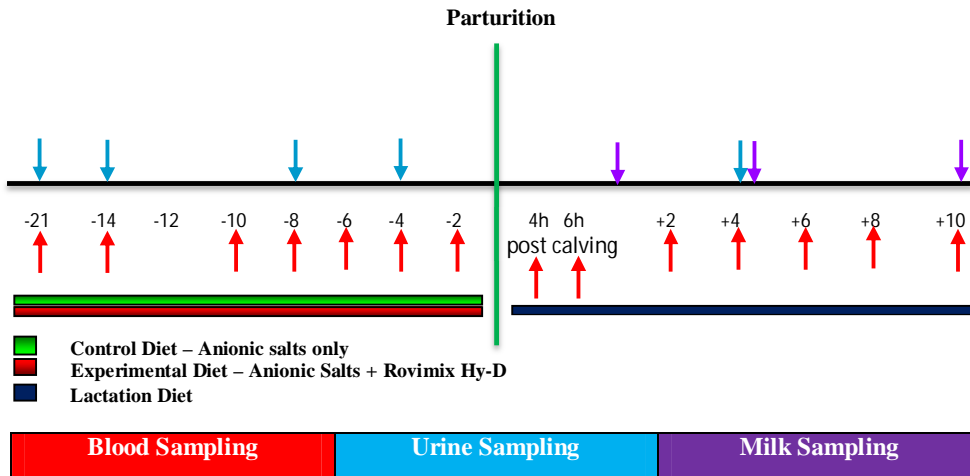


Figure 3.4 Summary of experimental layout and sampling schedule

### 3.3 Statistical Analyses:

Linear Mixed Model Analysis for repeated measurements was applied to all urine, milk and blood parameters. The fixed effects were specified as the group, day and group x day interaction. The random effects were specified as the cow and cow x day interaction and the correlation over days was fitted by the ante-dependence model of order 1. Significance was tested at the 5% level ( $P \leq 0.05$ ). Means were separated by applying Fisher's protected least significant difference (LSD) test at the 5% level. All data was analysed using the GenStat<sup>R</sup> statistical program (Payne, 2011). The urine parameters were log (base e) transformed to normalise the data and stabilise the group by day variance.

## **CHAPTER 4**

### **RESULTS AND DISCUSSION**

## Chapter 4: Results and discussion

### 4.1 Introduction:

Clinical hypocalcaemia (milk fever) is an economically important disease in dairy cow herds and significantly increases the cow's susceptibility to mastitis, retained foetal membranes, displaced abomasum, dystocia and ketosis and can therefore significantly reduce the cow's productive life (Reinhardt et al., 2010). Surveys in the USA suggest that around 5% of cows will develop milk fever annually and the incidence of subclinical milk fever is around 50% in mature cows (Goff, 2008; Reinhardt et al., 2010).

Several studies have been conducted over the years to help us understand how and why the homeostatic mechanisms in the cow fail and hypocalcaemia occurs. It has been demonstrated that low calcium diets fed during the dry period decreases the incidence of parturient paresis, as well as lead feeding and low phosphorus diets when calcium concentrations are high (Curtis et al., 1984). A further strategy implemented to reduce milk fever was to administer vitamin D and its metabolites (Hodnett et al., 1992; Okura et al., 2004. Rivera et al., 2005; Taylor et al., 2008).

The latter strategy in combination with anionic salts was investigated in this study and the results will be presented and discussed in detail in this chapter.

### 4.2 Feed Analyses:

Results of the Dairy Concentrate and TMR Analyses conducted by UP Nutrilab (University of Pretoria) and DSM Kaiseraugst, Switzerland, are shown in Table 4.1. The DCAD of the Afgri Animal Feeds Concentrate (R1001) fed to all animals were -160 mEq/DM. The only difference between the concentrate fed to the DCAD + HyD group and DCAD group animals was the supplementation of the Rovimix Hy-D at 240 mg per animal per day to the DCAD + HyD treatment group.

**Table 4.1** Nutrient composition of the dairy concentrate and TMR (DM basis) during the study

Nutrients	Average analyses values for all feed samples	Average analyses values for all concentrate samples
Protein (%)	12.2	18.4
NDF (%)	52.1	22.5
Calcium (Ca) (%)	0.54	0.83
Phosphorus (P) (%)	0.40	0.49
Sodium (Na) (%)	0.036	0.030
Potassium (K) (%)	1.12	0.91
Chlorine (Cl) (%)	0.84	1.85
Sulphur (S) (%)	0.35	0.64
Magnesium (Mg) (%)	0.31	0.33

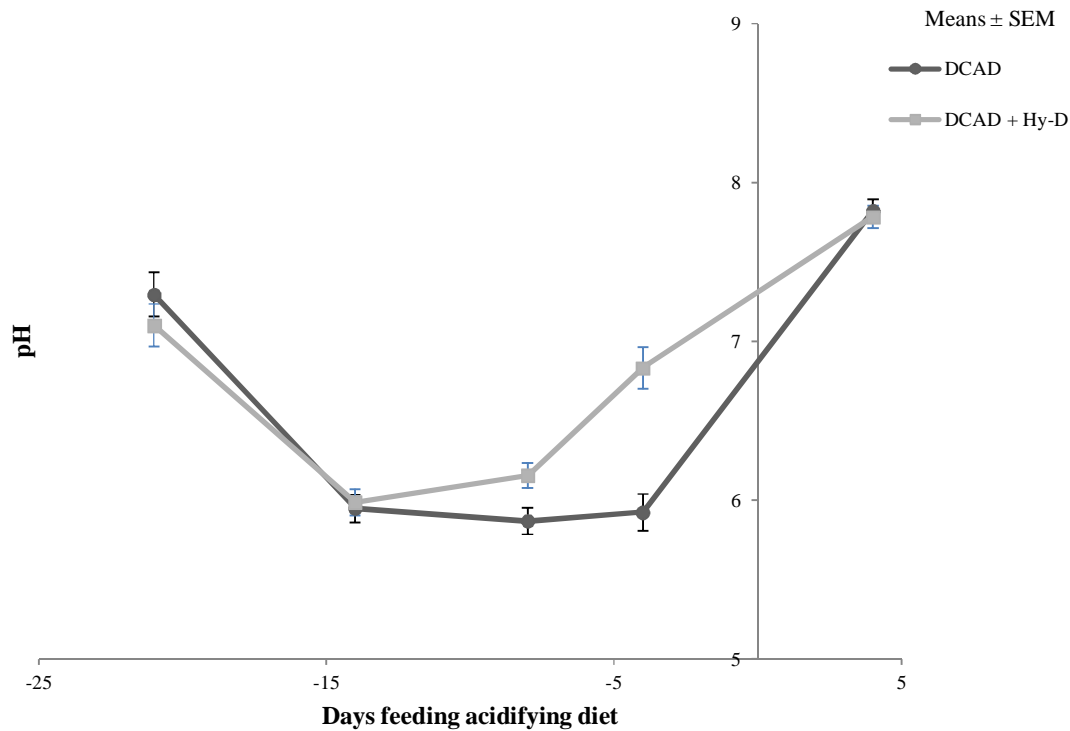
As previously indicated, individual feed intake was not determined due to constraints in the facilities and management practices. As feed was not supplemented to individual animals this could lead to significant differences in ruminal profiles and feed behaviour and intake. The ability to monitor the feeding patterns and/or intake may aid in identifying factors that could increase an animal's susceptibility to reduced performance and hypocalcaemia. Following similar feeding strategies as feedlots i.e. allowing a group/penned feeding situation may contribute to overcoming constraints at commercial institutions. Feeding strategies at feedlots may typically include e.g 120 animals allocated to two treatment groups and divided into 10 pens with six animals each. Such a model should provide enough statistical power to achieve significant differences between treatments.

### 4.3 Urine Analyses:

#### 4.3.1 Urine pH:

Urine samples were collected on day 21, 14, 8 and day 4 prepartum and day 4 postpartum. The urine pH was determined immediately after manual collection to control the feeding effect. Figure 4.1 shows the pH values for both experimental groups. There were no difference between treatments and no treatment by day interactions could be detected ( $P>0.05$ ). The treatment means for urine pH were  $6.570 \pm 0.127$  (DCAD group) and  $6.771 \pm 0.123$  (DCAD + HyD group) respectively. Table 4.2 shows the means ( $\pm$  SEM) for the experimental groups. Until day 14 prepartum urine pH values decreased linearly for both groups. Thereafter an increase in urinary pH values could be detected until day 4 postpartum when both experimental groups reached similar pH values.





**Figure 4.1** Urine pH in urinary samples taken from animals either on the DCAD or DCAD + HyD diet as a function of treatment duration; data presented as means ± SEM

**Table 4.2** Table reflecting the mean urinary pH values (±SEM)\* of urine samples collected from both the DCAD and DCAD + HyD treatment groups

Urinary pH values		
Day	DCAD Group	DCAD + HyD Group
-21	7.29 ± 0.281 (n <sup>#</sup> =12)	7.10 ± 0.269 (n=13)
-14	5.94 ± 0.176 (n=13)	5.98 ± 0.166 (n=15)
-8	5.86 ± 0.169 (n=11)	6.15 ± 0.158 (n=13)
-4	5.92 ± 0.234 (n=9)	6.83 ± 0.259 (n=6)
4	7.82 ± 0.143 (n=13)	7.78 ± 0.138 (n=14)

\*SEM is the Standard Error of the Mean

<sup>#</sup>n indicates the number of animals observed per time period

Most feedstuffs commonly used during the dry and lactation period often cause a moderate to high alkaline physiological effect on dairy cattle, which is associated with an increased milk fever risk (Seifi et al., 2004). The dietary cation-anion difference (DCAD) will increase when an abundance of sodium and potassium (cations) compared to sulphur and chlorides (anions) are present in the diet (Joyce et al., 1997). High cation levels in the diet will generally cause the urine pH to be alkaline (pH>8.0) (Goff, 2000). By supplementing these diets with anionic salts a state of metabolic acidosis

can be induced, thereby increasing two PTH-dependent functions: 1) bone resorption, and 2) renal production of  $1,25\text{-(OH)}_2\text{D}_3$ . Both of these functions increase the resistance of dairy cows to milk fever and hypocalcaemia (Wang and Beede, 1992; Block, 1994).

In the present study a midstream urine sample was collected via spontaneous urination, after gentle massaging of the perineum and the urine pH was determined immediately. A urine pH value of below 7 was indicative of a mild metabolic acidosis. Under normal physiological conditions the low urine pH is compensated by increased net acid excretion to maintain the normal acid-base equilibrium in blood. This net acid excretion (NAE) is an indicator of the effect of renal excretion and plasma strong ion difference (Mellau et al. 2002).

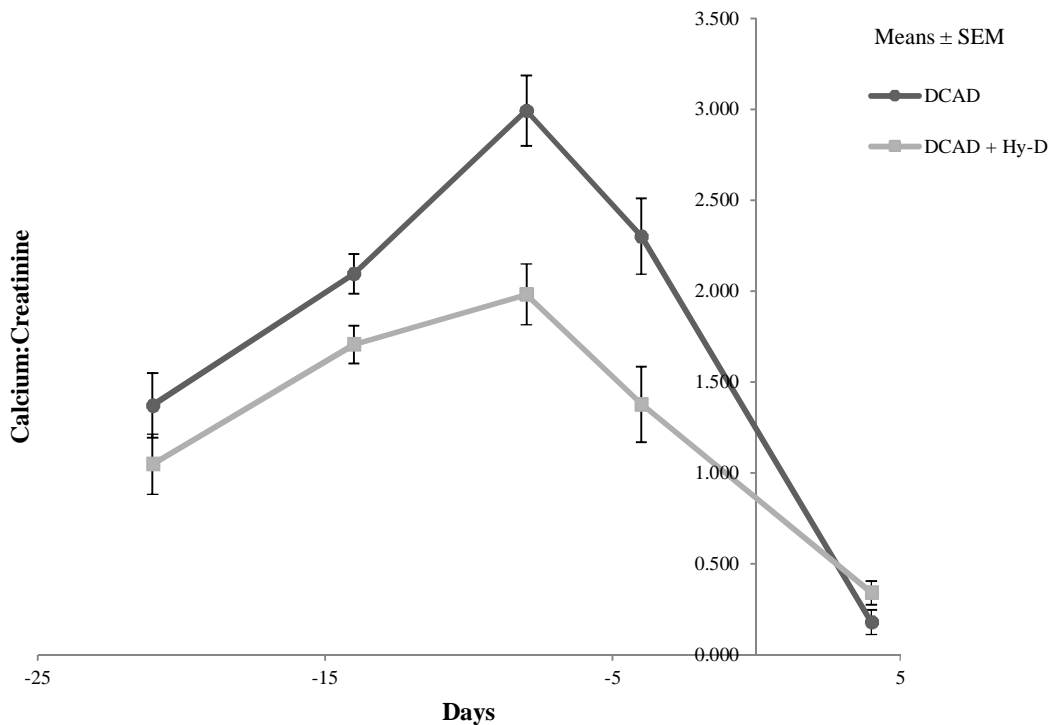
Van Dijk and Lourens (2001) demonstrated that in order for anionic salts to be effective, it should yield urinary pH levels below 6.5. In table 4.1 it is shown that this was indeed the case for both the DCAD and DCAD + HyD group although graphically (Figure 4.1) it appears that the urinary pH for cows supplemented with the DCAD + HyD concentrate increased much earlier than that of the DCAD group (day 4 prepartum). They further noted that an advantage of this method is that it accounts for inaccuracies in mineral analysis and for variable roughage mineral contents.

It is interesting to note from Figure 4.1, that cows supplemented with anionic salts only appeared to have a lower urine pH than cows supplemented with the DCAD + HyD concentrate although no differences per treatment or treatment by day interactions could be detected ( $P > 0.05$ ). Both the DCAD and DCAD + HyD treatment groups were fed exactly the same diet with the exception of Rovimix HyD being added to the DCAD + HyD group. Furthermore it is important to note that the DCAD treatment group received the diet as a TMR, while the DCAD + HyD treatment group received the anionic salts and HyD as part of a concentrate with all roughages supplied *ad lib*.

Block (2011) reported that it is important to note that although low DCAD diets will decrease urinary pH, a clinical finding of higher pH values may indicate that cows are not consuming the formulated DCAD it does not indicate that negative DCAD is not effective. The concentrate was supplemented at 4 kg per animal per day in the DCAD + HyD treatment group. Animals in this group were not separated during feedings and the possibility thus exists that some animals did not consume enough of the formulated DCAD. This may however be true for the DCAD group as well as some competition normally exists within a dairy cow herd.

#### 4.3.2 Minerals in Urine (Ca and P):

The urine samples collected on day 21, 14, 8 and 4 prepartum and day 4 postpartum were analysed for calcium and phosphorus and the calcium results were related to the creatinine excretion. No differences between treatments, or treatment by day interactions could be detected ( $P>0.05$ ) (Figure 4.2 and Table 4.3). The treatment means for the urinary calcium concentrations were  $1.788 \text{ mmol/L} \pm 0.197$  (DCAD) and  $1.291 \text{ mmol/L} \pm 0.182$  (DCAD + HyD). Before parturition a linear increase in the calcium excretion concentrations, could be observed for the DCAD group. The maximum level of calcium excretion was reached at day 8 prior to calving. The calcium excretion concentration started to decline hereafter with a sharp decrease observed from day 4 prepartum to day 4 postpartum. In contrast to this the DCAD + HyD did not show such a sharp increase in calcium excretion and the maximum level reached was about  $1.982 \text{ mmol/L}$  on day 8 prepartum. Thereafter a steady decline in these concentrations up to day 4 postpartum could be observed.



**Figure 4.2** Calcium:creatinine excretion in urinary samples taken from animals either on the DCAD or DCAD + HyD diet as a function of treatment duration; data presented as means ± SEM

**Table 4.3** Calcium excretion determined in urinary samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration from day 21 prepartum to day 4 postpartum; data presented as means  $\pm$  SEM\*

Urinary calcium concentrations (mmol/L)		
Day	DCAD Group	DCAD + HyD Group
-21	1.37 mmol/L $\pm$ 0.358 (n#=11)	1.04 mmol/L $\pm$ 0.330 (n=13)
-14	2.09 mmol/L $\pm$ 0.218 (n=13)	1.70 mmol/L $\pm$ 0.203 (n=15)
-8	2.99 mmol/L $\pm$ 0.387 (n=11)	1.98mmol/L $\pm$ 0.337 (n=15)
-4	2.30 mmol/L $\pm$ 0.417 (n=8)	1.37 mmol/L $\pm$ 0.414 (n=7)
<b>4</b>	0.17 mmol/L $\pm$ 0.135 (n=13)	0.34 mmol/L $\pm$ 0.126 (n=15)

\*SEM is the Standard Error of the Mean

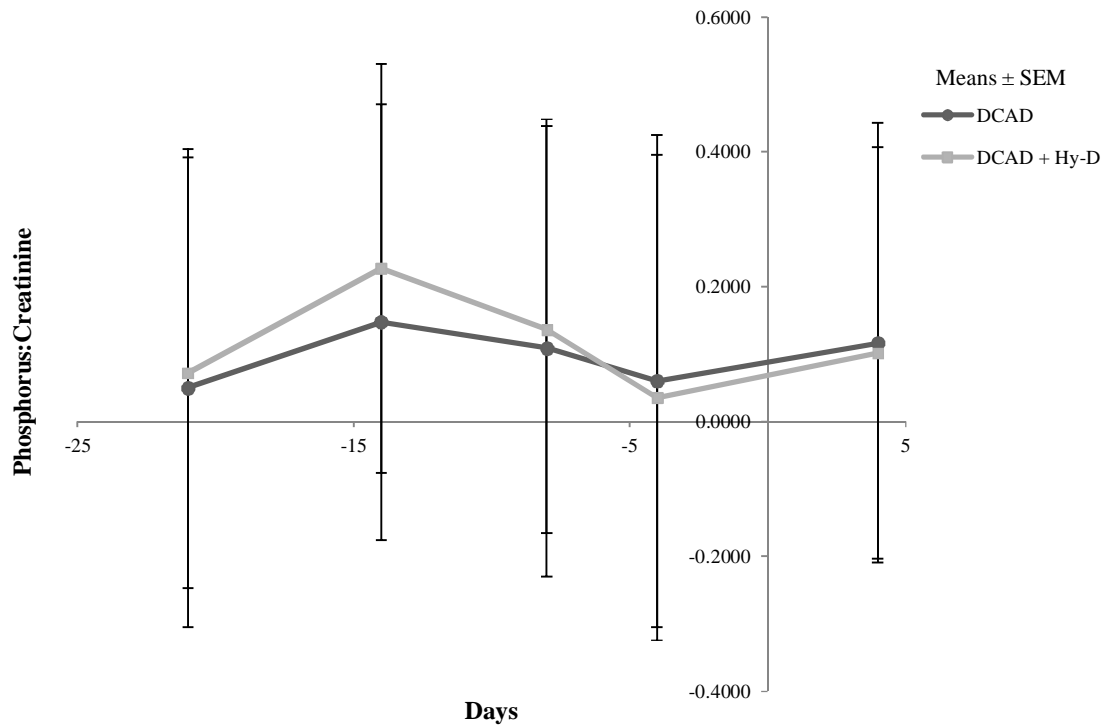
#n indicates the number of animals observed per time period

A number of authors have shown that the alteration of the DCAD had dramatic effects on the incidence of milk fever (Moore et al., 2000; Thilsing-Hansen et al., 2002 (a), Goff and Horst, 2003; Roche et al., 2003). It has been shown that by reducing the DCAD of a diet the urinary excretion of calcium is increased in ruminants (Van Mosel et al., 1993), excluding the stimulatory effect of a negative DCAD on calcium re-absorption in the kidney (Schönewille et al., 1994). Schönewille et al. (1994) further indicated an increased absorption of calcium when anionic salts were fed, but showed that only 60% of increased urinary calcium output could be accounted for by increased absorption. Block (1984) suggested that the additional urinary calcium in cows fed an anion-rich diet, originates from an increase in bone resorption. It was suggested (based on histological data) that cows fed an anion-rich diet showed a reduction in bone accretion. Unaltered bone resorption together with reduced bone accretion could thus explain an increase in urinary calcium excretion (Schönewille et al., 1994).

Although no treatment or treatment by day interactions could be detected ( $P > 0.05$ ) between the two experimental groups (Table 4.3), Figure 4.2 indicates that the negative DCAD of -160 mEq/kg DM that was used for this particular trial, caused more calcium to be excreted in the urine, supporting other studies (Vagnoni and Oetzel, 1998) and thus we can assume that increased calcium absorption occurred. The most likely reason that low DCAD diets can prevent peri-parturient hypocalcemia is an increased calcium flux due to the ingestion of the low DCAD diet. Lower urinary calcium concentrations after parturition results from an increased demand for calcium by the mammary gland for milk synthesis as well as a more alkaline or positive DCAD diet (Chan et al., 2006). The calcium homeostasis that thus occurs at the onset of lactation is due to the shift in calcium exit from the exchangeable calcium pool from the kidney to the mammary gland (Grünberg et al., 2011). This

means that the reduced urinary calcium concentrations was most likely a result of higher physiological pH, lactation demand for calcium and a changing diet.

The urine phosphorus is depicted in Figure 4.3. Repeated measurement analysis was applied to log (base e) P (mmol) values to normalise the data and stabilise the treatment and day variances. No treatment differences or treatment by day interactions could be detected ( $P>0.05$ ). The graph over days was created with the untransformed means and SEM's to indicate true trends.



**Figure 4.3** Phosphorus excretion determined in urinary samples taken from animals either on the DCAD or DCAD + HyD diet as a function of treatment duration: data presented as means ± SEM

**Table 4.4** Phosphorus excretion determined in urinary samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration from day 21 prepartum to day 4 postpartum; data presented as means  $\pm$  SEM\*

Urinary phosphorus concentrations (mmol/L)		
Day	DCAD Group	DCAD + HyD Group
-21	0.05 mmol/L $\pm$ 1.119 (n <sup>#</sup> =11)	0.07 mmol/L $\pm$ 1.619 (n=13)
-14	0.15 mmol/L $\pm$ 3.171 (n=13)	0.23 mmol/L $\pm$ 0.899 (n=15)
-8	0.11 mmol/L $\pm$ 1.492 (n=11)	0.14 mmol/L $\pm$ 0.507 (n=15)
-4	0.06 mmol/L $\pm$ 1.757 (n=8)	0.04 mmol/L $\pm$ 0.694 (n=7)
4	0.12 mmol/L $\pm$ 6.195 (n=13)	0.10 mmol/L $\pm$ 1.142 (n=15)

\*SEM is the Standard Error of the Mean

<sup>#</sup>n indicates the number of animals observed per time period

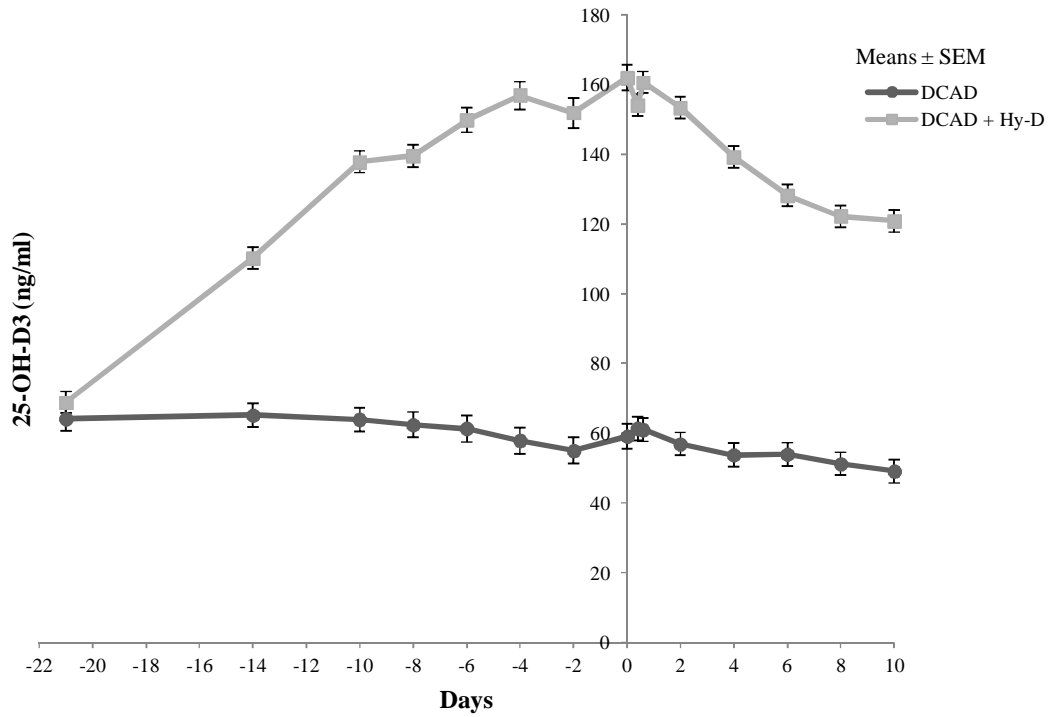
Phosphorus is of special interest among urinary minerals because the phosphate ion is a principle component of the titratable acidity in humans and swine (Vagnoni and Oetzel, 1998). Vagnoni and Oetzel (1998) further observed no effect of anionic salts on urinary phosphorus excretion. Similarly, in this study no treatment or treatment by day interactions were found ( $P > 0.05$ ) (Table 4.3). Goff (2000) indicated that PTH is secreted during periods of calcium stress and will increase renal and salivary excretion of phosphorus which can be detrimental to maintenance of normal blood phosphorus concentrations.

It can be concluded that a negative DCAD ration will lead to increased urinary calcium excretion and therefore it can be assumed that increased calcium absorption occurred. Negative DCAD rations do however not affect urinary phosphorus excretion.

#### 4.4 Blood Analyses:

##### 4.4.1 Plasma Hormones (25-OH-D<sub>3</sub> and 1,25-(OH)<sub>2</sub>D<sub>3</sub>)

Table 4.5 indicates a highly significant effect on treatment effect ( $P < 0.001$ ) and treatment by day interactions ( $P < 0.001$ ). Means for both treatment groups were 58.4 ng/ml  $\pm$  4.07 (DCAD) and 137.0 ng/ml  $\pm$  3.83 (DCAD + HyD) respectively ( $P < 0.001$ ). There was less variation in 25-OH-D<sub>3</sub> and for the duration of the sampling period the values did not deviate much from the average. Mean 25-OH-D<sub>3</sub> plasma concentrations for the DCAD + HyD group increased more or less linearly from day 14 prior to calving and were maintained at approximately 137.05 ng/ml. The plasma concentrations showed a continued decrease around 2 days postpartum. Plasma concentrations of up to 162 ng/ml were observed over a 21 day treatment period (Figure 4.4).



**Figure 4.4** Plasma concentrations of 25-hydroxyvitamin D<sub>3</sub> (25-OH-D<sub>3</sub>) of dairy cows as a function of treatment duration. Untreated cows on a control ration (DCAD group) and treated cows with 3 mg of 25-OH-D<sub>3</sub> daily (DCAD + HyD group) from day 21 prepartum until parturition; data presented as  $\pm$ SEM

**Table 4.5** 25-Hydroxyvitamin-D<sub>3</sub> plasma concentrations of samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration from day 21 prepartum to day 10 postpartum; data presented as mean ± SEM\*

Plasma 25-OH-D <sub>3</sub> concentrations (ng/ml)		
Day	DCAD Group	DCAD + HyD Group
-21	64.1 <sup>a</sup> ng/ml ± 6.69 (n <sup>#</sup> =14)	68.8 <sup>a</sup> ng/ml ± 6.26 (n=16)
-14	65.2 <sup>a</sup> ng/ml ± 6.69 (n=14)	110.2 <sup>b</sup> ng/ml ± 6.26 (n=16)
-10	63.9 <sup>a</sup> ng/ml ± 6.87 (n=13)	137.8 <sup>b</sup> ng/ml ± 6.26 (n=16)
-8	62.4 <sup>a</sup> ng/ml ± 7.19 (n=11)	139.5 <sup>b</sup> ng/ml ± 6.47 (n=14)
-6	61.3 <sup>a</sup> ng/ml ± 7.49 (n=10)	149.8 <sup>b</sup> ng/ml ± 6.96 (n=11)
-4	57.8 <sup>a</sup> ng/ml ± 7.52 (n=10)	156.9 <sup>b</sup> ng/ml ± 8.00 (n=7)
-2	55.0 <sup>a</sup> ng/ml ± 7.68 (n=8)	151.9 <sup>b</sup> ng/ml ± 8.61 (n=5)
0	59.0 <sup>a</sup> ng/ml ± 7.00 (n=5)	162.0 <sup>b</sup> ng/ml ± 7.32 (n=2)
4 hours p.p.	61.3 <sup>a</sup> ng/ml ± 6.69 (n=14)	154.0 <sup>b</sup> ng/ml ± 6.26 (n=16)
6 hours p.p.	61.0 <sup>a</sup> ng/ml ± 6.69 (n=14)	160.5 <sup>b</sup> ng/ml ± 6.26 (n=16)
2	56.9 <sup>a</sup> ng/ml ± 6.69 (n=14)	153.3 <sup>b</sup> ng/ml ± 6.26 (n=16)
4	53.7 <sup>a</sup> ng/ml ± 6.69 (n=14)	139.2 <sup>b</sup> ng/ml ± 6.26 (n=16)
6	53.9 <sup>a</sup> ng/ml ± 6.69 (n=14)	128.1 <sup>b</sup> ng/ml ± 6.26 (n=16)
8	51.2 <sup>a</sup> ng/ml ± 6.69 (n=14)	122.2 <sup>b</sup> ng/ml ± 6.26 (n=16)
10	49.1 <sup>a</sup> ng/ml ± 6.69 (n=14)	120.9 <sup>b</sup> ng/ml ± 6.36 (n=15)

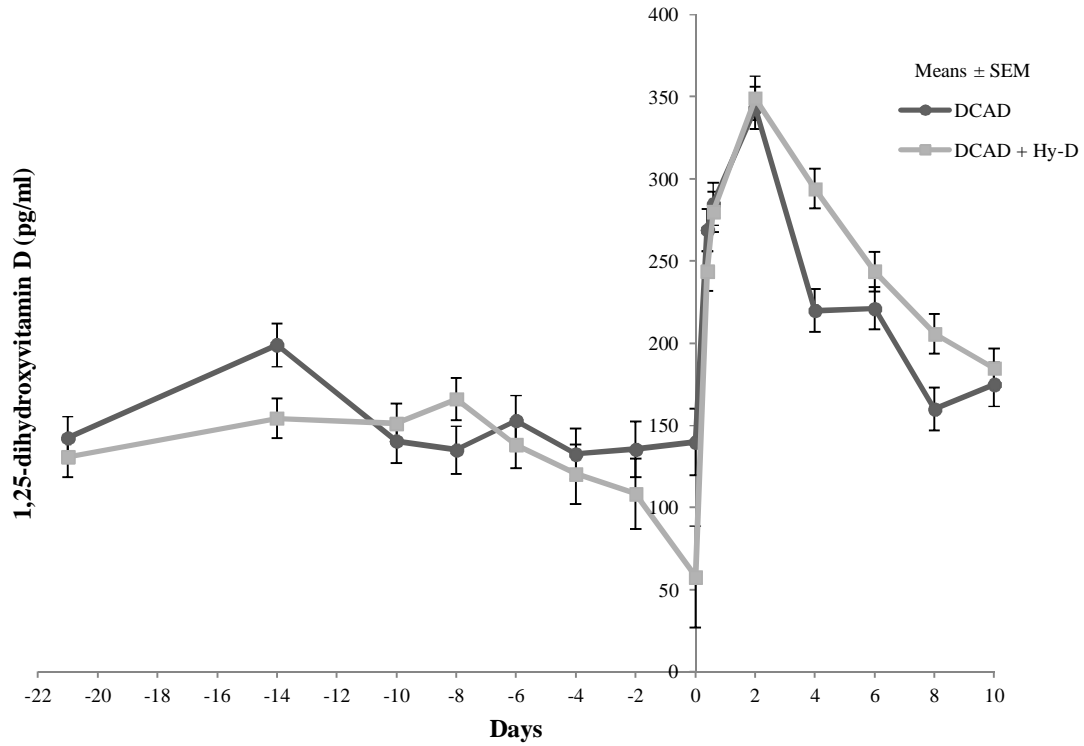
\*SEM is the Standard Error of the Mean

<sup>#</sup>n indicates the number of animals observed per time period

<sup>abc</sup> Row means (day) with different superscripts differ (P<0.05)

No treatment differences or treatment by day interactions could be detected (P>0.05) in the 1,25-(OH)<sub>2</sub>D<sub>3</sub> plasma levels (Figure 4.5 and Table 4.6) and the means for both treatments were 192.4 pg/ml ± 7.90 (DCAD) and 188.6 pg/ml ± 8.50 (DCAD + HyD) respectively. The plasma 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations remained on average approximately 150 pg/ml except for a decline in the concentration level of the DCAD + HyD group, around parturition. After parturition there was a sharp increase in the plasma concentration of both groups with the maximum levels reaching approximately 349.2 pg/ml at day 2 postpartum. Thereafter a linear decrease in the 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentration was observed.





**Figure 4.5** Plasma concentrations of 1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>] of dairy cows as a function of treatment duration. Untreated cows on a control ration (DCAD group) and cows treated with 3 mg of 25-OH-D<sub>3</sub> daily (DCAD + HyD group) from day 21 prepartum until parturition; data presented as means ±SEM

**Table 4.6** 1,25-Dihydroxyvitamin D<sub>3</sub> plasma concentrations (pg/ml) of samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration from day 21 prepartum to day 10 postpartum; data presented as means ± SEM\*

Plasma 1,25-(OH) <sub>2</sub> D <sub>3</sub> (pg/ml)		
Day	DCAD Group	DCAD + HyD Group
-14	198.9 pg/ml ± 26.70 (n=14)	154.3 pg/ml ± 24.10 (n=16)
-10	140.4 pg/ml ± 26.70 (n=13)	151.2 pg/ml ± 24.10 (n=16)
-8	135.0 pg/ml ± 29.10 (n=11)	166.0 pg/ml ± 25.80 (n=14)
-6	152.9 pg/ml ± 30.50 (n=10)	138.2 pg/ml ± 29.10 (n=11)
-4	132.6 pg/ml ± 30.50 (n=10)	120.4 pg/ml ± 36.40 (n=7)
-2	135.5 pg/ml ± 34.10 (n=8)	108.3 pg/ml ± 43.10 (n=5)
0	139.9 pg/ml ± 40.20 (n=5)	57.8 pg/ml ± 61.80 (n=2)
4 hours p.p.	268.9 pg/ml ± 25.80 (n=14)	243.9 pg/ml ± 24.10 (n=16)
6 hours p.p.	284.8 pg/ml ± 25.80 (n=14)	280.1 pg/ml ± 24.50 (n=16)
2	343.1 pg/ml ± 25.80 (n=14)	349.2 pg/ml ± 26.70 (n=16)
4	219.9 pg/ml ± 25.80 (n=14)	294.0 pg/ml ± 24.10 (n=16)
6	221.2 pg/ml ± 25.80 (n=14)	243.6 pg/ml ± 24.10 (n=16)
8	159.9 pg/ml ± 25.80 (n=14)	205.8 pg/ml ± 24.10 (n=16)
10	174.9 pg/ml ± 26.70 (n=14)	184.7 pg/ml ± 24.10 (n=15)

SEM is the Standard Error of the Mean

\*n indicates the number of animals observed per time period

In Figure 4.4 it is clearly shown that the 25-OH-D<sub>3</sub> plasma concentrations were greater for the DCAD + HyD treatment than the DCAD treatment. The 25-OH-D<sub>3</sub> concentrations increased linearly from day 21 prior to calving and peaked at 162 ng/ml. After reaching the maximum concentration, the 25-OH-D<sub>3</sub> concentrations remained stable after calving until the end of the observation period, 10 days postpartum. A significant effect on treatment and treatment by day interactions (P<0.001) were revealed. These stable plasma concentrations reflect the long half-life of 25-OH-D<sub>3</sub> of 15 days (Jones, 2008). The 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations remained relatively stable prior to calving and showed a sharp increase to a level of 350 pg/ml after calving. This sudden increase is perhaps a result of the regulation of 1 $\alpha$ -hydroxylase and the induced transformation of 25-OH-D<sub>3</sub> to 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Wilkins et al., 2012).

Previous research have shown that both feeding and injecting of 25-OH-D<sub>3</sub> and 1,25-(OH)<sub>2</sub>D<sub>3</sub> will increase serum and plasma concentrations of these metabolites. A study by Hove and Kristiansen (1982) reported peak plasma 1,25-(OH)<sub>2</sub>D<sub>3</sub> levels at 200 pg/ml when cattle was fed 500  $\mu$ g of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in a pelleted form and mixed into a daily ration, while an intramuscular injection of 1,25-(OH)<sub>2</sub>D<sub>3</sub> resulted in a peak concentration of approximately 1000 pg/ml within 12 hours (Rivera et al,

2005). Hodnett et al. (1992) found similar results when he injected 0.5 mg of  $1\alpha$ -dihydroxyvitamin  $D_3$  and 4 mg of 25-OH- $D_3$  into dairy cattle. In this particular study the serum 1,25-(OH) $_2D_3$  peaked at 125 pg/ml. These data sets clearly demonstrate that buccal dosing, injections, vaginal administrations (Okura et al., 2004) and feeding methods will increase plasma concentrations of 25-OH- $D_3$  and 1,25-(OH) $_2D_3$  in dairy cattle.

In the present study the elevated plasma hormone concentrations did however not lead to significantly increased plasma calcium concentrations. Taylor et al. (2008) experienced similar results when dosing 29 multiparous Jersey cows with oral boluses of corn starch, corn starch plus 15 mg of HyD or corn starch plus 15 mg of cholecalciferol (vitamin  $D_3$ ) administered as two gel capsules 6 days before expected calving. Taylor et al. (2008) suggested that the lack of response in serum calcium may be due to the cows not sufficiently converting 25-OH- $D_3$  into the active metabolite 1,25-(OH) $_2D_3$ , but rather that the hormone was catabolised.

Taylor et al. (2008) further commented that the theory can be substantiated by serum PTH concentrations tending to be lower in 25-OH- $D_3$  treated cows compared with the control cows. High circulating PTH concentrations depress catabolic enzymes in the kidney that metabolize 25-OH- $D_3$  to inactive metabolites (Goff et al., 1991 b).

Wilkens et al. (2012) conducted a study where a low DCAD diet and Rovimix HyD were combined. This is a similar study to the current study under discussion. It was found that both the PTH plasma concentrations and the bone resorption marker, CrossLaps, had the lowest concentrations for the combination of the low DCAD diet supplemented with Rovimix HyD, which is similar to the results found by Taylor et al. (2008). The serum CrossLaps<sup>®</sup> ELISA assay (Immuno Diagnostic Systems) measures degraded Type I collagen and small peptide fragments which is excreted into the bloodstream during renewal of the skeleton. Type I collagen accounts for more than 90% of the organic matrix of bone and is synthesised primarily in bone. The measurement of the specific degradation products of Type I collagen in both urine and serum are achieved with this particular assay and serves as an assessment of bone resorption during osteoclastic bone resorption. It was speculated that the 25-OH- $D_3$ -mediated increased gastro-intestinal absorption of calcium and phosphorus, prepartum in cows treated with Rovimix HyD, leads to improved mineralisation of the skeleton (Wilkens et al., 2012). Wilkens et al. (2012) further stated that as bone metabolism is characterised by continuous resorption and formation, a temporary decrease in resorption may result in higher mineral content.

It is speculated that the study conducted by Wilkens et al. (2012) may provide a possible answer as to why the elevated plasma hormone concentrations did not lead to increased plasma calcium concentrations. Plasma PTH concentrations were not determined in this study and therefore it is important that PTH concentrations, CrossLaps and mineral content of bone as well as direct measurement of bone mineral density should be included in future investigations.

It is expected from similar studies (Wilkens et al., 2012) that the negative DCAD + HyD treatment should cause significant increases in plasma 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations compared to the negative DCAD treatment. Although a visible increase of 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations were observed postpartum, both the negative DCAD and negative DCAD + HyD treatment groups achieved similar plasma concentrations (figure 4.5). It was previously demonstrated that the production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> was similar for both milk fever and non-milk fever cows and that peak plasma 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations were higher for milk fever than non-milk fever cows (Goff, 2000). Jorgenson (1974) indicated that the kidney mainly produces 1,25-(OH)<sub>2</sub>D<sub>3</sub> under hypocalcaemic conditions, while 24,25-(OH)<sub>2</sub>D<sub>3</sub> is the predominant metabolite produced under normal conditions in the kidney. 1,25-Dihydroxyvitamin D<sub>3</sub> is responsible for intestinal calcium absorption and bone calcium mobilisation. Jorgenson (1974) further suggested that it is possible that plasma calcium concentrations may function as a feedback system to regulate the production of 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Low calcium concentrations at calving will stimulate PTH secretion which in turn is responsible for 1,25-(OH)<sub>2</sub>D<sub>3</sub> production. If the plasma calcium concentration is within normal limits (2.1 – 2.5 mmol/L) PTH and 1,25-(OH)<sub>2</sub>D<sub>3</sub> synthesis is suppressed (Horst et al., 1994; Taylor et al., 2008). It can be assumed that as this study did not lead to increased plasma calcium concentrations the 1,25-(OH)<sub>2</sub>D<sub>3</sub> synthesis did not increase and therefore similar plasma concentrations were observed between the two treatment groups.

Previous studies have indicated that vitamin D and its metabolites and/or analogues are successful methods to prevent hypocalcaemia and milk fever. A reasonable approach is to supplement the dry cow with 20 - 30 000 IU vitamin D/animal/day (Goff, 2006). Jorgensen (1974) indicated that when 20-30 million units of vitamin D<sub>2</sub> were supplied 3-7 days prior to calving, the incidence of hypocalcaemia could be reduced by 70-80%. It was also mentioned that treatment should commence at least 3 days before parturition and should not be continued more than 7 days.

It was shown that feeding massive doses of vitamin D for longer than 7 days could be toxic and thus care must be taken when making use of this method. When 10 million IU of vitamin D<sub>3</sub> were administered intramuscularly within 10 days of parturition, a reasonable measure of protection against toxicity could be provided (Jorgensen, 1974). Many researchers have however indicated that vitamin D toxicity, soft tissue calcification as well as prediction of accurate calving dates can be

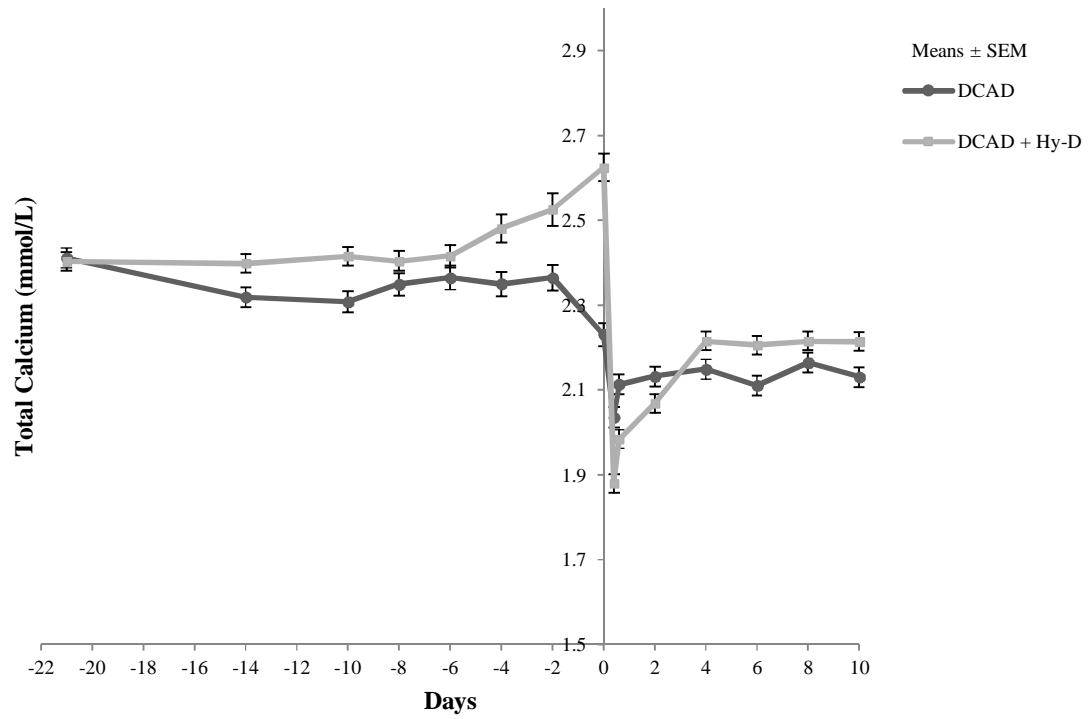
problematic when making use of this particular technique (Littledike and Horst, 1982; Thilising-Hansen et al., 2002 (a); Murray et al., 2008). Lower doses of between 500 000 to 1 million IU of vitamin D induced milk fever in some cases. This was due to the high levels of 25-OH-D<sub>3</sub> and 1,25-(OH)<sub>2</sub>D<sub>3</sub> resulting from treatment, actually suppressing PTH secretion and renal synthesis of endogenous 1,25-(OH)<sub>2</sub>D<sub>3</sub>. These animals become hypocalcaemic when the exogenous vitamin D source that had maintained elevated intestinal calcium absorption rates was cleared from the body (Goff, 2006). In certain cases, the ability to begin endogenous production of 1,25-(OH)<sub>2</sub>D<sub>3</sub> was suppressed for a week after calving (Goff, 2006).

The present study was conducted on a commercial dairy farm and therefore it was decided that current on-farm practices are not to be changed. Both the standard on-farm DCAD diet and the DCAD + HyD diet were fed from 21 days prior to the expected calving date up until calving. The HyD was supplied at an inclusion rate of 3mg/animal/day as 25-OH-D<sub>3</sub> (= 240 mg Rovimix HyD), which amounts to 120 000 IU vitamin D<sub>3</sub>/animal/day. This level is lower than the 20-30 million IU levels of vitamin D<sub>2</sub> supplied in the study by Jorgensen (1974) and no vitamin D toxicity was observed in a scenario where the diet was fed for a period of 21 days prior to calving. It can thus be concluded that longer feeding periods than the proposed 10 days prior to calving can safely be implemented when feeding Rovimix HyD. The advantage of longer supplementation periods is that the animals will receive the Rovimix HyD prior to calving even if it is a challenge to accurately predict the expected calving dates.

#### 4.4.2 Calcium:

##### a) Total and Ionized calcium ( $Ca_t$ and $Ca^{2+}$ )

Figure 4.6 and Table 4.7 shows that no treatment differences could be detected ( $P > 0.05$ ) for the total calcium concentrations in the plasma, although a treatment by day interaction was detected ( $P < 0.001$ ). Treatment means for the two treatment groups were 2.2 mmol/L  $\pm$  0.019 (DCAD) and 2.3 mmol/L  $\pm$  0.019 (DCAD + HyD) respectively. Mean  $Ca_t$  concentrations ranged between 2.3 mmol/L and 2.5 mmol/L from day 21 prepartum until parturition. At parturition,  $Ca_t$  concentrations decreased in both groups with a more pronounced decline observed in the DCAD + HyD group. The  $Ca_t$  concentration of the DCAD group was reduced to approximately 2 mmol/L while the  $Ca_t$  concentration of the DCAD + HyD group was reduced to about 1.8 mmol/L. An increase in the  $Ca_t$  concentrations was observed during the next 12 hours. The DCAD + HyD group showed an increase in the  $Ca_t$  levels to an average level of 2.2 mmol/L.



**Figure 4.6** Plasma concentrations of total calcium of dairy cows as a function of treatment duration. Untreated cows on a control ration (DCAD group) and treated with 3 mg 25-OH-D<sub>3</sub> daily (DCAD + HyD group) from day 21 prepartum until parturition; data presented as means ±SEM

**Table 4.7** Total calcium plasma concentrations of samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration from day 21 prepartum to day 10 postpartum; data presented as means  $\pm$  SEM\*

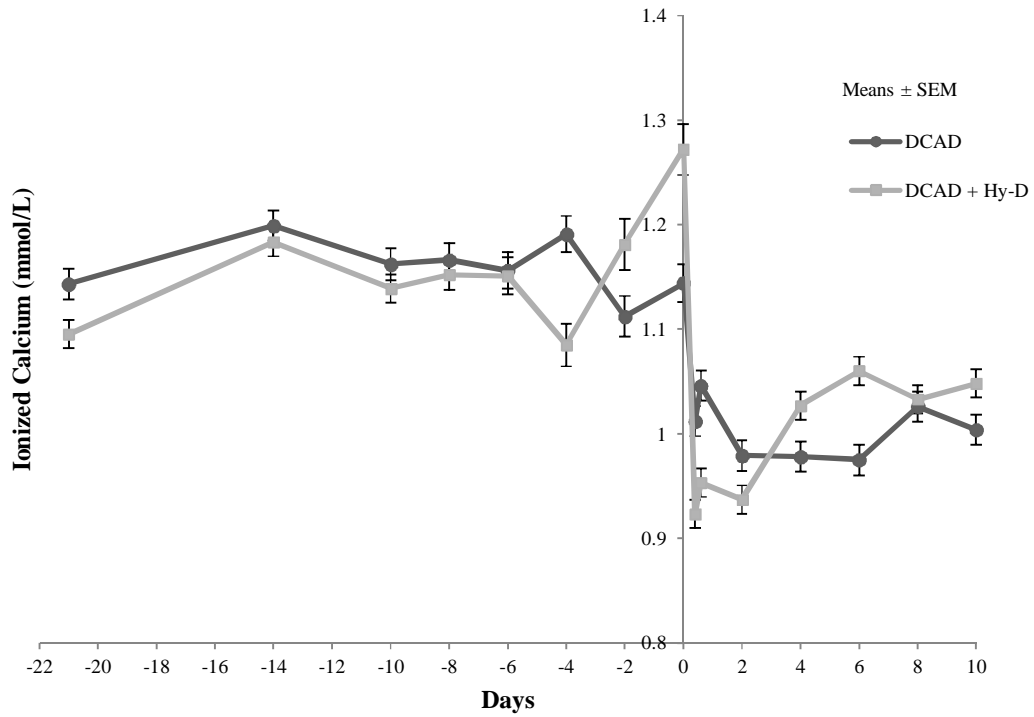
Total plasma calcium concentrations (mmol/L)		
Day	DCAD Group	DCAD + HyD Group
-14	2.31 mmol/L $\pm$ 0.047 (n = 14)	2.39 mmol/L $\pm$ 0.044 (n=16)
-10	2.30 mmol/L $\pm$ 0.049 (n=13)	2.41 mmol/L $\pm$ 0.044 (n=16)
-8	2.34 mmol/L $\pm$ 0.053 (n=11)	2.40 mmol/L $\pm$ 0.047 (n=14)
-6	2.36 mmol/L $\pm$ 0.056 (n = 10)	2.41 mmol/L $\pm$ 0.053 (n=11)
-4	2.35 mmol/L $\pm$ 0.056 (n = 10)	2.48 mmol/L $\pm$ 0.066 (n=7)
-2	2.36 mmol/L $\pm$ 0.061 (n=8)	2.52 mmol/L $\pm$ 0.076 (n=5)
0	2.23 <sup>a</sup> mmol/L $\pm$ 0.054 (n=5)	2.62 <sup>b</sup> mmol/L $\pm$ 0.066 (n=2)
4 hours p.p.	2.03 <sup>a</sup> mmol/L $\pm$ 0.047 (n=14)	1.87 <sup>b</sup> mmol/L $\pm$ 0.044 (n=16)
6 hours p.p.	2.11 <sup>a</sup> mmol/L $\pm$ 0.047 (n=14)	1.98 <sup>b</sup> mmol/L $\pm$ 0.044 (n=16)
2	2.13 mmol/L $\pm$ 0.047 (n=14)	2.06 mmol/L $\pm$ 0.044 (n=16)
4	2.14 mmol/L $\pm$ 0.047 (n=14)	2.21 mmol/L $\pm$ 0.044 (n=16)
6	2.11 mmol/L $\pm$ 0.047 (n=14)	2.20 mmol/L $\pm$ 0.044 (n=16)
8	2.16 mmol/L $\pm$ 0.047 (n=14)	2.21 mmol/L $\pm$ 0.044 (n=16)
10	2.13 mmol/L $\pm$ 0.047 (n=14)	2.21 mmol/L $\pm$ 0.044 (n=16)

\*SEM is the Standard Error of the Mean

<sup>#</sup>n indicates the number of animals observed per time period

<sup>ab</sup> Row means (day) with different superscripts differ (P<0.05)

No treatment differences could be detected in the plasma concentrations for ionized calcium (P>0.05), while the treatment by day interaction was significant (P<0.05) (Figure 4.7 and Table 4.8). Treatment means for the groups were 1.08 mmol/L  $\pm$  0.010 (DCAD) and 1.08 mmol/L  $\pm$  0.010 (DCAD + HyD) respectively. Mean Ca<sup>2+</sup> concentrations ranged between 1.1 mmol/L and 1.15 mmol/L from the commencement of the trial up to day 6 before calving. Cows on the DCAD treatment showed a slight increase to approximately 1.19 mmol/l on day 4 prepartum. After calving there was a decrease in the Ca<sup>2+</sup> concentrations from 1.14 mmol/L to 1.0 mmol/L, 6 hours postpartum. The Ca<sup>2+</sup> concentrations did not increase postpartum and settled at approximately 1 mmol/L. The DCAD + HyD treatment showed a slight decrease in the Ca<sup>2+</sup> levels, but it increased dramatically from 1.08 mmol/L on day 4 before calving to 1.27 mmol/L at calving. A sudden decrease can be observed 4 hours postpartum and the Ca<sup>2+</sup> concentrations settled and were maintained at approximately 1 mmol/L postpartum.



**Figure 4.7** Plasma concentrations of ionized calcium ( $\text{Ca}^{2+}$ ) of dairy cows as a function of treatment duration. Untreated cows on a control diet (DCAD group) and treated cows with 3 mg 25-OH- $\text{D}_3$  daily (DCAD + HyD group) from day 21 prepartum until parturition; data presented as means  $\pm$ SEM



**Table 4.8** Ionized calcium plasma concentrations samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration from day 21 prepartum to day 10 postpartum; data presented as SEM\*

Ionized plasma calcium concentrations (mmol/L)		
Day	DCAD Group	DCAD + HyD Group
-21	1.14 mmol/L $\pm$ 0.029 (n <sup>#</sup> =14)	1.09 mmol/L $\pm$ 0.027 (n=16)
-14	1.19 mmol/L $\pm$ 0.029 (n=14)	1.18 mmol/L $\pm$ 0.027 (n=16)
-10	1.16 mmol/L $\pm$ 0.030 (n=13)	1.13 mmol/L $\pm$ 0.027 (n=16)
-8	1.16 mmol/L $\pm$ 0.033 (n=11)	1.15 mmol/L $\pm$ 0.029 (n=14)
-6	1.15 mmol/L $\pm$ 0.035 (n=10)	1.15 mmol/L $\pm$ 0.035 (n=10)
-4	1.19 mmol/L $\pm$ 0.035 (n=10)	1.08 mmol/L $\pm$ 0.041 (n=7)
-2	1.11 mmol/L $\pm$ 0.039 (n=8)	1.18 mmol/L $\pm$ 0.049 (n=5)
0	1.14 <sup>a</sup> mmol/L $\pm$ 0.036 (n=5)	1.27 <sup>b</sup> mmol/L $\pm$ 0.049 (n=2)
4 hours p.p.	1.01 <sup>a</sup> mmol/L $\pm$ 0.029 (n=14)	0.923 <sup>b</sup> mmol/L $\pm$ 0.027 (n=16)
6 hours p.p.	1.04 <sup>a</sup> mmol/L $\pm$ 0.029 (n=14)	0.953 <sup>b</sup> mmol/L $\pm$ 0.027 (n=16)
2	0.979 mmol/L $\pm$ 0.029 (n=14)	0.937 mmol/L $\pm$ 0.027 (n=16)
4	0.979 mmol/L $\pm$ 0.029 (n=14)	1.02 mmol/L $\pm$ 0.027 (n=16)
6	0.975 mmol/L $\pm$ 0.029 (n=14)	1.06 <sup>b</sup> mmol/L $\pm$ 0.027 (n=16)
8	1.02 mmol/L $\pm$ 0.029 (n=14)	1.03 mmol/L $\pm$ 0.027 (n=16)
10	1.00 mmol/L $\pm$ 0.029 (n=14)	1.04 mmol/L $\pm$ 0.027 (n=16)

\*SEM is the Standard Error of the Mean

<sup>#</sup>n indicates the number of animals observed per time period

<sup>ab</sup> Row means (day) with different superscripts differ (P<0.05)

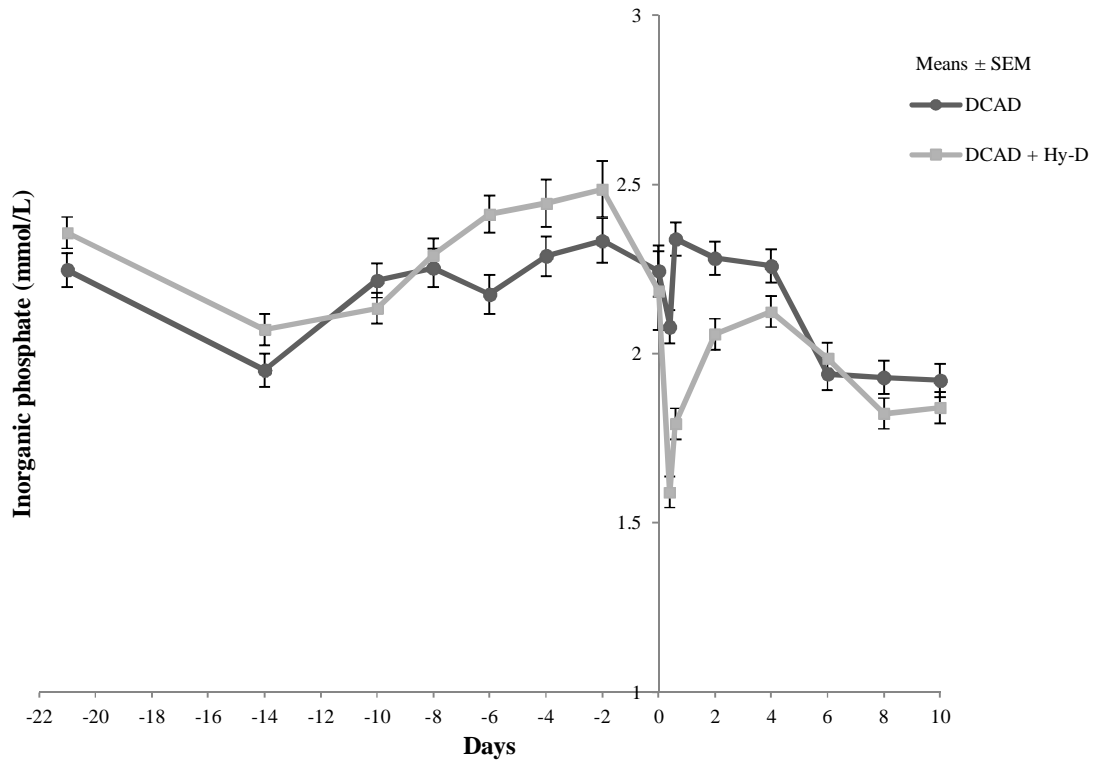
Total calcium consist of free or ionized calcium (50%), calcium bound to protein (40-45%) and albumin and calcium to anions (5-10%) e.g. citrate, lactate or bicarbonate (French et al., 2010 b). In this study both total and ionized calcium in plasma were analysed. Total calcium concentration does not give an indication of what is available at the cellular level. Total calcium is absorbed in the small intestine and is dependent on functioning homeostatic mechanisms for increased absorption (Ferneborg, 2010). Ionized calcium is readily available for absorption in the rumen and abomasum and determination of ionized calcium gives a more accurate reflection of the physiological calcium state (French et al., 2010 b; Ferneborg, 2012).

Blood calcium concentrations in the adult dairy cow is maintained at around 2.1 – 2.5 mmol/L (Goff, 2006). Inadequate amounts of plasma calcium, phosphorus, magnesium and potassium can cause a cow to lose the ability to rise after calving as these minerals are necessary for nerve and muscle function (Goff, 2006). The mean calcium (total) concentrations varied between 2.36 mmol/L  $\pm$  0.061 to 2.62 mmol/L  $\pm$  0.076 from 21 days prepartum until parturition for both experimental groups

(Table 4.7). These levels were clearly within the levels as stated by Goff (2006). Four hours postpartum the mean plasma concentration declined to 2.04 mmol/L  $\pm$  0.047 for the DCAD group and 1.87 mmol/L  $\pm$  0.044 for the DCAD + HyD group. Based on these calcium concentrations animals in the DCAD + HyD treatment group could have been diagnosed as hypocalcaemic and it would be suggestive of milk fever, although no clinical signs of milk fever were observed. The negative DCAD treatment group maintained higher blood calcium concentrations around calving and it can be concluded that these animals did not experience hypocalcaemia. Bigras-Poulin and Tremblay (1998) stated that oestrogens are higher during the last 5 days prepartum and decrease feed intake and tend to force the animal on the first day postpartum to change from passive to active intestinal absorption of calcium and phosphorus. They further stated that oestrogens also inhibit bone resorption, diminish bone responsiveness to PTH and decrease the intestinal calcium absorption. As with the study by Bigras-Poulin and Tremblay (1998) these effects coupled with the increased calcium demand from the mammary gland, could thus explain why some of the animals in the DCAD + HyD treatment group appeared to be hypocalcaemic.

#### **4.4.3 Phosphate ( $P_i$ ):**

No treatment differences could be found in the plasma phosphorus concentrations of the cows supplemented with the negative DCAD or negative DCAD + HyD rations ( $P > 0.05$ ). Treatment by day interactions was however indicated ( $P < 0.05$ ) (Figure 4.8 and Table 4.9). Treatment means for the two groups were 2.16 mmol/L  $\pm$  0.030 (DCAD) and 2.11 mmol/L  $\pm$  0.032 (DCAD + HyD) respectively. Inorganic phosphorus ( $P_i$ ) levels remained relatively constant between 2.15 mmol/L and 2.45 mmol/L up to parturition. At parturition  $P_i$  concentrations displayed a more substantial decline in the DCAD + HyD treatment group than the DCAD treatment group.



**Figure 4.8** Plasma concentrations of inorganic phosphate of dairy cows as a function of treatment duration. Untreated cows on a control ration (DCAD group) and treated with 3mg of 25-OH-D<sub>3</sub> daily (DCAD + HyD group) from day 21 prepartum until parturition; data presented as means ±SEM

**Table 4.9** Inorganic phosphorus plasma concentrations of samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration from day 21 prepartum to day 10 postpartum; data presented as means  $\pm$  SEM\*

<b>Inorganic phosphorus plasma concentrations (mmol/L)</b>		
<b>Day</b>	<b>DCAD Group</b>	<b>DCAD + HyD Group</b>
-14	1.95 mmol/L $\pm$ 0.098 (n=14)	2.07 mmol/L $\pm$ 0.092 (n=16)
-10	2.21 mmol/L $\pm$ 0.102 (n=14)	2.13 mmol/L $\pm$ 0.092 (n=16)
-8	2.25 mmol/L $\pm$ 0.111 (n=14)	2.29 mmol/L $\pm$ 0.098 (n=16)
-6	2.17 <sup>a</sup> mmol/L $\pm$ 0.116 (n=14)	2.41 <sup>b</sup> mmol/L $\pm$ 0.111 (n=16)
-4	2.28 mmol/L $\pm$ 0.116 (n=14)	2.44 mmol/L $\pm$ 0.139 (n=16)
-2	2.33 mmol/L $\pm$ 0.130 (n=14)	2.48 mmol/L $\pm$ 0.164 (n=16)
0	2.24 mmol/L $\pm$ 0.152 (n=14)	2.18 mmol/L $\pm$ 0.233 (n=16)
4 hours p.p.	2.07 <sup>a</sup> mmol/L $\pm$ 0.098 (n=14)	1.59 <sup>b</sup> mmol/L $\pm$ 0.092 (n=16)
6 hours p.p.	2.33 <sup>a</sup> mmol/L $\pm$ 0.098 (n=14)	1.79 <sup>b</sup> mmol/L $\pm$ 0.092 (n=16)
2	2.28 <sup>a</sup> mmol/L $\pm$ 0.098 (n=14)	2.05 <sup>b</sup> mmol/L $\pm$ 0.092 (n=16)
4	2.25 mmol/L $\pm$ 0.098 (n=14)	2.12 mmol/L $\pm$ 0.092 (n=16)
6	1.94 mmol/L $\pm$ 0.098 (n=14)	1.98 mmol/L $\pm$ 0.092 (n=16)
8	1.92 mmol/L $\pm$ 0.098 (n=14)	1.82 mmol/L $\pm$ 0.092 (n=16)
10	1.92 mmol/L $\pm$ 0.098 (n=14)	1.84 mmol/L $\pm$ 0.092 (n=16)

\*SEM is the Standard Error of the Mean

<sup>#</sup>n indicates the number of animals observed per time period

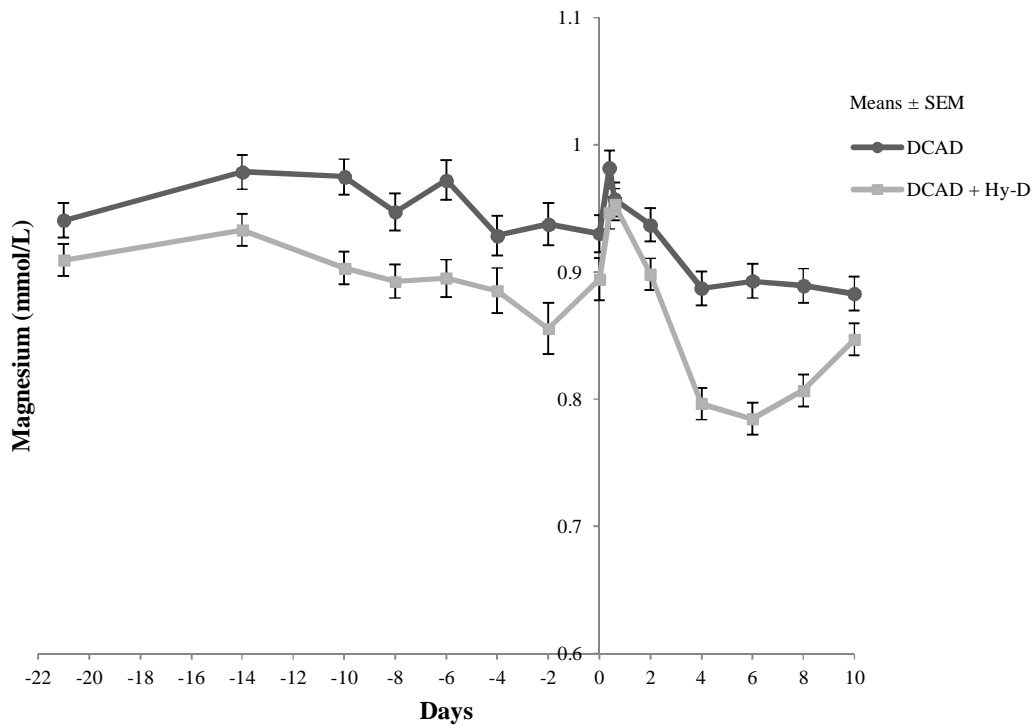
<sup>ab</sup> Row means (day) with different superscripts differ (P<0.05)

Plasma phosphorus concentrations are normally maintained between 1.3 and 2.6 mmol/L (Goff, 2000). Approximately 1-2 g phosphorus is present in the plasma inorganic phosphorus pool and 4.7g phosphorus is generally present in the extracellular phosphorus pool of a 500 kg dairy cow (Goff, 2000). Maintaining the extracellular phosphorus pool involves replacing phosphorus removed from bone and muscle growth, endogenous faecal loss, urinary phosphorus loss and milk production with phosphorus absorbed from the diet or via bone resorption (Goff, 2000). Phosphate is absorbed in the small intestine (especially the jejunum) by both paracellular and active transport and is enhanced by low dietary calcium, increased dietary acidity, growth hormone and vitamin D (French et.al., 2010 c). Figure 4.8 shows that the inorganic phosphorus ( $P_i$ ) levels in this particular study were maintained between 2.17 mmol/L  $\pm$  0.116 (DCAD treatment) and 2.48 mmol/L  $\pm$  0.164 (DCAD + HyD treatment). The nadir in  $P_i$  concentration was a level of 1.59 mmol/L  $\pm$  0.092 in the DCAD + HyD treatment. This level is however still above the lowest level indicated by Goff (2000). Grünberg et al. (2011) commented that mild metabolic acidosis during late gestation does not alter  $P_i$  concentration or balance. This is confirmed by the slight variation shown in Figure 4.8.

Furthermore a correlation can be drawn between plasma calcium and phosphorus levels as disturbances in phosphorus metabolism can occur secondary to disturbances in calcium metabolism (Goff, 2000) and it is clear from this study that oral supplementation of 25-OH-D<sub>3</sub> did not cause significant increases in either plasma calcium or phosphorus levels.

#### 4.4.4 Magnesium (Mg):

The mean plasma concentration of magnesium for both treatment groups was 0.9359 mmol/L ± 0.0126 (DCAD) and 0.8800 mmol/L ± 0.0121 (DCAD + HyD) respectively. No treatment by day interactions could be detected (P>0.05) (Figure 4.9 and Table 4.10). Postpartum magnesium levels showed an inverse profile to plasma calcium and phosphorus with an increase at parturition and maximum levels were reached around 6 hours postpartum. Cows fed the negative DCAD ration had consistently higher plasma magnesium concentrations cows fed the negative DCAD + HyD ration.



**Figure 4.9** Plasma concentrations of magnesium (Mg<sup>2+</sup>) of dairy cows as a function of treatment duration. Untreated cows on a control ration (DCAD group) and treated with 3 mg of 25-OH-D<sub>3</sub> daily (DCAD + HyD group) from day 21 prepartum until parturition; data presented as means ±SEM

**Table 4.10** Plasma magnesium concentrations of the samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration from day 21 prepartum to day 10 postpartum; data presented as means  $\pm$  SEM\*

Plasma magnesium concentrations (mmol/L)		
Day	DCAD Group	DCAD + HyD Group
-14	0.978 mmol/L $\pm$ 0.0268 (n = 14)	0.933 mmol/L $\pm$ 0.0251 (n = 16)
-10	0.974 mmol/L $\pm$ 0.0277 (n = 14)	0.903 mmol/L $\pm$ 0.0251 (n = 16)
-8	0.947 mmol/L $\pm$ 0.0296 (n = 14)	0.892 mmol/L $\pm$ 0.0264 (n = 16)
-6	0.972 mmol/L $\pm$ 0.0312 (n = 14)	0.895 mmol/L $\pm$ 0.0293 (n = 16)
-4	0.928 mmol/L $\pm$ 0.0314 (n = 14)	0.885 mmol/L $\pm$ 0.0356 (n = 16)
-2	0.937 mmol/L $\pm$ 0.0332 (n = 14)	0.855 mmol/L $\pm$ 0.0400 (n = 16)
0	0.930 mmol/L $\pm$ 0.0293 (n = 14)	0.894 mmol/L $\pm$ 0.0333 (n = 16)
4 hours p.p.	0.982 mmol/L $\pm$ 0.0268 (n = 14)	0.946 mmol/L $\pm$ 0.0251 (n = 16)
6 hours p.p.	0.957 mmol/L $\pm$ 0.0268 (n = 14)	0.953 mmol/L $\pm$ 0.0251 (n = 16)
2	0.937 mmol/L $\pm$ 0.0268 (n = 14)	0.898 mmol/L $\pm$ 0.0251 (n = 16)
4	0.887 mmol/L $\pm$ 0.0268 (n = 14)	0.796 mmol/L $\pm$ 0.0251 (n = 16)
6	0.892 mmol/L $\pm$ 0.0268 (n = 14)	0.784 mmol/L $\pm$ 0.0251 (n = 16)
8	0.889 mmol/L $\pm$ 0.0268 (n = 14)	0.806 mmol/L $\pm$ 0.0251 (n = 16)
10	0.882 mmol/L $\pm$ 0.0268 (n = 14)	0.846 mmol/L $\pm$ 0.0251 (n = 16)

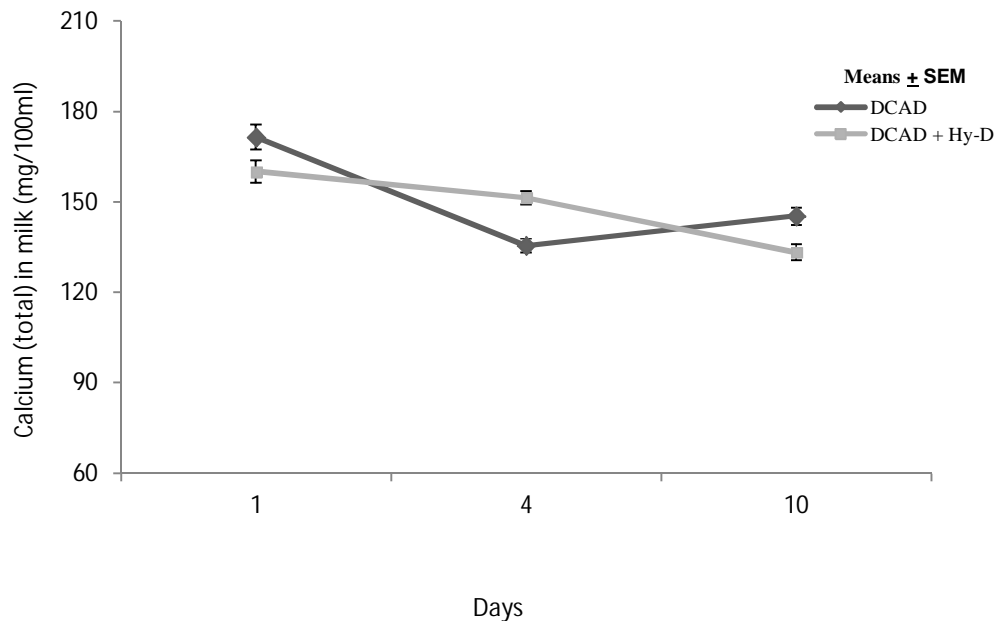
\*SEM is the Standard Error of the Mean

#n indicates the number of animals observed per time period

Magnesium is a major intracellular cation and a necessary cofactor for enzymatic reactions vital to every major metabolic pathway. Magnesium homeostasis is maintained mainly by the balance between intestinal absorption and renal excretion. Determination of plasma magnesium concentrations is important as very little is known about the control of magnesium homeostasis. Factors involved in homeostasis include dietary content, hormones (e.g. PTH, calcitonin, aldosterone and others) and serum levels of magnesium and calcium (French et al., 2010 a). Cow plasma magnesium concentrations normally range between 0.75 and 1.0 mmol/L and maintenance of normal plasma magnesium concentrations is almost completely dependent on a constant influx of magnesium from the diet (Goff, 2006). In this particular study the mean plasma magnesium concentrations were maintained between  $0.9359 \pm 0.0126$  (DCAD treatment) and  $0.88 \pm 0.0121$  (DCAD + HyD treatment) respectively. It is thus clear that the plasma magnesium concentration was not affected by treatment.

#### 4.5 Milk Analyses:

The total calcium concentration in the milk of cows supplemented with either the negative DCAD ration or the negative DCAD + HyD ration is shown in figure 4.10 and table 4.11. Total calcium concentrations were linearly reduced from day 1 to 10 postpartum in cows receiving the negative DCAD ration. The negative DCAD + HyD supplemented cows showed a more pronounced reduction around day 4 postpartum. Significant treatment differences could not be detected ( $P > 0.05$ ). Treatment by day interaction were however indicated ( $P < 0.05$ ). Treatment means for both the groups were  $150 \text{ mg}/100\text{ml} \pm 3.4$  (DCAD treatment) and  $148.3 \text{ mg}/100 \text{ ml} \pm 3.2$  (DCAD + HyD treatment) respectively.



**Figure 4.10 Milk concentrations of total calcium of dairy cows as a function of treatment duration. Untreated cows on a control ration (DCAD group) and treated with 3 mg of 25-OH-D<sub>3</sub> daily (DCAD + HyD group) from day 21 prepartum until parturition; data presented as means  $\pm$ SEM**

**Table 4.11** Total calcium concentrations (mg/100ml) of the milk samples collected from cows fed either the negative DCAD ration or a combination of the negative DCAD + HyD ration on day 1, 4 and 10 postpartum; data presented as means  $\pm$  SEM\*

Total calcium concentrations (mg/100ml) in milk		
Day	DCAD Group	DCAD + HyD Group
1	171.5 mg/100ml $\pm$ 8.3 (n <sup>#</sup> =13)	160.1 <sup>a</sup> mg/100ml $\pm$ 7.5 (n=16)
4	135.5 <sup>a</sup> mg/100ml $\pm$ 4.5 (n=14)	151.4 <sup>b</sup> mg/100ml $\pm$ 4.4 (n=15)
10	145.3 mg/100ml $\pm$ 5.6 (n=14)	133.3 mg/100ml $\pm$ 5.3 (n=16)

\*SEM is the Standard Error of the Mean

<sup>#</sup>n indicates the number of animals observed per time period

<sup>ab</sup> Row means (day) with different superscripts differ (P<0.05)

As mentioned previously the dairy cow faces unique challenges with calcium homeostasis. The dairy cow lactates for a period of 10 months. She is usually bred during the third month of lactation and gives birth approximately 8 weeks after the cessation of lactation. Unlike the rat and human, the dairy cow lactates into late gestation and calcium demands on the cow decrease significantly upon cessation of lactation, despite increasing foetal requirements (Horst et al., 1997 b).

It is clear that the 25-OH-D<sub>3</sub> serum levels are increased after oral, intramuscular and intravenous administration of 25-OH-D<sub>3</sub> and Koshy and VanDerSlik (1979) decided to determine if there is any corresponding increase in the 25-OH-D<sub>3</sub> concentration in milk. They developed a high-performance liquid chromatographic (LC) procedure for the determination of 25-OH-D<sub>3</sub> in cow milk. It was found that this method was quantitative at the 10 ppb level and capable of detection at the 2-3 ppb level. The method was applicable to both milk and colostrum. The endogenous 25-OH-D<sub>3</sub> levels in milk were however below the detection limit (Koshy and VanDerSlik, 1979).

It was interesting to note that even in cows whose serum concentrations were elevated fivefold after treatment with 25-OH-D<sub>3</sub>, the concentration in milk was below the detection level (Koshy and VanDerSlik, 1979). A study was conducted by Bar et al. (1986) to determine the content of vitamin D metabolites and the vitamin D<sub>3</sub> equivalence of milk produced by 1 $\alpha$ -hydroxyvitamin D<sub>3</sub> treated cows. Two studies were conducted where 1 $\alpha$ -hydroxyvitamin D<sub>3</sub> was injected to the trial animals at 700  $\mu$ g. Milk was collected from animals that calved 36-43 hours after treatment in the first study and 37-60 hours after the second injection (2 injections of 350  $\mu$ g 1 $\alpha$ -hydroxyvitamin D<sub>3</sub> was given at 72 hour intervals) in the second study. Concentrations of vitamin D metabolites in milk of the treated cows did not differ significantly from those of controls (Bar et.al., 1986). The concentration of 25-OH-D<sub>3</sub> in milk for this particular study have not been determined and from the above



mentioned studies it is expected that there are no significant differences in the concentrations of vitamin D metabolites in milk, between the DCAD and DCAD + HyD treatment groups.

Hidiroglou and Proulx (1982) conducted a study where vitamin D<sub>3</sub> and 25-OH-D<sub>3</sub> were administered to 27 gestating Shorthorn heifers. It was found that the colostrum calcium (%) was higher on day 2 and 3 postpartum for all 25-OH-D<sub>3</sub> treated animals. It was concluded that the higher calcium concentrations in colostrum from day 1-3 postpartum could be attributed to increased intestinal absorption and most likely followed by higher trans-mammary calcium transfer than in the other treatment groups (Hidiroglou and Proulx, 1982).

According to South African Guidelines the calcium content of a whole milk sample should be approximately 113 mg/100g. During this particular study it was found that the mean calcium concentrations were 171.5 mg/100ml for the DCAD treatment group and 160.1 mg/100ml for the DCAD + HyD treatment group respectively. Milk samples were collected on day 1, 4 and 10 postpartum and thus only one day of colostrum production was taken into account. Cows receiving the DCAD + HyD treatment however showed the highest levels of calcium (151.4 mg/100ml) on day 4 postpartum as well with a reduction to 133.3 mg/100ml thereafter. It can thus be concluded that as with the study of Hidiroglou and Proulx (1982), the high calcium levels in colostrum could be due to increased calcium absorption.

#### **4.6 Characteristics of calving and diseases around parturition:**

The characteristics of calving were actively observed during this trial. Spontaneous calving was classified as “non-complicated”, while any manipulations and/or difficult calvings were classified as “complicated”. The majority of trial animals (80%) calved normally, while only 20% of the animals in both experimental groups required assistance during calving. All trial animals close to calving were observed on a daily basis for labour signs. The animals were examined if they did not make “normal” progress once in labour and timely intervention was taken if an animal was identified as having a difficult birth. No caesarean sections were performed during the trial period. The following factors play a role in calving difficulty: Age of cow, sire used, condition, health and nutrition of cow prior to calving (Seykora, 2000).

No cases of clinical milk fever were observed during the course of this trial. A total of 6 animals were diagnosed with placental retention and 33.3% belonged to the DCAD + HyD treatment group while 66.67% belonged to the DCAD treatment group. A total of 6 animals were diagnosed with mastitis, with 83.3% and 16.67% of cows belonging to the DCAD + HyD and DCAD treatment groups respectively. These cows all received mastitis treatment during the first 3 weeks postpartum. Several associations between retained foetal membranes and hypocalcaemia have been made (Melendez et al., 2004; Beagley et al., 2010). It has been found that in cows fed anionic diets, those with retained foetal membranes had significantly lower total plasma calcium concentrations than cows without retained foetal membranes (Melendez et al., 2004; Beagley et al., 2010).

In the study by Melendez et al. (2004), the hypocalcaemia experienced by cows with retained foetal membranes was subclinical. It is thus clear that there is a link between milk fever and the occurrence of retained placenta. It is important to note that the study by Melendez et al. (2004) mainly focused on total calcium levels, rather than the biologically active ionized calcium (Beagley et al., 2010). Total calcium levels can be affected by other factors, e.g. hypo-albuminemia, in the face of normal ionized calcium concentrations (Beagley et al., 2010).

In the present study total plasma calcium concentrations of between 1.71 and 2.03 mmol/L were observed for the individual cows that experienced retained foetal membranes, confirming the link between milk fever and the incidence of retained placenta. Mulligan et al. (2006) indicated that cows that suffered clinical milk fever in previous lactations were eight times more likely to develop mastitis than normal cows. It is hypothesized that the reasons for this occurrence is (a) a reduction in smooth muscle function at the teat sphincter and hence an easy route for infection after milking and (b) an exacerbated suppression of immunity in milk fever cows when compared with normal cows (Mulligan et al., 2006). Kimura et al. (2006) demonstrated that hypocalcaemia is associated with reduced intracellular calcium concentrations in peripheral blood mononuclear cells and that this intensifies peri-parturient immunosuppression. The epidemiological association found between milk fever and mastitis incidence is thus supported by several potential biological mechanisms, some of which have been reported to be more relevant in peri-parturient dairy cows (Mulligan et al., 2006).

Mastitis cases observed during this trial may potentially be related to subclinical milk fever, as well as environmental conditions. Dairy farming in the South African interior relies mostly on Total Mixed Ration (TMR) systems, extensive farming or a combination of TMR and extensive farming. Heat is a problem for most of the year, while rain can lead to muddy conditions. These environmental conditions lead to the occurrence of mastitis and high somatic cell counts. The trial was conducted during the summer rainy season and as this commercial dairy farm has no cow housing available, the animals are exposed to both heat and rain. It is likely that these conditions also had an impact on the mastitis cases observed during the trial.

## **CHAPTER 5**

### **CONCLUSION AND RECOMMENDATIONS**

## Chapter 5: Conclusion and recommendations

The transition period of the dairy cow is still one of the principle research fields as many have realized the potential of a more productive life and improved lactational performance if the dairy cow manages to make a smooth transition to lactation during this period. Hypocalcaemia and its correlation to numerous homeostatic disorders, including metritis, ketosis, retained placenta and abomasal displacement emphasize the importance of a good dry cow program in any dairy herd. Many established programs are currently implemented to ensure the smooth transition into lactation e.g. low calcium prepartal diets or reducing the DCAD of the diet. It is however difficult to achieve truly low calcium diets (<20 g calcium/day) with available feedstuffs. Cows fed the low DCAD diet appeared to have a less pronounced drop in calcium plasma levels postpartum, suggesting that enough calcium was mobilised to meet the demands of early lactation. Reducing the DCAD of a diet has proven to be a successful method to counteract hypocalcaemia and/or clinical milk fever postpartum, by inducing mild metabolic acidosis which stimulates bone and renal sensitivity to PTH. It has however been found in certain cases that a reduced DCAD may influence DM intake.

Several authors have made use of vitamin D and its metabolites and/or analogues as a possible method to improve calcium homeostasis and reduce the incidence of hypocalcaemia and/or clinical milk fever postpartum. Administration of large doses of vitamin D (20 – 30 million IU of vitamin D per day) could lead to toxicity if administered for more than 7 days. Further studies proved that reasonable protection against hypocalcaemia could be achieved by supplementing 10 million IU of vitamin D intramuscularly 10 days prior to parturition. In addition to the possibility of toxicity another major difficulty of using vitamin D and its metabolites is to determine the accurate calving date as it was found that treatment is generally most effective between 1 and 4 days prior to parturition. It was however demonstrated in this study that longer feeding periods ( $\pm$  21 days) can safely be achieved when feeding a dosage of 3 mg 25-OH-D<sub>3</sub> (=240 mg Rovimix HyD 1,25%) per animal per day in combination with anionic salts. No cases of clinical milk fever were observed during the trial period although some cases of hypocalcaemia at parturition could not be avoided completely.

The study failed to prove that a combination of anionic salts and 25-OH-D<sub>3</sub> could be more effective than anionic salts alone to improve the calcium status of a group of close-up dairy cattle. Plasma calcium concentrations were not significantly increased in the DCAD + HyD treatment group. It would appear that the animals included in this trial were not sufficiently challenged i.e. the negative DCAD diet supplied to both the treatment groups were effective in maintaining the plasma calcium concentrations at higher levels around parturition, thus masking the expected effect of a combination of anionic salts and 25-OH-D<sub>3</sub>.

Several recommendations should be considered for future investigations and includes the following:

1. Due to the failure to prove the effectiveness of a combination of anionic salts and 25-OH-D<sub>3</sub> to increase plasma calcium concentrations, future research could further investigate the use of different value ranges of low DCAD diets and combinations with 25-OH-D<sub>3</sub>. These DCAD value ranges could be used to establish the exact level of mEq/kg DM for a low DCAD diet and 25-OH-D<sub>3</sub> combination to be effective in increasing plasma calcium concentrations postpartum.
2. It is important to note from this study that pseudo-replication occurred. Pseudo-replication refers to observations that are not statistically independent, but treated as if they are (Hurlbert, 1984) and occurs when there are multiple observations on the same subjects, the data have a hierarchical structure and observations are correlated in time or space (Lazic, 2010). This particular aspect is an influential methodological issue in ecological and animal behaviour research at present. The researcher used 30 Holstein type dairy cows – 15 cows per treatment representing 15 blocks. Only two treatment groups (DCAD and DCAD + HyD) were used during this trial resulting in a single series of observations to be made on groups of animals with a repeatability of one each. Analysis of this data was done without taking into consideration the dependencies mentioned earlier and thus could lead to statistically meaningless results. It is therefore recommended that more replicates and additional treatment groups, e.g. a control treatment group, a low DCAD group and a low DCAD + HyD group be used should this study be repeated, thus providing more comparable information to clearly demonstrate the differences between groups.
3. Individual feed intake could not be determined due to constraints in the facilities and management practices, leading to variable feed intake. It is recommended that, similar to feedlot trials, group feeding is allowed as this may contribute to overcoming the above mentioned constraints at commercial institutions. Feeding strategies at feedlots may typically include e.g 120 animals allocated to two treatment groups and divided into 10 pens with six animals each. Such a model should provide enough statistical power to achieve significant differences between treatments.
4. As this study was conducted on a commercial dairy farm it was important to maintain existing management practices. Furthermore, full access to dietary information of dairy concentrates utilized for this study was denied and thus the repeatability of this particular study may be questionable. It is thus recommended that similar studies be conducted at universities or research institutions as it will enable the researcher to study parameters without considering on-farm practices and commercial constraints such as protection of intellectual property. Universities and research institutions are however urged to maintain and increase the size of their dairy herds to conduct meaningful dairy cattle research.

5. It is further recommended that plasma PTH concentrations, mineral content of bone, measurement of bone mineral density and the bone resorption marker CrossLaps (a measurement used for the assessment of bone resorption) be determined. These assessments may increase our understanding of the results obtained in this study and possibly explain why increased plasma calcium concentrations were not achieved as expected.

In conclusion the results from this study provide us with valuable information regarding the use of Rovimix HyD 1.25% on a commercial dairy farm. It is demonstrated that longer feeding periods ( $\pm 21$  days) than the proposed 10 days prior to calving (as suggested by a number of studies) can safely be achieved when feeding 3 mg 25-OH-D<sub>3</sub> per animal per day (=240 mg Rovimix HyD 1,25%) in combination with anionic salts. Rovimix HyD 1,25% (DSM Nutritional Products, Basel, Switzerland) could thus be introduced to commercial dairy farms as part of normal feeding practices.

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