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LIVESTOCK RESEARCH
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Samenvatting Het doel van deze literatuurstudie is de onzekerheden en aannames binnen de huidige P behoeftemodellen in kaart te brengen, suggesties te doen voor verbetering om nauwkeuriger en met meer zekerheid naar behoefte te kunnen voeren, en biomarkers te zoeken om de P balans bij levende dieren te monitoren. Het P-gehalte van melk is een belangrijke factor op de totale P balans, maar kan sterk variëren tussen individuele koeien. In het skelet ligt een zeer groot aandeel van de totale P voorraad opgeslagen, en botweefsel kan een belangrijke invloed hebben op de P balans en de plasma P regulatie. Plasma P is nog altijd de meest gebruikte diagnostische parameter om de P status te beoordelen, ondanks alle beperkingen die een goede interpretatie moeilijk maken; nieuwe biomarkers zijn daarom hard nodig. Verder onderzoek naar achterliggende mechanismes met de bijbehorende kwantificering is noodzakelijk om de kennis over de P stofwisseling te verbeteren.

Summary Goal of this literature study is to define the uncertainties related to P requirement models and ways to make them more precise and more reliable than current systems and to find biomarkers to monitor P balance in living animals. Milk P is an important factor in P balance, and concentration may vary between individual cows. Bone represents a large P reserve and can have a profound impact on cow P balance and plasma P regulation. Plasma P concentration is still the most commonly used diagnostic measurement available to judge the P status of an animal, with all restrictions for good interpretation; new biomarkers defining P status are needed. Further research into mechanisms and quantification of the is needed to improve our understanding of P metabolism.

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Foreword

This study was conducted within the research programme "Feed4Foodure": a public-private partnership between the Dutch Ministry of Economic Affairs and a consortium of various organizations within the animal feed industry and the animal production chain. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening our competitive position on the global market.

The research programme comprises three main research lines: socially responsible livestock farming; nutrition, gut health and immunity; and "more-with-less" by efficient nutrient use. The aim of this third research line, "More with Less", is to reduce the footprint of the Dutch livestock sector in the field of phosphate, nitrate, copper, zinc, ammonia and greenhouse gases. New nutritional models and measurement techniques will be developed to improve efficient use of nutrients in livestock farming.

The present literature study entitled "Phosphorus metabolism in dairy cattle: a literature study on recent developments and missing links" was written within research line "More with Less", theme 4: "Reduction of phosphorus losses". Main aim of this subproject is to gain insight in the dynamics and regulation of phosphorus absorption, mobilisation and utilization in farm animals to update the current phosphorus requirements for farm animals, thereby improving phosphorus efficiency.

For dairy cattle, much work has been done to understand phosphorus digestion, absorption and metabolism, but insight in some underlying factors, limits in P regulatory mechanisms, and consequences of nutritional history and P dynamics over time seems lacking. With this literature study we aim to further delineate the facts and figures on these aspects, and to indicate how their representation can be improved in extant models of phosphorus metabolism in dairy cattle.

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Summary

Phosphorus (P) is an important macromineral to all forms of life as we know. Our sources of P for feedstuffs and other use are not infinite, making P efficiency more and more important. On dairy farms, the efficiency of P utilization (P in animal product leaving the farm as % of total P inputs at farm gate) varies substantially. The goal of this literature study is to define the uncertainties related to P requirement models and ways to make them more precise and more reliable than current systems and to find biomarkers to monitor P balance in living animals.

Phosphorus balance in adult ruminants is mainly determined through regulation of apparent P digestion, P retention with milk and regulation of exchange of P between blood and bone. There are a number of endocrine factors involved in the regulation of P metabolism, many of which are interregulated with feedback loops.

In animal feed, P is present in inorganic P and organic P. In feedstuffs of plant origin, a high fraction of P is present in the form of phytate. Monogastrics are not able to digest phytate, but ruminants have an extensive microbial activity in the rumen with a large mass of microorganisms in the possession of enzymatic phytase to break down phytate, releasing P in a form that can be absorbed along the gastrointestinal tract. The source of P is therefore far less important in ruminants as compared to monogastrics.
In ruminants, P recycling through salivation is also an important factor determining P balance and excretion. Endogenous P excreted in saliva becomes available in the rumen for microbial utilisation and can be reabsorbed again along the gastrointestinal tract.

As efficiency of P utilization by dairy cattle is defined as the amount of P incorporated in milk and body growth relative to the amount of P ingested, any variation in milk P retention is a significant factor determining P efficiency of high-yielding dairy cows. Genetic or nutritional factors may influence the amount of P excreted in milk, but these factors have not been thoroughly investigated yet. In current P requirements systems for dairy cow nutrition, a fixed milk P concentration is adopted to calculate net P requirements. In reality, P concentration in milk may vary at least from 0.7 to 1.2 g P/kg milk between individual cows.

The skeleton represents another buffer of P balance. About 80-85% of total body P is present in bone in the form of hydroxyapatite within a collagen matrix. Bone P can thus have a profound impact on cow P balance, in the regulation of P concentration in blood, in P supply for milk P secretion and in P recycling for rumen P availability for rumen micro-organisms. This buffer is well-used in early lactation, to support milk production and milk P excretion. The calculated P balance is already negative at the end of the dry period, extending until the first weeks of lactation.

Plasma P concentration is still the most commonly used diagnostic measurement available to judge the P status of an animal, with all restrictions for good interpretation. Several biomarkers in plasma samples now become available to evaluate the amount of bone turnover, which may help in defining P balance and buffering in a larger time frame.

Further research into mechanisms and quantification of the following topics is needed to improve our understanding and modelling of P metabolism in dairy cattle:
- P metabolism in the transition period - challenge with minimal P allowance
- P metabolism in long-term challenges
- P metabolism in bone: how is P resorption from bone regulated (in the transition period)?
- Individual variation in milk P: how can this be explained and how is it affected by P balance
- Other biomarkers for P status: what can faecal P tell us?
- How is salivary P regulated?
- Does P balance during growth and development affect adult P metabolism?

1 Introduction

Phosphorus (P) is an important macromineral to all forms of life as we know. Combined with oxygen atoms forming phosphate (PO_4), the biological presence of P in organic (P_O) and inorganic (P_I) forms is ubiquitous. Phosphorus has a very broad functionality, being part of many essential molecules/structures ranging from nucleic acids (DNA, RNA), to cell membranes (phospholipids), to bone (calcium phosphate), as well as being an essential element in molecules that affect metabolic activity (protein phosphorylation affecting enzyme functionality and post-translational effects affecting gene expression) and are responsible for energy transmission (ATP).

1.1 Body distribution of P

Grünberg (2014) stated that about 80-85% of total P in the animal body is present as insoluble salts in the skeleton (mainly $Ca_3[PO_4]_2$ and $Ca_{10}[PO_4]_6[OH]_2$). The other 15-20% of total body P forms the more readily available P pool present in soft tissues and body fluids, which is partly P_I (HPO_4^{2-}, H_2PO4^-) and partly P_O (bound to carbon-containing components).
Most of the P available in the non-bone P pool is present intracellular (>99%), estimated to be approximately 100 mmol/L, of which only 1 mmol/L is P_I while the rest is incorporated in organic molecules. In the extracellular space, P is present both as P_I (free, bound to carrier proteins or complexed with cations) as well as P_O in lipoprotein molecules. Serum P_I concentration in dairy cattle is on average 1.5 mmol/L and is in close equilibrium with extracellular P status.

Input of P comes from feed (Figure 1.1) and absorption takes place in the gastrointestinal tract. P is excreted mainly by faeces and milk (when producing); urinary excretion is hardly relevant in ruminants.

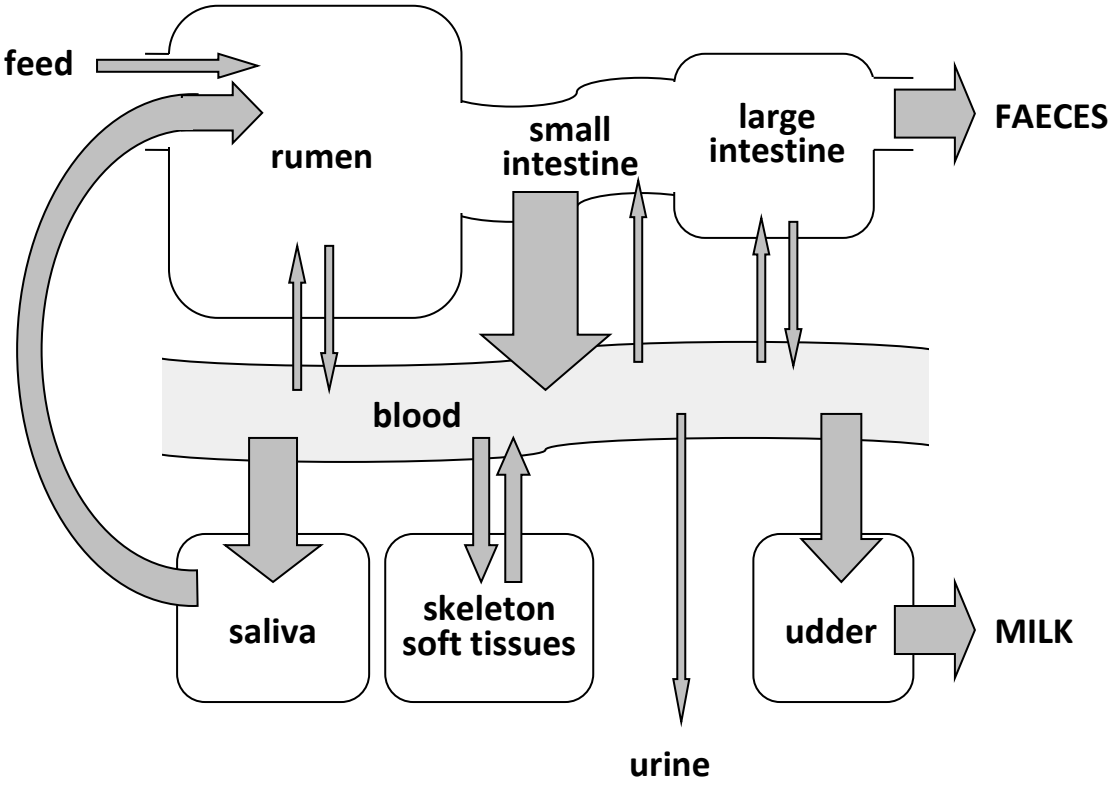

Figure 1.1 *Simplified scheme of phosphorus pools (boxes drawn) and transport routes (arrows) in the ruminant.*

1.2 Objectives

The efficiency of P utilization (P in animal product leaving the farm as % of total P inputs at farm gate) varies substantially between dairy farms; from 20 to 40% in the Netherlands (Schröder et al., 2005). Many farms should be able to reach an improvement in P utilization to decrease P losses in the dairy sector. P requirements have so far been consolidated in requirement models (e.g. COMV, 2005; NRC, 2001; AFRC 1991). These models still have room for improvement regarding some uncertainties and assumptions made to be able to calculate the net P requirement of dairy cows. Some reviews have been published and the reader is referred to these for more extensive background information on P metabolism and P balance in dairy cattle (Klop et al., 2013; Bannink et al., 2010; Van Straalen & Bruinenberg, 2007). In the Netherlands, subsequent to the extensive and long-term studies (two lactations) on the effect of reduced dietary P on P balance and performance of dairy cows by Valk et al. (1999a, 1999b, 2002), more short-term studies were done by Van Straalen et al. (2009b) on cow performance, and by Van Straalen et al. (2009a) on the in vitro and in situ degradability of dietary P and protein. The present review is not summarizing the measurements performed in these Dutch experimental studies on P metabolism in dairy cows.

The goal of this literature study is to define the uncertainties related to extant P requirement models and ways to improve these, to make them more precise and more reliable than current systems with built-in safety margins, to make them more dynamic by taking the nutritional and performance history into account, and to find biomarkers to monitor P balance in living animals.
Specific parts of the P balance have been elaborated separately (Chapter 2: extracellular and serum P; Chapter 3: milk; Chapter 4: saliva; Chapter 5: bone; Chapter 6: other tissues), followed by Chapter 7 evaluating P intake on P utilization and Chapter 8 describing possibilities to use biomarkers to determine the cow P status in practice. Chapter 9 will summarize the gaps in our current state of understanding of cow P metabolism and will review directions for future research.

2 Regulation of extracellular P

Phosphorus balance in (adult) ruminants is mainly determined through regulation of apparent P digestion, P retention with milk and regulation of exchange of P between blood and bone. For ruminants, regulation of P digestion is oriented at gastrointestinal absorption together with the secretion of P with saliva re-entering the gastrointestinal tract (rumen). Parathyroid hormone (PTH) and active vitamin D_3 (also known as calcitriol, 1,25-dihydroxyvitamin D or $1,25(OH)_2D$) are the main regulating hormones of gastrointestinal absorption of Ca and P. Their role in maintaining Ca homeostasis is very prominent. In contrast with monogastrics, studies in ruminants indicated that intestinal P absorption may be up-regulated in states of P deficiency independently of vitamin D3, suggesting the presence of an alternative regulatory circuit of intestinal P uptake (Pfeffer et al., 2005). This leads to the general notion that Ca and P absorption are regulated independently in ruminants (Pfeffer et al., 2005). Nevertheless, Horst (1986) indicated that low P concentrations in blood may stimulate the synthesis of vitamin D3, independently of Ca influences, and that vitamin D3 increase may promote a more efficient intestinal P absorption.
The P_I is transported across all types of cell membranes by sodium coupled facilitated transport. These NaP_I co-transporters also mediate P transport with absorption in the gastrointestinal tract and with P_I reabsorption in the proximal tubules of the kidneys.

2.1 Endocrine regulation

There are a number of endocrine factors involved in the regulation of P metabolism, many of which are interregulated with various feedback loops (Figure 2.1).

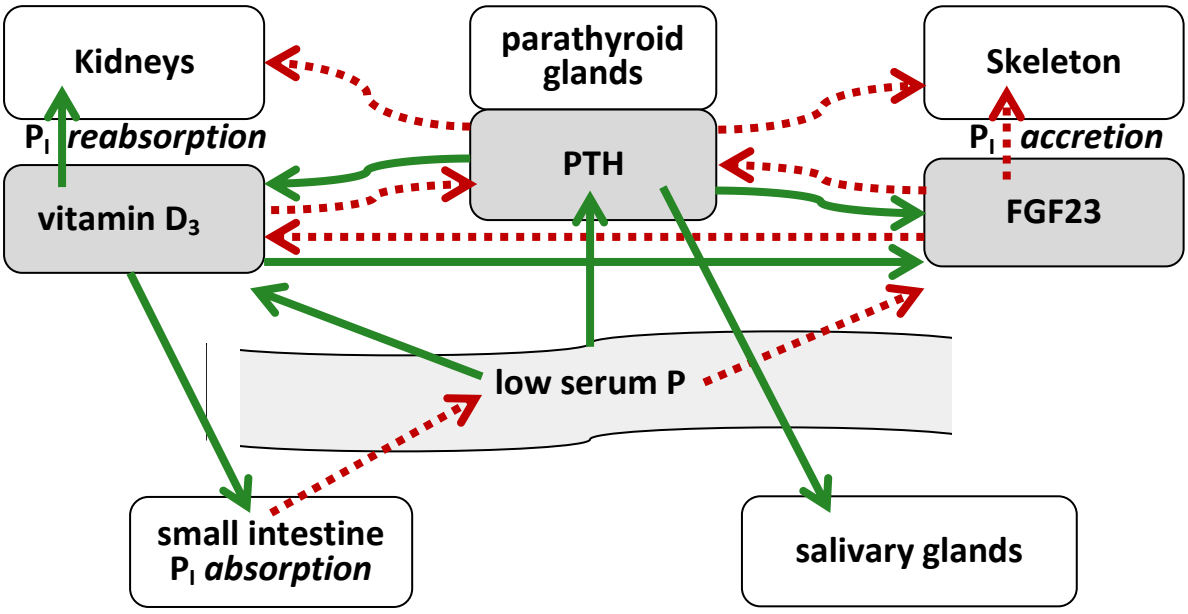

Figure 2.1 *Simplified scheme of regulation of phosphorus metabolism in case of decreased extracellular phosphorus. Positive actions (activation) are shown as green solid arrows; negative actions (inhibition) in red, dotted arrows.*
PI - inorganic phosphorus; PTH - parathyroid hormone; FGF23 - fibroblast growth factor 23

2.1.1 Vitamin D_3

In contrast with monogastrics, studies in ruminants indicated that intestinal P absorption may be up-regulated in states of P deficiency independently of vitamin D3, suggesting the presence of an alternative regulatory circuit of intestinal P uptake (Pfeffer et al., 2005). Also the stimulatory effect of low P on vitamin D3 is described (Horst, 1986; Puggaard, 2012) and P depletion was found to increase intestinal vitamin D3 receptor binding affinity in lactating goats (Breves and Schröder, 1991). Vitamin D_3 is produced by the kidney and the main regulator of active intestinal (Ca and) P absorption, stimulating P uptake in the jejunum by activating NaP_I transporters. It also increases renal reabsorption of P by increased NaP_I transporter activity in the kidney. Vitamin D_3 production is induced by hypophosphatemia, hypocalcemia and PTH; PTH secretion is further controlled by a negative feedback mechanism of vitamin D_3 reducing PTH production in the parathyroid glands. As UV light is needed in the final step to produced vitamin D_3, grazing or artificial UV light indoors stimulates vitamin D_3 concentration in plasma and milk (Jakobsen et al., 2015).

2.1.2 PTH

The parathyroid glands directly respond to changes in serum Ca by a Ca-sensing receptor, resulting in increased PTH production. It is not directly responsive to intravenous P infusion but indirectly responds to extracellular P concentrations by its effect on mRNA stability coding for PTH synthesis which destabilizes in cases of hypophosphatemia (Bergwitz and Jüppner, 2011). Once released in the circulation, PTH affects the renal excretion of P by reducing tubular reabsorption through internalization of the NaP_I transporters, thus increasing urinary P concentration (Berndt et al., 2005). Additionally, PTH stimulates bone turnover and Ca and P flux from the skeleton and increases vitamin D_3 production (Bergwitz and Jüppner, 2011). It also stimulates active P secretion in the salivary glands (Wright et al., 1984).

2.1.3 Calcitonin

Calcitonin is also produced in the parathyroid glands, and mainly involved in Ca balance. With the current state of knowledge its direct role in P homeostasis is moderate. However, calcitonin is found to increase and decrease with high and low extracellular P concentrations, respectively, leading to a lower and higher resorption of bone (Puggaard, 2012).

2.1.4 Phosphatonins

Parallel to calcitonin reducing serum Ca, a group of substances for P regulation has been named 'phosphatonins' based on their effect on decreasing serum P levels (Berndt et al., 2005). Fibroblast growth factor 23 (FGF-23) and secreted frizzled related protein 4 (sFRP-4) are the most relevant factors in this group, reducing P balance by increasing renal excretion and reducing vitamin D_3 synthesis while leaving Ca balance unaffected (Berndt et al., 2005).
FGF23 is produced mainly in osteoblasts and osteoclasts and the local activity of FGF23 was shown to be dependent of the expression of co-receptor α-klotho, reducing P reabsorption and vitamin D_3 production in the kidneys and PTH production in the parathyroid glands. FGF23 secretion was increased in human and mouse with increased dietary P intake, but not directly affected by plasma P_I concentration after intravenous injection; FGF23 production may therefore be related to other intermediates (Sapir-Koren and Livshits, 2014). FGF23 expression can be stimulated by vitamin D_3 as well as PTH activity (Tan et al., 2014; Sapir-Koren and Livshits, 2014).
Fibroblast growth factor 7 (FGF-7) and matrix extracellular phosphoglycoprotein (MEPE) also reduce reabsorption of P from urine, but their effect is compensated by increased vitamin D_3 activity (Berndt et al., 2005).

2.2 Gastrointestinal absorption

Most dietary P is absorbed in the small intestines (Breves and Schröder, 1991). Passive transport can occur when P concentration in the digesta is very high, especially in the first part of the small

intestine; at low P concentration, active transport takes place by NaP_I co-transporters, especially in the jejunum and ileum as shown by gene-expression of different intestinal sections in dairy cattle (Foote et al., 2011).
It is generally accepted that P absorption in mammals is enhanced through vitamin D_3 activity, stimulating P transporters. For ruminants however, not vitamin D_3 concentration increases during dietary P depletion but vitamin D_3 receptor affinity is increased (Breves et al., 1985; Schröder et al., 1995). Other (local) factors may also be relevant in increasing P absorption and should not be ruled out. For example, in mice on deprived diets and with a suppressed intestinal vitamin D receptor activity the absorption of P remained to be increased despite of absence of vitamin D_3 (Berndt et al., 2005).
In young, growing animals, intestinal absorption is higher than in older cattle. Serum levels of P_I are also higher in young animals (Bide and Tumbleson, 1976), presumably to provide sufficient P_I for the higher level of bone mineralization.
Another difference between ruminants and monogastrics regarding P absorption is that in ruminants, P and Ca absorption are not coupled (Breves et al., 1985). This means that, if P status of the dairy cows is low, P absorption can be increased independently of Ca status of the cow, and independently of the nutritional strategy to affect Ca metabolism. Nevertheless, with nutritional strategies to stimulate Ca resorption from bone, such as diets with a low cation-anion difference as tested by Block (1994), also bone P metabolism is affected and both Ca and P become available according to the Ca:P ratio in the bone resorbed. As bone has a higher Ca:P ratio than milk, Bannink et al. (2010) suggested that relatively more Ca than P becomes mobilized around or after calving, or with Ca-promoting nutritional strategies, than required for milk synthesis. Therefore bone resorption post calving possibly reduces extracellular P concentration instead of increasing it. The Ca from resorbed bone or of dietary origin does not affect P absorption, not even in high concentrations (Pfeffer et al., 2005). A topic requiring further investigation perhaps is which Ca:P ratio applies for the various sources of bone resorbed, and how regulatory mechanisms of P metabolism discriminate between the Ca and P reserves in different bones. Moreover, as Ca dynamics seem to rule the regulatory mechanisms of bone metabolism during early lactation, the dynamics of Ca metabolism and Ca generated from resorbed bone needs to be taken into account when evaluating the effect of P intake on dynamics of P metabolism and P status of dairy cows in lactation (Bannink et al., 2010). Current recommendation systems do not consider such a relationship between Ca and P metabolism however. Also, mechanistic dynamic models of P metabolism (Hill et al., 2008; Feng et al., 2015) do not contain any representation of Ca metabolism.

2.3 Renal reabsorption

In the kidney, virtually all plasma P_I passing the glomerulus is filtered into the tubular fluid (pre-urine). This is opposite to plasma Ca which is largely bound to plasma proteins and therefore becomes filtered only partly. Next, the tubular fluid passes the proximal tubules where a regulated reabsorption of P takes place by NaP_I transporters. In monogastrics and preruminating (young) ruminants, the regulation of renal absorption is an important factor for P homeostasis. In ruminants, tubular reabsorption is very active resulting in low urinary P losses (usually below 1% of dietary intake). Renal reabsorption can be decreased by factors such as metabolic acidosis (probably to provide buffer to the acidic urine) or PTH activation (Table 2.1), thereby leading to substantial urinary P excretion in ruminants as well. This means that nutritional strategies expected to lead to a metabolic acidosis (or a severe rumen acidosis causing a metabolic acidosis), may inflate renal P excretion. Also, PTH activation by a low Ca status may indirectly affect renal P excretion by reduced reabsorption. In cases of severe P depletion however, PTH action on the renal reabsorption may be overruled locally, thus reducing urinary excretion (Grünberg, 2014). However, under normal physiological conditions and under normal feeding conditions of dairy cows, reabsorption by the kidneys is fairly complete.

Table 2.1

Factors influencing phosphorus reabsorption in the kidneys (based on Berndt et al., 2005).

Factors decreasing P reabsorption	Factors increasing P reabsorption
Increased serum P	Decreased serum P
Increased PTH	Increased vitamin D_3
Increased volume	Decreased volume
Hypercalcemia	Hypocalcaemia
Metabolic acidosis	Growth hormone
Respiratory acidosis	Respiratory alkalosis
Increased dopamine	Increased serotonin
FGF-23, sFRP-4, MEPE, FGF-7	

2.4 Cellular transport

Although passive transport of P_I is possible across cell membranes, cells use active or facilitated transport to reach a sufficient and fast P_I supply to regulate intracellular P_I concentration. However , regulation of P exchange between intracellular and extracellular fluid is still poorly understood. Until today, no specific 'sensors' for extracellular P are known in mammalian cells regulating cellular P balance. Single cellular organisms such as bacteria and yeast are able to sense external phosphate concentration by use of a multi-protein complex in their plasma membrane, resulting in modulation of gene expression for P uptake and processing. However, comparable transmembrane sensors in mammals have not been found yet (Bergwitz and Jüppner, 2011), suggesting different regulation mechanisms of P. According to current knowledge, intracellular uptake of P_I is needed to sense P_I status and to regulate the stimulation of gene expression and protein functionality, as a high extracellular P_I concentration with blocked NaP_I transporters is not able to deliver a comparable effect on gene expression (Bergwitz and Jüppner, 2011).

Changes in the equilibrium between intracellular and extracellular P_I concentration can occur suddenly, affecting extracellular P_I concentration. These sudden changes in extracellular P concentration may be initiated by insulin and catecholamine secretion or a sudden change in acid-base status.
Insulin is an important factor stimulating a shift of P_I from the extracellular to the intracellular space. Plasma P concentration decreases rapidly after starting a dextrose infusion, due to changes in plasma glucose and insulin concentration (Grünberg et al. 2006). This increased intracellular P concentration may be relevant to support the increased carbohydrate metabolism (Grünberg, 2014).
A second factor stimulating P_I uptake by cells are catecholamines like adrenalin. In dairy cattle, catecholamines have also shown to increase PTH secretion, thereby increasing P excretion in saliva and urine (Blum et al., 1978).
It is likely that the labile P reserves (discussed later) will at least partly buffer these sudden changes in extracellular P status on the short term. Information on these dynamics seems useful to understand consequences of sudden changes in the equilibrium between intracellular and extracellular P_I concentration as this aids in understanding the risks involved for the dairy cow, and the effect on bone metabolism.

3 Regulation of P in milk

As efficiency of P utilization by dairy cattle is defined as the amount of P incorporated in milk and body growth relative to the amount of P ingested, any variation in milk P content is a significant factor determining P efficiency of high-yielding dairy cows. Genetic or nutritional factors may influence the amount of P excreted in milk, but these factors have not been thoroughly investigated yet.

In current P requirements systems for dairy cow nutrition, a fixed concentration of 0.9 (NRC, 2001; Valk and Beynen, 2003) or 1.0 g P/kg milk (COMV, 2005) is adopted to calculate net P requirements. In reality, P concentration in milk may vary at least from 0.7 to 1.2 g P/kg milk between individual cows (Pfeffer et al., 2005; Bannink et al., 2010; Klop et al., 2014). Part of this variation may be explained by protein content (Wu et al., 2001), and protein and lactose content (Klop et al., 2014; Shennan and Peaker, 2000). Perhaps also P availability and P balance affect milk P content, as data from Valk et al. (2002) suggest a numerically reduced P content per kg corrected milk (under assumption of identical protein, fat and lactose content; Valk et al. 1999b) at 67% compared to 100% of previous Dutch P requirements (previous to COMV, 2005). Although this reduction was numerically consistent through two subsequent lactations, the relevance of this observations needs to be confirmed the exact regulation mechanisms underlying these effects are still unclear.

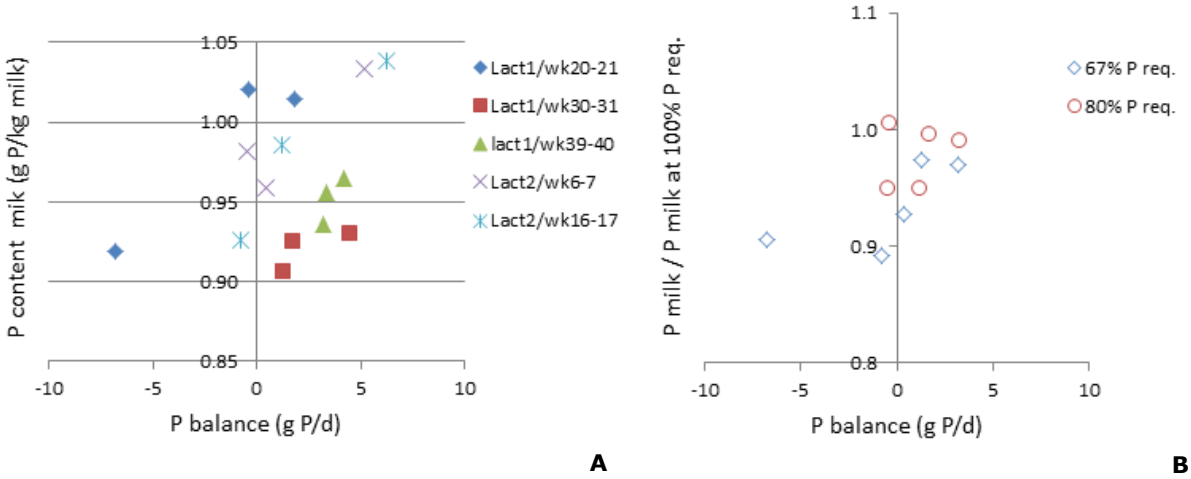

Figure 3.1 *Relationship between reported (Valk et al., 2002) phosphorus balance and phosphorus content in fat-corrected milk (A) for dietary treatments with either 67, 80 or 100% of phosphorus allowance according to previous Dutch requirements (previous to COMV, 2005), and (B) the same relationship with results for 67 or 80% expressed relative to those for 100% of previous Dutch requirements.*

3.1 Milk composition

In milk, about 70% of P is in inorganic and 30% in organic form. About 50% of P_I and 70% of calcium is located in the casein micelles, giving micelles structural stability and preventing precipitation of calcium phosphate (Holt, 2004). The ratio between casein and P is rather constant, providing a physiological base for a (partial) relationship between milk protein content and milk P content. Milk serum is supersaturated with calcium phosphate, indicating that increases in milk P_I content are mainly related to increases in casein content, increasing P_I secretion capacity (Bijl et al., 2013). The P_O in milk is for about two third present in the casein fraction and for one third in the lipid fraction (NRC, 2001).

In a recent study, milk production data of 121 cows have been combined to examine the relationship between milk composition (fat, protein, lactose) and P content (Klop et al., 2014). Secretion of P_I is related to synthesis of casein and lactose in the endoplasmic reticulum and Golgi complex. This physiological background of P_I and the pathway for milk casein and lactose synthesis was confirmed by the fact that good correlations could be established between milk protein, lactose and milk P content. Further research is needed to improve prediction of milk P from milk constituents as measured with regular milk control. This may be achieved by increasing the data set and perhaps by attempts to make a distinction between P_I and P_O and by identifying different fractions of P_I and P_O associated with the milk constituents protein (casein), lactose and fat (and other if required). Also other factors may be included in these studies to address the fraction of variation in milk P that remained unexplained. These can be cow factors such as parity or stage of lactation, but also genetic factors (van Hulzen et al., 2012) causing differences in protein/casein, lactose and fat synthesis, or even in general udder metabolism and regulation of milk synthesis by alveoli cells in the mammary gland. It is unknown how udder health affects milk P content and how this can be related to changes in milk constituents. Specific datasets would have to be generated to investigate the existence of any relationships.

4 Regulation of P in saliva

Rumination is the most effective stimulus of secretion of saliva in cattle. P_I concentrations in ruminal fluid are determined principally by the extent of salivary P secretion (Pfeffer et al., 2005). The salivary glands actively concentrate P_I obtained from blood into saliva to reach saliva P_I concentrations which are always far higher than plasma concentration (between 4-15 mmol/L in saliva compared to 1-3 mmol/L in blood plasma). This saliva P becomes available in the rumen for microbial utilisation and after outflow to the intestine can be reabsorbed again along the gastrointestinal tract, just as efficient as dietary P. This P recycling provides both a buffer to rumen contents to a minor extent (Counotte, 1981), and a highly available P source for rumen microflora growth and metabolism.

Concentration of P in saliva depends on plasma concentration and saliva production rate (Breves and Schröder, 1991; Valk et al., 2002). In current Dutch requirements (COMV, 2005), the amount of P needed for maintenance requirements is calculated by an average concentration of 8 mmol P/L saliva (Valk et al., 2002) and an average saliva production of 13 liter per kg of DM intake is assumed for lactating dairy cattle, and 15 liter per kg of DM intake for dry cows (Valk and Beynen, 2003). In the detailed model representation of P digestion published by Hill et al. (2008), saliva production is represented in a rather simple manner. A fixed amount of saliva is assumed of 239 L/d, a fixed rumen liquid passage of 198 L/d and a fixed fraction of blood P_I concentration (delivering a unit of mmol P/L blood x L saliva/d, which inherently assumes that P concentration in saliva is a constant, fixed fraction of P concentration in blood). It seems there is room for improvement to represent the amount of P recycled with saliva when both variation in saliva production and variation in the P concentration in saliva are taken into account. Current requirement calculation rules do not yet take full account of these sources of variation. Furthermore, specific dietary factors may have a modulating effect on these variable such as dietary fibre content, or regulatory mechanisms may modulate saliva P excretion if rumen P content is low or conversely blood P content is low.

Increased PTH levels stimulate P secretion in the salivary glands and a decrease in salivary bicarbonate, as the sum of $[HCO_3^-]$, $[Cl^-]$ and $[HPO_4^{2-}]$ remains fairly constant (and equal to the sum of $[Na^+]$ and $[K^+]$) in saliva (Wright et al., 1984). In cases of severe P depletion and low serum P levels however, PTH was somehow overruled and could not exert the same effect on salivary P secretion (Wright et al., 1984).

Valk et al. (2002) clearly demonstrated a relationship between blood and saliva P concentrations. However, much variation remains. This indicates that either the measurement method leads to much variation, or that other (regulatory) factors may be involved, making saliva P not directly proportional to blood P. In a study of Maekawa et al. (2002) on salivation, the level of roughage (40-60% of DM) did not seem to influence total daily saliva production in dairy cattle at equal DMI. Total saliva production was calculated based eating time, rumination time and resting time in 24h, multiplied by the respective rates of saliva production as measured by intraruminal collection of feed boluses and saliva. For higher levels of roughage, eating and rumination time were longer with higher rates of saliva production, but this was compensated by lower resting saliva production reaching equal total saliva production on a daily base (Maekawa et al., 2002).
Interesting factors to be investigated are the intake of cations and anions (salts) and their effect on fluid dynamics, the effect of specific dietary factors such as dietary NDF content or feed particle size on saliva production, or the existence of compensatory regulatory mechanisms for saliva production rate with low extracellular P concentration (and perhaps rumen P concentrations).

5 Regulation of P in bone

About 80-85% of total body P is present in bone in the form of hydroxyapatite within a collagen matrix. At normal P balance, there is virtually no net exchange of P between bone structure and blood however turnover of bone tissue remains under these conditions with extensive bidirectional exchange rates of P (Hill et al., 2008; France et al., 2010; review Bannink et al., 2010). Growing animals have a net accretion of P in bone, while in case of a negative P balance during early lactation in dairy cows bone can deliver an extra contribution of P of about 15-25% of daily P intake by resorption of P from bone reserves (Valk et al., 2002; Taylor et al., 2009). This indicates that bone P has a profound impact on cow P balance, in the regulation of P concentration in blood, in P supply for milk P secretion and in P recycling for rumen P availability for rumen micro-organisms. Bone balance is however difficult to measure separately in trials, and often calculations or extrapolations from other species have been used to estimate bone P balance (Klop et al., 2013).

5.1 Anatomical structure

Turnover may differ between bones in different anatomical locations, having different bone structure depending on their function. Weight bearing, long bones such as the bones of the limbs are much heavier and more resistant to breaking than for example the caudal (tail) vertebrae (Keene et al., 2005). Long bones contain more cortical (or 'compact') bone in the shaft than vertebral bones, which contain more trabecular (or 'cancellous') bone. Cortical bone gives hardness and structure to the bone, being much more rigid with a porosity of 5-30%. Trabecular bone is less hard but gives a much higher surface area per unit of volume due to a porosity of 30-90%, thereby playing a role in metabolic function and having a more extensive exchange with animal metabolism.
Bones with high fraction of trabecular over cortical bone are therefore most relevant in cow P metabolism, with porosity facilitating the exchange of P_I between bone and the rest of the body. Long bones only start to show loss of mineral bone at severe dietary P deficiency, while cancellous bones already have been much more affected as shown in sheep (Benzie et al., 1959) and sows (Giesemann et al., 1998). A similar process takes place in lactating cows which will be discussed further below.

5.2 Bone mineralization

Bone mineralization and resorption are mediated by two cell types: osteoblasts, promoting calcification and bone formation; and osteoclasts, demineralizing and absorbing bone. The mineralization by osteoblasts in the process of bone formation is regulated by a tight balance between activators and inhibitors. Osteoblasts are presumed to have a (still not fully understood) extracellular P_I sensing mechanism, as specific genes are more expressed when P_I influx is increased at high extracellular P_I levels; but when NaP_I transporters are blocked, the effect of extracellular P_I on osteocyte gene expression disappears (Bergwitz and Jüppner, 2011). In response to increasing extracellular P_I levels the expression of FGF23 and its co-receptor α-klotho is increased (Sapir-Koren and Livshits, 2014). Sclerostin is another osteocyte produced factor regulating bone mineralization by upregulation of FGF23 production. FGF23 inhibits mineralization by stimulating the production of inorganic pyrophoshate (PP_I). A low P_I/PP_I ratio (low P_I availability) inhibits hydroxyapatite formation, while a high ratio promotes mineralization.

5.3 Factors affecting bone metabolism

5.3.1 Lactation stage

The reduction of bone mineral content in early lactation has commonly been described as a natural process in many mammal species. In rats, bone strength decreases during lactation but is fully restored by eight weeks after weaning (Vajda et al., 2001). In sheep, bone mineralization decreases

during lactation while after mid lactation, mineralization increases again (Benzie et al., 1959). In primiparous and multiparous sows the same observations have been described, with decreasing bone weight and strength during lactation, increasing during subsequent gestation (Giesemann et al., 1998).
In dairy cattle, P balance is negative at the end of the dry period at about -4 to -14 g/d (Elizondo Salizar et al., 2013) and in the first weeks of lactation (Knowlton and Herbein, 2002), even at high dietary P diets. This is not only due to the loss of P with the onset of milk production, as the same drop also occurs in mastectomized cows around parturition, but may be related to the reduction in feed intake, an increase in corticosteroids and a redistribution of intracellular and extracellular P_I (Grünberg, 2014). The P content of colostrum is far higher than that of milk (Pfeffer et al., 2005) and this may add to the negative P balance immediately after calving.
In cows fed a diet with 0.36% P in DM during lactation, P balance is negative in early lactation (wk 1-5 after calving) but restores again in late lactation (Elizondo Salizar et al., 2013), whereas a diet with 0.43% P in DM resulted in a positive P balance in early lactation. Bone thickness and strength has shown to be higher in early lactating (0-90 DIM) and midlactating (150-250 DIM) cows, than in cows between 90 to 150 DIM or cows >250 DIM; in this study however, there was no correlation found between bone strength and bone mineral content (Keene et al., 2005). In a study with 16 cows followed during a full lactation, P content of cortical rib bone showed a tendency to increase during the first 60-90 days of lactation while Ca content decreased between 30 and 60 days postpartum; towards the end of lactation and during the dry period, P content increased, while in the last weeks before parturtion, P content was reduced again (Beighle, 1999). From this study of Beighle (1999) it was also concluded that P and Ca resorption involve separate regulation mechanisms, as changes in P and Ca content do not fully occur in parallel even though this might be expected base on the elemental composition of hydroxyapatite in bone. Therefore, a similar situation exists for bone compared to intestinal P absorption in that separate regulation mechanism are in place for Ca and P.

Both PTH and 1,25-dihydroxyvitamin D_3 are involved in the regulation of bone metabolism. PTH secretion is stimulated by hypocalcaemia. The onset of milk production around parturition involves a decrease of plasma Ca, thereby increasing PTH secretion. PTH will increase vitamin D activation in the kidneys producing calcitriol, and both PTH and vitamin D will stimulate bone resorption, releasing Ca and P.
In early lactation however, other factors may determine bone activation. In rat studies, PTH knock-out, vitamin D depleted, adrenectomized or ovariectomized animals showed an equal level of bone resorption during early lactation as others (Brommage and DeLuca, 1985). Prolactin is suggested to be the major activator of bone resorption in early lactation, while PTH and vitamin D further increase bone resorption when serum Ca levels decrease (Brommage and DeLuca, 1985). Also parathyroid hormone-related protein (PTHrP) may be involved according to human and rodent studies, as serum PTHrP concentration increases postpartum and spikes during suckling, just like prolactin and oxytocin (Kovacs, 2012). PTHrP acts through the same receptor as PTH and is undetectable in the circulation of non-pregnant, non-lactating adults, but acts as a paracrine or autocrine messenger. During pregnancy and lactation, PTHrP reaches detectable levels in the circulation and regulates mineral and bone homeostasis of mother and child (Kovacs, 2014). Also in dairy cattle, PTHrP has been detected in milk and lower concentrations in serum; approximately 2% of PTHrP produced in the mammary gland can be found in serum (Sato et al., 2014; Filipovic et al., 2008). PTHrP is produced in the mammary gland in response to local factors such as suckling, calcitonin, prolactin, calcium receptors expressed in the mammary epithelium, and the fluid content of milk (Kovacs, 2012), but the exact role of PTHrP in dairy cattle mineral metabolism during lactation is still unknown (Sato et al., 2014; Filipovic et al., 2008).
Oestradiol is known to decrease bone turnover, and in dairy cows, oestradiol levels sharply decrease after calving in an inverse relationship with bone resorption biomarker CTX (Filipovic et al., 2008).

At dry-off, the mammary gland involutes, PTHrP production decreases, and Ca and P excretion to milk stops, generally supporting mineral balance and accretion of bone minerals. Postweaning rats fully regain their bone mineralization within 10 to 21 days, also in case of simultaneous pregnancy; neither PTH, PTHrP, calcitonin or calcitriol are required to stimulate this bone formation (Kovacs, 2012).

5.3.2 Interaction bone and energy metabolism

Bone turnover and energy metabolism are closely interacting. Osteocalcin for example is present in an active and inactive form; the active form is involved in bone metabolism, while the inactive (carboxylated) form stimulates β-cell proliferation, insulin secretion and adiponectin secretion in murine studies. On the other hand, adiponectin secreted by adipose tissue promoted osteoblast proliferation, increasing bone mineral accretion. Insulin directly inhibits osteoblast activity, stimulating bone resorption, thereby increasing decarboxylation of osteocalcin to the active form. The same relationships may be valid in dairy cattle during the transition period, as postulated by Lean et al. (2014).
Insulin has also been shown to reduce extracellular P_I concentration due to active P uptake in insulin responsive cells, parallel with glucose uptake (Grünberg et al., 2006; Grünberg, 2014).

5.3.3 Pregnancy

During pregnancy, plasma calcitriol is increased in mammals, even without a dietary Ca deficiency or an increased level of PTH. Calcitonin levels also increases due to its production in the thyroid gland, mammary gland and placenta. PTHrP is produced in the developing mammary gland and placenta, stimulating bone accretion in the fetus (Kovacs, 2014). Renal activation of vitamin D is directly stimulated by certain pregnancy factors such as prolactin or placental lactogen. Prolactin and placental lactogen are also involved directly in increasing intestinal calcium absorption, without presence of calcitriol (Kovacs, 2012). All these factors activate bone metabolism and increase bone resorption, as shown by increased levels of bone resorption markers, especially increases during the third trimester which coincides with the largest mineral accretion of the growing foetus.

Total P requirements for pregnancy are considered virtually zero in the first two third of pregnancy (NRC, 2001). In the last 190 to 270 days, P accretion in the growing calf and uterine structures was investigated by House and Bell (1993) for a calf reaching a birth weight of approximately 46 kg at 280 days. If these results are extrapolated to a birth weight of 40 or 50 kg, net P requirements for pregnancy in the last weeks before calving may vary between 4.6 and 5.8 g/d; in case of a twin pregnancy with 2 calves of 40 kg each, requirements could reach 9 g/d (Figure 5.1).

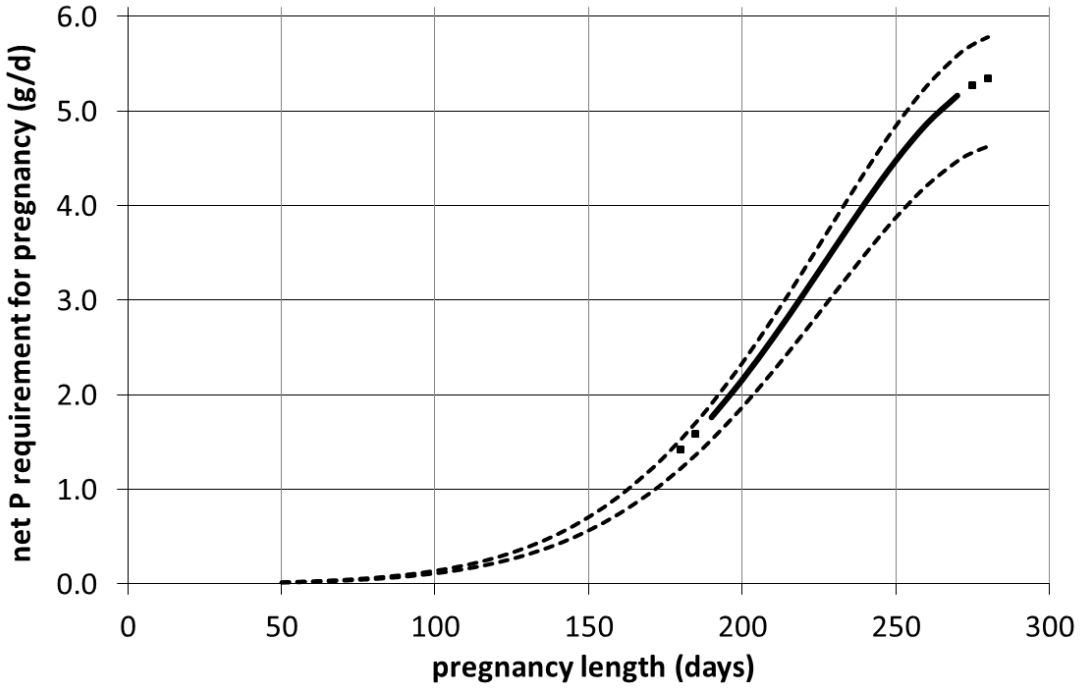

Figure 5.1 *Calculated net P-requirement for pregnancy (y-as) from 190 to 270 days after insemination for a calf weighing approximately 46 kg at 280 days (derived from House and Bell, 1993), extrapolated to a calf of 50 kg (upper broken line) or 40 kg (lower broken line) at 280 days.*

5.3.4 Parity

Bone strength of metacarpal bone or caudal vertebrae did not differ between parity 1 to 4 (Keene et al., 2005). Bone mineral content did not correlate with breaking strength either, but variability in mineral content of the samples used was quite low (Keene et al., 2005). In sows, bone weight and mineralization was lower for first parity relative to second parity or older sows (Giesemann et al., 2008). Fluctuations in bone strength during lactation were also higher in primiparous sows, which may be caused both by a lower Ca and P intake as by an increased bone metabolism (Giesemann et al., 2008).
In dairy cattle, older cows are more prone to hypocalcaemia and hypophosphatemia than heifers (Shappell et al., 1987). Heifers produce less milk and have therefore lower Ca and P outputs; but also have a more active bone metabolism, giving easier access to bone reserves, during their growing period as shown for example by higher blood concentrations of osteocalcin (Sato et al., 2011).

5.3.5 Dietary factors

An acidogenic diet facilitates the demineralization of bone, releasing Ca and P_I. A state of metabolic acidosis caused by rumen acidosis may do the same.

Dietary P concentration below requirements (2.3 g P/kg DM during lactation) decreased feed intake, milk yield and plasma P_I concentration, and increased the bone marker for resorption CTX (Puggaard et al., 2014). Low dietary P intake may thus influence bone mineralization and even bone strength. At dietary P levels of 0.31% relative to 0.39% or 0.47%, total ash and P content tended to be lower in rib bone sections of dairy cattle; but bone strength was not significantly affected (Wu et al., 2001). Studies that do find a reduced bone strength are associated with severe mineral deficiencies but do not show a linear effect of P supplementation on bone strength (Keene et al., 2005).

Low levels of dietary Ca in early lactation and the incidence of milk fever (hypocalcaemia) promotes hypophosphatemia through PTH release. At low serum Ca, PTH is secreted to mobilise Ca from bone. Another effect of PTH is increasing P excretion in saliva and urine, thereby increasing P losses and decreasing plasma P_I. Good feed intake and prevention of hypocalcaemia are therefore vital to prevent losses of P (Goff, 2006).

6 Regulation of P in other tissues

Evidence on the impact of P allowance on cow health and performance is scarce as not many studies have been conducted recently with really challenging dietary P contents. Indications from the studies of Valk et al. (2002), and recently Puggaard et al. (2014), with dietary P content of 2.3 to 2.4 g P/kg DM fed to lactating cows indicate that detrimental effects on cow health probably should not be attributed to an impaired diet digestibility. Both Valk et al. (2002) and Puggaard (2012) found no effect on DM digestibility. Nevertheless, Puggaard et al (2014) did demonstrate a numerical drop in NDF digestibility of 4% units when reducing dietary P content from 2.5 to 1.7 g P/kg DM during the dry period (P=0.14), in contrast to the lack of a drop in NDF digestibility when reducing dietary P from 3.4 to 2.3 g P/kg DM during lactation (P=0.61). This might indicate that P was limiting for rumen digestion with 1.7 g P/kg DM. With the onset of lactation and 2.3 g P/kg DM this apparent limitation of rumen digestion did not occur anymore. Nevertheless, DM intake and milk yield with the lowest P level of 2.3 g P/kg DM during lactation were strongly reduced. Because of the number of health issues with the cows this P regime could not be continued after 12 weeks of lactation and dietary P concentration was increased again. These results indicate that metabolic problems and clinical effects do occur if dietary P content becomes as low as 2.3 g P/kg DM, even though Valk et al. (2002) only had health incidences with some cows after feeding 2.4 g P/kg DM for a prolonged period (during the second lactation). In a study with lactating cattle (30 kg milk/d) at restricted DMI (20 kg DM/d) with either 2.4 or 3.4 g P/kg DM fed during 14 days, Puggaard et al. (2011) found a reduction in rumen fluid P_I and a reduction in NDF digestibility (52.6% vs. 47.8%) at the lowest dietary P concentration. Current Dutch P recommendation for a milk yield of 20 kg/d or more is a far higher value however of 2.9 g P/kg DM (COMV, 2005). Such low P values can only be achieved when feeding lactating cows predominantly maize silage as a roughage supplemented only with low-P by-products and concentrates. For comparison, Puggaard et al. (2014) fed 1.7 g P/kg DM with the dry period ration by combining low-P concentrate (2.6 g P/kg DM), barley straw (0.4 g P/kg DM), maize silage (1.9 g P/kg DM), grass clover silage (3.1 g P/kg DM) and beet molasses (0.4 g P/kg DM) at 33, 29, 20, 12 and 6% of dietary DM, resp., and they fed 2.3 g P/kg DM with the lactation diet by combining at 46, 0, 29, 16 and 9% of dietary DM, resp. (i.e. maize silage : grass silage at roughly 2 : 1).

6.1 Immune system

In human and rat studies, hypophosphatemia has been shown to reduce immune cell activity. In dairy cattle, one study is known in which lymphocyte and granulocyte function was tested at extremely low P intake (0.2%). Granulocyte counts were reduced but lymphocyte activity was independent of the low P status (Eisenberg et al., 2014).

6.2 Liver

In the first weeks of lactation, liver cytosolic P_I concentration was shown to be reduced (Grünberg et al., 2009). Clinical or metabolic relevance of low liver P_I remains to be elucidated. Extracellular P is predominantly regulated by P absorption in the gastrointestinal tract, P mobilized next to Ca with bone resorption during early lactation, and extensive P secretion with saliva production, and a main regulatory role for the liver seems unlikely. It is unknown what minimum P status is required for optimal functioning of the liver, and whether other metabolic functions are more sensitive to extracellular P depletion than the liver or not.

6.3 Labile P reserves

Model simulations for P metabolism in various ruminants and derived with various model schemes (overview given by France et al. 2010) indicates that with low dietary P content there may a substantial exchange of P between blood and soft tissues. This rather labile P is an important reserve

which can be quickly depleted and replenished with sudden variations in blood P concentration. Understanding these dynamics is important when aiming at a quantification of blood P variation (on the short-term) with sudden dietary P depletion, drop in DM intake or increase in milk yield for example. This flow may be larger than that of P recycling with saliva, which emphasizes the importance to take into account the contribution of soft tissue P, or the labile P reserves, when studying the dynamics of P metabolism and P balance in dairy cows. Exchange of the more labile P reserves may therefore affect the time course of the cow's response in resorbing P from bone under conditions of dietary P depletion.

The role of this labile P pool seems to be quite important as it may act as a buffer against sudden fluctuations in extracellular P. Only with prolonged depletion, and a low reserve of labile P, a depletion of P would lead to metabolic problems because of the fundamental role of P in various biochemical processes and energy metabolism.

The authors are unaware however of measurements in lactating cows of the dynamics of P exchange between the labile P reserves and blood P, in combination of the dynamics of replenishment of blood P by P originating from resorbed bone. Risk of hypophosphatemia would be expected in cases where P depletion is sudden, severe and prolonged (for example by too low dietary P content during lactation); the P depletion is larger in size than can be replenishment by labile P reserves in combination with increased P absorption from the gastrointestinal tract; and the P depletion is faster and stronger than what can be met by P mobilisation from bone.

7 Effect of P intake on utilization

7.1 Type of P

In animal feed, P is present as P_I and P_O. In feed stuffs of plant origin, a high fraction of P is present in the form of phytate. Monogastrics are not able to digest phytate, but ruminants have an extensive microbial activity in the rumen with a large mass of microorganisms in the possession of enzymatic phytase to break down phytate, releasing P in a form that can be absorbed along the gastrointestinal tract.

Comparing different concentrates with P_O vs. P_I sources, the P source did not seem to influence the total level of absorption in ruminants (Ekelund et al., 2003; Knowlton et al., 2001). This research has however been conducted at a positive P balance and results may be different in a situation with a negative P balance. If dietary P concentration is below requirements, the P source may become relevant. If for example the ratio of undegradable to degradable protein is high in a certain diet, while phytate is the main P source, phytate may be present in rumen undegradable ingredients and thus escape rumen fermentation which is needed to release P (Bravo et al., 2002). Decreasing rumen degradability of rice hulls by formaldehyde treatment, increasing rumen undegradable dry matter from 37% to 60%, increased the level of rumen undegradable phytate from 8% to 32% (Martín-Tereso et al., 2009). Rumen by-pass phytate may be degraded in the large intestine due to microbial activity. The release of P here is only relevant if P can be absorbed from the large intestine. Even though the extent was highly variable, disappearance of P from the large intestine has been reported (Scharrer, 1985; Breves and Schroder, 1991).
Phytase supplementation may increase P digestibility in specific cases as demonstrated by Kincaid et al. (2005); in this study however, high concentrate TMR diets resulted in high feed intake levels (4% of body weight) accompanied by rapid rumen turnover. Moreover, dietary P levels were high (4.6 to 5.5 g P/kg DM) as well as the amount of phytate P within total dietary P (33% to 52%). Other studies evaluated the effect of exogenous phytase with variable success. Jarrett et al. (2014) evaluated the effect of phytase and forage particle length in a 2x2 study at 4.3 g P/kg DM; reduced particle length increased digesta passage rate and thereby faecal P excretion, but exogenous phytase did not affect P digestibility. Brask-Pedersen et al. (2013) observed an increase in rumen phytate degradability with phytase addition (from 86.4% to 96.3%) and also in total tract phytate degradability (from 90.1 to 94.2%), but total faecal P flow actually increased with phytase addition. Winter et al. (2015) did not find an effect of exogenous phytase on P digestibility at low or high dietary P input (1.9 vs. 4.9 g P / kg DM). Ray et al. (2013) demonstrated that P digestibility was unaffected by the form of P in the diet, and hence it can be concluded that the proportion of dietary phytate-P in total dietary P or phytase activity is not much importance for P digestibility. In contrast, total dietary P content is the main determining factor for P digestibility.
Puggaard et al. (2013) suggested that microbial P is relatively low digestible (43%) and performed a trial to influence microbial P incorporation. A reduction in P supply through saliva or a reduction in rumen degradable protein would decrease rumen microbial growth and P incorporation, thereby decreasing microbial P losses in faeces. This concept could however not be proven; faecal excretion was not reduced, but other factors may have interfered such as a difference in total DMI (Puggaard, et al. 2013).

In a recent model simulation study by Dijkstra et al. (2014), based on a dynamic model adapted from that published by Hill et al. (2008), dietary P content had most impact on P digestion, whereas phytate fraction in total dietary P, NDF content, fermentable OM content, and roughage proportion of the diet hardly affected P digestibility. Next most important factor affecting P digestibility was milk composition (a 0.2% and 0.1% change of milk protein and lactose content, respectively, was tested). These simulation results indicate that milk production, milk composition and dietary P content have a large impact on P digestibility, whereas diet composition and type of dietary P do not. This finding is relevant with respect to the general emphasis put on digestive factors affecting P digestibility (Hill et al., 2008; Van Straalen & Bruinenberg, 2007; Feng et al., 2015). For delineating the consequences of dietary measures on P metabolism in dairy cattle, more emphasis is warranted on quantifying

regulatory mechanisms involved in recycling of P with saliva secretion, P metabolism in bone and the dynamics of exchange with the labile P reserves affecting the long-term dynamics of P metabolism in dairy cows.

7.2 Rumen flora

Rumen concentration of available P must be sufficient to rumen flora requirements to prevent any limitations on rumen fermentation. In vitro rumen incubations have shown that fermentation rate is reduced at concentrations below 0.5 mmol P/L (Komisarczuk-Bony and Durand, 1991). This suggests that in vivo rumen P concentration will rarely become limiting microbial fermentation, even at low dietary P concentration. Bannink et al. (2010) supported this with three arguments. First of all, saliva contribution to rumen fluid is about three times the volume of drinking water; rumen P concentration will therefore rather closely match that of saliva. Saliva P content depends on plasma P concentration but stays at relatively high levels at low dietary intake (Puggaard, 2012); at a low dietary P level of 2.4 g/kg DM, saliva P concentration does not drop below 5 mmol/L (Valk et al. 2002). Secondly, microbial growth requires a maximum of 70 g P/d, which will be easily met with feed intake and saliva production. Finally, a dietary P level below 2.4 g P/kg DM is very hard to achieve with common roughage-based diets in Netherlands, unless the diet consists of maize silage mainly (on average 2.0 g P/kg DM). In practical situations with sufficient rumination activity a P deficiency at rumen level is hence very unlikely (Meschy and Ramirez-Perz, 2005). Van Straalen and Bruinenberg (2007) indicate that a minimal rumen P concentration of 2-3 mmol P/L is necessary for optimal microbial activity and cellulolytic activity, and base their presumption on in vitro studies from Durand & Kawashima (1980) and Komisarczuk et al. (1987). In contrast, Van Straalen et al. (2009a) adopted a rather high P requirement for optimal rumen cellulolytic activity of 7.7 g/kg fermentable organic matter. With 15 kg/d of fermentable organic matter this would be 3.7 mol P/d, which also seems easily to be met by a saliva production with 5 mmol P/L saliva and a lower estimate of production rate of 150 L/d (i.e. 8.3 mol P/d). It seems likely that rumen P concentration levels of 2-3 mmol P/L can easily be met by saliva production which seldom contains less than 5 mmol/L. On a diet containing only 2.4 g P/kg DM (about 25% below current P recommendations), rumen P concentration remains around 3 mmol/L, which appears to be sufficient still for optimal microbial activity.

7.3 Other dietary factors

High dietary Fe content, especially ferrous Fe as present in drinking water, may form insoluble complexes with PO_4 which in turn could reduce P absorption. In cows however, this negative effect on P digestibility could not be confirmed with ferrous lactate infused abomasally up to 1,250 mg of Fe/d. (Feng et al., 2013). This might be caused by the acidic rumen environment, and the large fluid volume leading to lower concentrations, and hence solubilisation of otherwise most insoluble ferrophosphate complexes. This would require further investigation and it is not known to what extent complex formation plays an important role in ruminant nutrition as in nutrition of monogastrics.
Other dietary factors (P_I fraction in total dietary P, NDF content, fermentable OM content, roughage proportion of the diet) were considered in the simulation study performed recently by Dijkstra et al. (2014) but appeared to be relative unimportant in explaining variation in P digestibility and P excretion.

8 Potential biomarkers

Biomarkers for P balance are not easily obtained, and a golden standard to obtain P balance is still not present for *in vivo* experiments.

8.1 Blood

Serum P_I concentration in dairy cattle is on average 1.5 mmol/L ranging from 1.4 to 2.6 mmol/L in adult cattle and 1.9 to 2.6 mmol/L in growing cattle. The location of blood sampling and the type of coagulant used can affect the concentration. In general, blood samples from the jugular vein have 4-19% lower P_I concentration than blood samples from mammary vein or tail vein; this is suggested to be due to the extraction of P_I in the salivary glands; use of NaF as a coagulant may reduce PI by 10% relative to the use of heparin plasma or serum tubes (Grünberg, 2014). Growing animals have generally higher serum P_I, presumably to provide for the higher level of bone mineralization.

Hypophosphatemia (blood P_I below reference values) has been regularly found around parturition and is sometimes suggested to be a contributing factor in the pathophysiology of periparturient paresis or downer cow. Induction of hypophosphatemia with normal Ca levels however does not result in signs of milk fever. Treatment of hypocalcaemia with intravenous Ca generally improves blood P_I levels due to the negative feedback on PTH and the resumption of normal feed intake.

Serum P_I gives an indication of the non-bone, *extracellular* P status. However, most non-bone P is present intracellular (about 99%); blood P concentration is therefore not representative of total P status of a cow (Grünberg, 2014). Moreover, changes in the balance between intracellular and extracellular [P_I] can occur suddenly, thus affecting extracellular [P_I] and blood P as described in paragraph 2.4, further complicating the reliability of an instantaneous blood test for P status. During a period of depletion with a diet containing 1.8 g P per kg DM, plasma PI may first decrease and then increase again after ±9 days, without clinical signs of a P deficiency; electromyography may however reveal (subclinical) muscle malfunctioning (Grünberg et al., 2015b).
Preanalytical factors also affect the result: the *type* of blood sample (plasma vs. serum), the sampling *location* (jugular vs. coccygeal vene) and most important the *timing* of sampling (after a meal or after fasting) influence the P_I concentration measured (Montiel et al., 2007).

Bone P balance is also not sufficiently monitored by analysis of serum P_I. In severely restricted ewes (fed at 1.5 g P per day), a low serum P_I concentration was detected together with bone resorption (Benzie et al., 1959). However, when additional P was fed (towards 4.5 g of P per day), blood P_I values increased within 4 weeks while bone mineralization was still inadequate (Benzie et al., 1959). Given that mobilization of P reserves from bone compensates for long-standing inadequate P supply, blood plasma P_I poorly reflects states and degree of chronic P deficiency (Grünberg, 2014).

The P_I in red blood cells may be used to estimate the intracellular P_I concentration. It is often suggested that a situation of severe P deficiency may induce haemolysis as erythrocytes have insufficient ATP to maintain membrane stability. At this stage it is likely that other cell types are also dysfunctional due to low ATP availability. It is however unclear to what extent red blood cells represent the average intracellular P_I concentration in the body and whether such a severe case of P deficiency serves monitoring of interest at more moderate P deficiency levels. Other factors than P status must however be involved, as an induced P depletion alone did not result in haemolysis or a change in osmotic resistance or P_I content of erythrocytes (Grünberg et al., 2015a).

As an alternative to monitoring P, there might also be scope to follow Ca levels in blood in conjunction with P, as bone Ca is coming available together with bone P when there is a net resorption of bone. The mobilisation of Ca might therefore be used as a proxy of extent of P mobilisation based on known bone Ca : P ratio. Ca regulation is however more dependent on bone turnover than P regulation: 98-99% of total body Ca is present in bone, compared to only 80-85% of total body P.

It requires further investigation however to what extent these ratios are constant during various stages of bone resorption and during different statuses of P in dairy cows, and how homeostatic regulatory mechanisms may interfere.

Plasma P_I is still the most commonly used diagnostic measurement available, with all restrictions for good interpretation. In cases of low plasma P_I, P supplementation may be given although the effect and relevance is still unclear. Intravenous therapy is indicated in cases of extreme hypophosphatemia leading to intravascular haemolysis; an inorganic PO_4 source (7-12 g P_I per adult cow) is indicated because organic sources or other P substances first need to be metabolized to PO_4 to be effective (Grünberg, 2014). In all other cases where P supplementation is indicated, oral or parenteral dosage of PO_4 (40-60 g of P_I per adult cow) is more effective on the long term (Grünberg, 2014).

Finally, blood may also contain biomarkers for the P status of specific organs, which are described below in separate paragraphs.

8.2 Milk

As discussed in Chapter 3, about 70% of P in milk is in inorganic and 30% in organic form, and about 50% of P_I is located in the casein micelles, giving micelles structural stability and preventing precipitation of calcium phosphate (Holt, 2004). Total milk P concentration may vary at least from 0.7 to 1.2 g P/kg milk between individual cows (Pfeffer et al., 2005; Bannink et al., 2010; Klop et al., 2014).
The ratio between casein and P is rather constant, providing a (partial) relationship between milk protein content and milk P content (Wu et al., 2001), but also lactose content is correlated to milk P (Klop et al., 2014). Not all variation in milk P can be explained by protein and lactose and therefore other factors must be involved as well. These can be cow factors such as parity or stage of lactation, but also genetic factors (van Hulzen et al., 2012) causing differences in protein/casein, lactose and fat synthesis, or even in general udder metabolism and regulation of milk synthesis by alveoli cells in the mammary gland.
Dietary intake of P hardly affects total milk P concentration. In a study of Peterson et al. (2005) a negative relationship was found between dry cow dietary P supply (2.1, 3.1 or 4.1 g P / kg DM) and postpartum milk P concentration during the first four weeks after calving. Other studies however did not find any effect of dietary P and milk P content (Wu et al., 2001; Winter et al. 2015). In extreme situations, feeding P depleting diets at 67% of the requirements for two lactations, a reduction of milk P content may be found (Valk et al. 2002) together with other signs of P deficiency (reduced DMI, reduced milk yield).
Other indicators for P balance in milk have not been developed so far. Individual monitoring of milk P in time may help to find irregularities relative to the individual "normal" average P concentration. The remaining variation after correcting for protein (casein) and lactose concentration may be specifically relevant to monitor. Further determination of the location of P in milk (in milk serum, in casein micelles, phosphorylated structures, etc.) may help to investigate changes depending on parity, lactation stage, P intake or udder health.
Fat-soluble vitamin D_3 is excreted to milk, but concentrations vary irrespective of P balance, for example due to season (grazing) or exposure to UV light (Jakobsen et al., 2015). PTH-related peptide (PTHrP) can also be detected in milk. It is produced in the mammary gland in response to local factors such as suckling, calcitonin, prolactin, calcium and phosphorus receptors (Kovacs, 2014) and approximately 2% of PTHrP produced in the mammary gland can be found in serum (Sato et al., 2014; Filipovic et al., 2008). The exact role of PTHrP in dairy cattle mineral metabolism during lactation is still unknown (Sato et al., 2014; Filipovic et al., 2008).

8.3 Bone

Representative bone biopsies are difficult to take on a regular base in the living animal in practice, but may give an indication of long term P balance. In contrast to the very high turnover rates of bone P reported in literature (up to 10% of bone P turned over daily; studies reviewed by France et al. 2010), bone seems to respond only slowly to actual P balance by either increasing demineralization (osteoclast activity) or calcification (osteoblast activity) which may vary over time depending on the P requirements. It is therefore not possible to use P content in an instantaneous sample of bone as a marker for the actual changes occurring in the body P pool, but more as the long-term cumulative effect of factors affecting P balance and thereby bone P content.

Several biomarkers in plasma samples have been used to evaluate the amount of bone turnover. In humans, the most commonly used marker of bone resorption is CTX (carboxyterminal cross-linked telopeptide of type I collagen), a degradation product of type I collagen. For bone formation, the osteoblast enzyme BSALP (bone specific alkaline phosphatase) is commonly used as marker or osteocalcin as an osteoblast-specific protein which is not essential for bone formation but reflects osteoblast activity (Sato et al., 2011).
During bone resorption also bone collagen is degraded. The carboxyterminal telopeptide of type I collagen can be analysed in serum as a marker for bone resorption (Liesegang et al., 1998). Pyridinoline and deoxypyridinoline are two collagen breakdown products that can be found in urine, deoxypyridinoline (DPD) being the most specific for bone collagen breakdown. The ratio of DPD to creatinine concentration in urine may help to determine the level of bone degradation in dairy cows (Liesegang et al., 1998) already after 4 days feeding a 0.18% P (depletion) diet (Grünberg et al., 2015b).

It is not known whether such biomarkers give any relevant information on P status or P balance of a dairy cow. This would have to be investigated specifically in combination with accurate determination of P balance and controlled P intake.

8.4 Saliva and rumen fluid

The P_I concentration in saliva is mainly dependent on the P_I concentration in plasma and total saliva production, but is never reported to be lower than approximately 5 mmol/L, even at a low dietary P level of 2.4 g/kg DM (Valk et al. 2002). Taking a representative sample from the salivary glands may be quite difficult in practice. Not only should contamination with feed particles be prevented, but there is also a difference in saliva and P_I production between different salivary glands, thereby likely inducing variation over time (Grünberg, 2014).
The P_I content of rumen fluid depends on dietary P intake, saliva production and blood P concentration. Decreasing the level of dietary P showed to stimulate salivary P_I excretion, preventing a linear reduction of rumen fluid P_I (Valk et al., 2002). Under practical circumstances, rumen fluid P_I concentration therefore does not seem to give any additional information on cow P status.

8.5 Urine and faeces

The P_I concentration in urine is very low in adult ruminants and is not representative of P status; it can be increased by other factors such as aciduria (increased excretion to provide additional phosphate buffer to the urine) or hypocalcemia (increased excretion through the increase in PTH in response to low serum Ca) (Grünberg, 2014), but these must be considered as rather abnormal conditions.
As mentioned in paragraph 8.2, analysis of urinary deoxypyridinoline may be useful to determine the level of bone resorption (Liesegang et al., 1998) as an indicator of Ca and P balance.

Under normal feeding conditions faecal P is the most important route for P leaving the cow's body next to milk synthesis. In contrast to incidental sampling of blood P with substantial variation obscuring the significance of such observations, defeacation is a rather constant and continuous output. Moreover it delivers the more interesting unit of P to obtain an indication of cow P balance, presuming the amount of faeces produced can be estimated accurately (kg faeces/d x g P/kg faeces = g P/d). A blood sample delivers a unit (mmol P/l) which cannot easily be translated into a P balance, and furthermore blood P is highly regulated and kept within narrow limits which leaves it useful as an indicator for a P balance.

With variation in dietary P content, there is variation not only in the amount of P excreted (and hence in faecal P content) but also in the chemical form in which P is excreted (P_I vs. P_O). Dou et al. (2002) investigated the effect of P allowance on faecal P excretion, and clearly established that mainly P_I, or the water-soluble fraction of P excreted, increases with increasing P allowance. They argued that about 2 g P/kg faecal DM would indicate adequate P status of the cow, with higher values indicating excess P feeding. Although faecal P excretion was considered an indicator of cow P status which has high potential, Dou et al. (2002) also concluded that more work needs to be done to investigate reliability and accuracy of this method. Nordqvist et al. (2014) analysed faecal samples from cows in different stages of lactation (mid-lactation to end-lactation, to prevent interfering effects of bone mobilization in early lactation) to analyse the P balance of a dairy farm. They concluded that average soluble P as well as total P in pooled faecal samples of lactating cows were good indicators to estimate the level of P feeding (Nordqvist et al., 2014). The minimum dietary P levels investigated in these studies were not beneath 3 g P/kg DM which is around the current P recommendation for lactating cows in The Netherlands. It would be more interesting to investigate the potential of faecal P as an indicator of cow P status of more challenging levels of dietary P (between 2 and 3 g P/kg DM). The authors are unware of such research however in combination with a detailed characterisation of faecal P_I and P_O fractions. Also Valk et al. (2002) and more recently Van Straalen et al. (2009b) did not perform such measurements.
Although there is a good correlation between P intake with feed and total P excreted with faeces, it is noted that this only holds for the full range of dietary P contents.

8.6 Tissue biopsies

By taking biopsies of tissue (e.g. liver, muscle), intracellular P status may be assessed. In cases of depletion however, mobilization from bone may compensate resulting in a relatively constant tissue P concentration. An organ can apparently be affected by a P deficiency while tissue concentrations of P are within reference range. During a short period of P depletion with a diet containing 1.8 g P per kg DM for example, muscle P content was constant and within reference range, while muscle function was already subclinically hampered when judged by electromyography (Grünberg et al., 2015b).

9 Future research

In relation to P metabolism in dairy cows the following topics require further experimental and modelling work. Also an indication is given of the type of experimental work that is needed, i.e. what experimental set-up is required to investigate the research questions formulated. And an indication is given of what type of model is needed to come to a representation of the mechanisms involved in the dynamics and regulation of P metabolism and P balance in dairy cows.
The topics are prioritized based on required costs, and on the contribution to achieving a more efficient P utilization by dairy cattle or a more accurate estimation of P requirements of dairy cattle.

9.1 Experimental research

Challenge with minimal P allowance (2.0 - 2.5 g P/kg DM) in the transition period
Experiments need to be conducted in which dairy cows are challenged in the short-term. These experiments should focus on challenging dairy cows with minimal P allowance just below the calculated net requirements for maintenance, pregnancy and milk production according to the CVB (COMV, 2005), and test for the effect on cow P metabolism, including feed intake, digestive functions (e.g. rumen fermentation), energy metabolism and productive functions.
Short term effects need to be separated from long-term effects, and focus should be primarily on the transition period (late dry period until mid-lactation). Cause and effect need to be delineated with the potential reduction in feed intake at low dietary P (2.3 g/kg DM) despite maintaining equal NDF digestibility, as demonstrated in such a study by Puggaard et al. (2012), and this may need to be related to the situation at calving. The practical relevance is high.

Long-term challenge with minimal P allowance (2.0 - 2.5 g P/kg DM)
Short term effects need to be separated from long-term effects. To ensure health and proper functioning of dairy cows a minimum P status needs to be maintained with low dietary P content (P is core to many metabolic functions, structural compounds and energy metabolism). The long-term effects and capacity of the dairy cow to replenish P reserves after depletion during the close-up period and following calving needs to be investigated and quantified over 1-2 lactations.

Bone metabolism, including bone P status
As classical bone biopsies and their chemical analysis deliver highly variable results and are not easily obtained in living animals, they cannot be used to identify short-term effects in the regulation of bone metabolism, and hence they cannot give a clear indicate of the P status of bone and cow P balance. New experimental work is needed that focuses on the introduction of novel techniques such as micro-array testing up-or down-regulation of genes involved with bone resorption. Such techniques may help to better distinguish the various specific physiological functions in bone tissue and reveal important biomarkers for actual P status.
The relationship between energy metabolism and mineral (bone) metabolism and factors involved (osteocalcin, insulin, adiponectin) also requires further investigation specifically in the transition period.

Explaining variation in milk P and challenge effects of low P allowance on milk P content
Because secretion of P in milk has such a large impact on cow P status, more experimental work is needed on the effect of low P allowance on milk synthesis and P secretion with milk. It needs to be investigated whether a P deficiency affects milk synthesis and to what extent regulatory mechanisms determine the eventual milk P content.

xperimental basis for the use of content and type of faecal P as a proxy for cow P allowance and/or P status.
Because with low dietary P content almost all P excreted by the dairy cow ends up in faeces (and not in urine), analysis of faecal P might have great potential for monitoring P allowance and status of (mobilisation) of P reserves. Only faecal P concentration can be determined and therefore experimental research should be aimed at deriving relationships for total faecal P concentrations and the various P fractions it is composed of. Furthermore, experimental work should allow to derive relationships that enable to translate analysis of faecal samples (g P/kg faeces DM for example) to the unit of daily P excretion rate (g P/d).

Intestinal P digestion and regulation of salivary P
In vivo investigation of the effects of rather extreme diets with mainly bypass protein-associated phytate P on the extracellular P concentration, under conditions of minimal P allowance, testing on various aspects of P metabolism (P digestion, salivary P content and rumen P content).

Requirements during growth and development
Experimental work in youngstock on P requirements can help to define unknown factors of P metabolism in the first years of life. The effect of dietary P on bone development and the implications for P balance during adult life are still unknown.

9.2 Modelling P metabolism

Quantifying endocrine regulation of Ca and P metabolism in bone tissue as affected by Ca and P status and nutritional measures
Information on the mechanisms involved in P metabolism has become available lately, and the most prominent mechanisms in combination with a representation of Ca nutrition and metabolism have to be modelled in a more mechanistic manner encompassing recent literature.

Quantifying effects of P status and nutrition on milk P synthesis
Relationships have been established between the organic and inorganic P fraction in milk and milk constituents. Further efforts may explain a higher fraction of variation in P_O and P_I content of milk. Also effects of P status on milk synthesis may be explored further with quantitative methods.

Quantifying P recycling with saliva secretion.
The relationship between P concentration in saliva and blood P is close, but a large variation remains. Understanding this relationship in a more quantitative manner is prerequisite for predicting the effects of P recycling with saliva for blood P and extracellular P concentrations and the course of regulatory mechanisms for P metabolism of P (and Ca) in bone. Main focus is needed for the periparturient period with respect to sensitivity of the dairy cow to Ca metabolism. However, the whole dry and lactation period requires attention to be able to predict long-term consequences for the development of the P status of the dairy cow.

Quantifying the effects of history of P nutrition
A method needs to identified to be able to let a model of Ca and P metabolism take into account the history of Ca and P allowance, including nutritional measures to affect Ca metabolism, on extracellular P concentration and cow P status and balance. The aim is to allow quantification of the effects of nutritional history of dairy cattle in an integrated manner, taking various nutritional aspects and the time course of their effect into account.

Quantifying effects of Ca and P allowance around parturition on rumen P status and rumen microbial activity
A representation is needed of how microbial activity reacts on rumen P concentrations as an outcome of P allowance and P recycling with saliva production.

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