

Reappraisal of the phosphorus requirement of lactating dairy cows



Pornsin Keanthao

Reappraisal of the phosphorus requirement of lactating dairy cows

Pornsin Keanthao

2022

Colofon

Reappraisal of the phosphorus requirement of lactating dairy cows

ISBN: 978-90-393-7513-6

Copyright © 2022 Pornsin Keanthao

Printed by Proefschrift-aio.nl

The research described in this thesis was performed at Dairy Campus, Wageningen University & Research, Leeuwarden, and the Faculty of Veterinary Medicine, Utrecht University, the Netherlands

Reappraisal of the phosphorus requirement of lactating dairy cows

Herwaardering van de fosforbehoefte van melkgevende melkkoeien
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof. dr. H.R.B.M. Kummeling
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

maandag 7 november 2022 des middags te 2.15 uur

door

Pornsin Keanthao
geboren op 20 September 1985
te Udon Thani, Thailand

Promotor:

Prof. dr. ir. W. H. Hendriks

Co-promotor:

Assoc. Prof. dr. J. Th. Schonewille

Assoc. Prof. dr. ir. J. Dijkstra

This thesis was accomplished with financial support from the Ministry of Agriculture, Nature and Food Quality (the Hague, the Netherlands) within the framework of the Policy Support Research theme ‘Feed4Foodure’ (BO-31.03-005-001; TKI-AF12039), the Vereniging Diervoederonderzoek Nederland (Rijswijk, the Netherlands) and the Ministry of Higher Education, Science, Research and Innovation, Bangkok, Thailand.

Assessment Committee:

Prof. dr. T.J.G.M. Lam

Prof. dr. Y.H. Schukken

Prof. dr. D. Salvatori

Prof. dr. G. Janssens

Dr. M.M. Hostens

Content

	Page
Chapter 1 General introduction.....	7
Chapter 2 Effects of dietary phosphorus concentration during the transition period on plasma calcium concentrations, feed intake, and milk production in dairy cows.....	17
Chapter 3 Effects of low phosphorus diets on phosphorus balance and plasma concentrations of PTH, 25(OH)D ₃ , and CTX during the transition period in dairy cows.....	39
Chapter 4 Plasma concentrations of 1,25 vitamin D ₃ are not upregulated during phosphorus deficiency in lactating dairy cows	59
Chapter 5 Variation in macronutrients and minerals in Dutch bovine milk and their relationships with milk phosphorus content.....	81
Chapter 6 General discussion.....	99
Summary.....	111
Samenvatting.....	115
Acknowledgement	119
Curriculum Vitae	120
List of publications	121

Chapter 1

General introduction

Biological Function and Distribution of Phosphorus within the Animal Body

Phosphorus (P) is an essential macro-mineral necessary for many body functions and needs to be supplied in sufficient quantities to safeguard animal health and production. Phosphorus is of critical importance in energy metabolism because energy exchange inside living cells involves the synthesis and breakdown of high energy bounds such as ATP (Grünberg, 2014). In addition, P is essential for transfer of genetic information (i.e. DNA and RNA), and it is a vital component of the bodies' various buffering systems. Phosphorus is also an integral part of phospholipids, and these compounds are necessary for the integrity of cell membranes and they also serve as integral components of myelin which sheaths the nerves (Dowhan, 1997).

The total amount of P in a 650 kg cow is estimated to be 4.8 kg and ~ 80% of total body P is located in the skeleton and teeth (Figure 1). The remaining ~ 20% of total body P is almost completely located within cells. Thus, only a very small proportion of total body P is found in the extracellular space, i.e. < 0.2%.

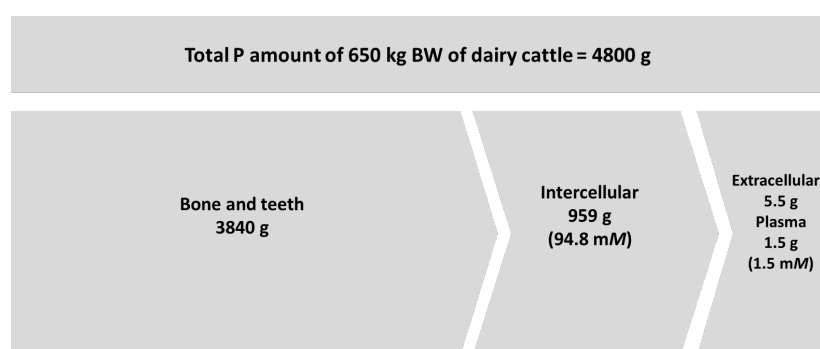


Figure 1. Distribution of phosphorus (P) in the body of a 650 kg cow (Goff, 2000; Valk, 2005; Grünberg, 2014).

Phosphorus Balance in Dairy Cows

The P content of grass and grass silages in the Netherlands ranges from 2.9 to 5.1 g/kg dry matter (DM), whereas corn silage contains 1.6-2.0 g/kg DM. Commercial concentrates generally contain ~ 4.1 g P/kg DM (CVB, 2016; CBS, 2019). On average, the Dutch dairy cow produces around 30 kg of milk/day (i.e. 9000 kg of milk/yr) which is associated with an P excretion of ~ 30 g/day (CVB, 2005). On average, cows ingest ~ 20 kg DM/d which is associated with a P intake ~ 70 g/day. Unlike monogastric animals, P excretion with urine is considered negligible in ruminants under practical feeding conditions (Grünberg, 2014). Thus, the remaining 40 g of P is excreted via feces so as to maintain zero P balance (adult, non-pregnant cow).

In ruminants, the gut-parotid gland axis typically plays a predominant role in maintaining the P balance. In ruminants consuming 20 kg DM, it can be estimated that ~ 80 g of salivary P enters the rumen (CVB, 2005; Valk, 2005). Thus, the daily amount of P that enters the ileum is ~ 2 times greater than the amount of P ingested through the diet (Hill et al., 2008). The intensive recycling of P is held responsible for the fecal excretion of excess P because the recycled P (re-) enters the gut prior to the main side of P absorption, i.e. the proximal part of the small

intestine (Valk et al., 2002; Puggaard et al., 2010). The salivary gland extracts inorganic phosphorus (Pi) from plasma in proportion to the plasma Pi concentration (Valk et al., 2002), thereby, maintaining maximum plasma Pi levels of ~ 2.3 mM (Grünberg, 2014). Such plasma Pi levels are below the renal threshold (Widiyono et al., 1998), thereby, virtually preventing the urinary excretion of P.

Retention of P (i.e. P intake > total P excretion with milk and feces) typically occurs only in growing and pregnant animals and thus can be considered physiologically normal in these conditions. A negative P balance is believed to be common during the first weeks after calving (Grünberg, 2014) because during the periparturient period, dry matter intake (DMI) is compromised in combination with rapid increase in milk production. In the condition of a negative P balance, dairy cows mobilize P from the skeleton (Grünberg et al., 2019).

Phosphorus Mobilization

In the condition of a negative P balance at the onset of lactation, P is not mobilized from the intracellular pool (Grünberg, 2014), most likely due to the physiological need for maintaining adequate concentrations of Pi to safeguard for instance cellular energy metabolism. The extracellular P pool does not contain enough P to compensate for the P losses with milk (Figure 1), thus quantitative important amounts of P can only be mobilized from the skeleton. Indeed, Satter (2002) indicated that the skeleton functions as an important P reservoir for resorption when P requirements temporarily exceed dietary intake. In the skeleton, hydroxy-apatite is considered the main P containing mineral where P is bound to Ca (Ca : P = 1 : 1.67 (molar basis), Ternouth, 1990; Grünberg, 2014). In cattle, Little et al. (1978) indicated that rib bone P could be reduced by 17-42%, while Ternouth (1990) and Valk (2005) suggested that up to 40% of the total bone P in cattle could be reabsorbed during periods of P depletion. In case P mobilization of bone occurs during the periparturient period, skeletal P stores should be replenished during the subsequent period of the lactation.

Clinical Signs of Phosphorus Deficiency

The earliest clinical signs related to P deficiency are not specific and involve a reduced feed intake, body weight loss and a decrease in milk production in lactating dairy cows (Goff, 1998; NRC, 2001; Valk and Sebek, 1999). Symptoms related to chronic, severe P deficiency include muscle weakness (recumbency), fertility problems and intravascular hemolysis (Goff, 2000; Grünberg et al., 2015), the latter causing decolorization (i.e. brownish) of the urine. Overall, none of the indicated clinical signs are specific to indicate P deficiency. Grünberg (2014) extensively reviewed potential biomarkers related to P deficiency, and concluded that “accurately assessing the P status of an individual animal remains a challenge”. Although widely used in practice, serum or plasma concentrations of Pi are considered unreliable to detect cases of P deficiency (Grünberg, 2014).

Phosphorus Absorption

In both ruminants and nonruminants, the major site for P absorption is the small intestine (Breves and Schröder, 1991). In case of high concentrations of P in digesta, P is passively transported across the gut wall, especially the duodenum. In the condition of low concentrations of P in the digesta, P is actively transported across the gut epithelial cells by means of a sodium/phosphate co-transporter (Foote et al., 2011). It is generally accepted that the active transport component of P is stimulated by $1,25(\text{OH})_2\text{D}_3$ in most mammalian species (Rizzoli et al., 1977). For ruminants, however, the role of $1,25(\text{OH})_2\text{D}_3$, if any, is not completely settled yet (Host, 1986; Cohrs et al., 2018).

Excess P Excretion and Environment

The dairy cattle sector in the Netherlands excretes large amounts of P (Figure 2), thereby, causing eutrophication of surface- and ground water (Knowlton et al., 2004). Moreover, phosphate rock reserves are expected to run out in 50-100 years (Cordell et al., 2009). Thus, in view of these concerns, the amount of P ingested with the diets should not exceed the amount of P required by the animals.

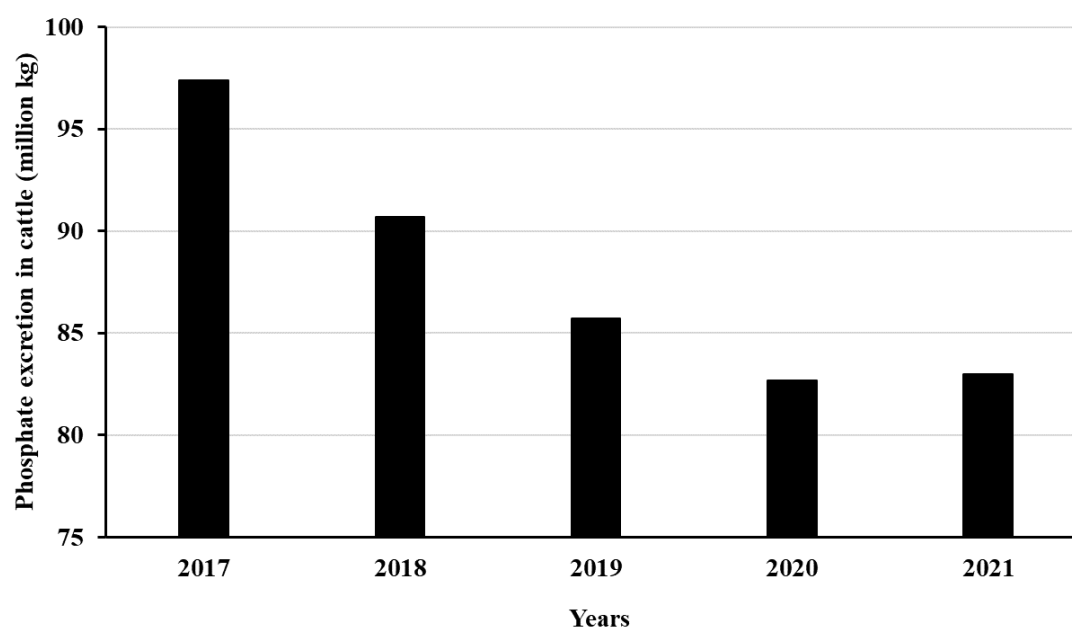


Figure 2. Fecal phosphate excretion of cattle in the Netherlands from 2017 to 2021 (modified from CBS, 2022).

Several countries, including the Netherlands, have implemented regulations aimed at reducing the amount of P applied onto farmland with manure, in order to decrease excess P excretion into the environment (e.g., EU Water Framework Directive; EC, 2000). Despite these efforts, however, under practical feeding conditions on farms, cows generally ingest an amount of P in excess of their requirement (Valk, 2005; Kebreab et al., 2008).

Phosphorus Requirements of Dairy Cows

In Table 1, an overview is provided of the P recommendations as set by different national authorities from the United Kingdom, Germany, the United States of America and the Netherlands. Considerable variation exists in dietary P recommendations between the various international authorities (Table 1), thereby, indicating that the P requirement of dairy cows as such, is a matter of debate. The Dutch CVB (Valk, 2005) recommends the lowest levels of dietary P compared to the AFRC (1991), GfE (1993) and the NRC (2001).

Table 1. Overview of phosphorus (P) recommendations as set by different authorities¹.

Milk (kg/d)	Dry matter intake (kg/d)	Recommended dietary P content (g/kg dry matter)			
		AFRC ²	GfE ³	NRC ⁴	CVB ⁵
0, dry cow	11.0	-	-	2.3	2.0
15	17.0	3.3	2.7	3.0	2.2
25	20.3	3.8	3.2	3.2	2.7
35	23.6	4.2	3.6	3.5	3.1
45	26.9	4.5	3.8	3.6	3.3
55	30.0	4.7	4.0	3.8	3.5

¹ Adapted and modified data originally reported by Valk (2005).

² AFRC, 1991; Agricultural and Food Research Council-Technical Committee on Responses to nutrients, 1991. A reappraisal of the calcium and phosphorus requirements of sheep and cattle, report 6. Nutrition Abstracts and Reviews (Series B) 61: 573-608.

³ GfE, 1993; Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie, 1993. Überarbeitete Empfehlungen zur Versorgung von Milchkühen mit Calcium und Phosphor. Proceedings of the Society of Nutrition Physiology, 1: 108-113.

⁴ NRC, 2001; National Research Council, 2001. Nutrient requirement of dairy cattle. Seventh revised edition.

⁵ Valk, 2005; Reviews on the mineral provision in ruminants (II): Phosphorus metabolism and requirements in ruminants. CVB (Centraal Veevoederbureau) documentation rapport 34, Central Bureau for Livestock Feeding, Lelystad, the Netherlands.

Despite the fact the recommended levels of P for dairy cows are already quite low in the Netherlands, it is still considered opportune to fine tune the current recommendations on dietary P contents towards lower values. There are values reported in literature that the efficiency of P absorption can be as high as 90% of intake (Martz et al., 1999) whereas a value of 75% is adopted by the Dutch Central Bureau for Livestock Feeding (CVB, 2005). Furthermore, there are also indications that dietary P contents below current CVB recommendations are instrumental to prevent milk fever (Puggaard et al., 2014). With respect to the latter, it is of interest to note that Dutch veterinarians in the field express their concerns, via technical papers, on the current low P recommendations. This concern was also one of reasons to conduct the research described in the current thesis.

Outline of Experimental Work

For obvious reasons, the cow's health and production performance are important principles to determine the minimum dietary P content. Thus, the aim of the first experiment described in this thesis (Chapter 2) was to evaluate the effects of a low or high dietary P content during the dry period, followed by either a high or low dietary P content during the first 8 weeks of lactation, on plasma Ca concentrations, feed intake and lactational performance of dairy cattle. It was hypothesized that the feeding of low vs. high P diets during both the dry period and thereafter, cause greater plasma Ca concentrations. Furthermore, it was hypothesized that feed intake and milk production would not be reduced by moderately lowering the dietary P content below requirements from CVB (2012) in early lactation. The results of the study were interpreted in that current CVB recommendations might indeed be too high, but hypophosphatemia occurred after calving and this observation warranted further investigation. Therefore, P balances of the cows were determined, and markers associated with P absorption and P mobilization from bone were investigated (Chapter 3). The results of that study indicated that the periparturient cows were in negative P balance which was associated with P mobilization from bone. However, next to hypophosphatemia, the periparturient cows also showed hypocalcemia after calving. The concomitant occurrence of both hypophosphatemia and hypocalcemia hindered the interpretation of the biomarkers related to P absorption and P mobilization. Thus, a second experiment was designed (Chapter 4) to investigate the effects of a low P diet on P absorption in mid lactation cows fed sufficient amounts of Ca to prevent hypocalcemia. In this experiment, one of the low P treatments was offered in combination with pelleted grass hay instead of coarse artificial dried grass in an attempt to reduce rumination time. It was anticipated that the reduction in rumination time in combination with a low P diet would trigger an extra stimulus to upregulate P absorption. In the last study of the current thesis (Chapter 5), it was attempted to gain more insight into the factors that explain the variation of the P content of milk. This issue was considered important in view of the current regulations on P excretion into the environment.

REFERENCES

- Breves, G., and B. Schröder. 1991. Comparative aspects of gastrointestinal phosphorus metabolism. *Nutr. Res. Rev.* 4: 125-140.
- CBS. 2019. Phosphate output from livestock manure down again. CBS, the Netherlands. <https://www.cbs.nl/en-gb/news/2019/07/phosphate-output-from-livestock-manure-down-again>.
- CBS. 2022. Lower nitrogen and phosphate output from animal manure. CBS, the Netherlands. <https://www.cbs.nl/en-gb/news/2022/07/lower-nitrogen-and-phosphate-output-from-animal-manure>.
- Cohrs, I., M. R. Wilkens, and W. Grünberg. 2018. Short communication: Effect of dietary phosphorus deprivation in late gestation and early lactation on the calcium homeostasis of periparturient dairy cows. *J. Dairy Sci.* 101: 9591-9598.
- Cordell, D., J. O. Drangert, and S. White. 2009. The story of phosphorus: Global food security and food for thought. *Global Environ. Change* 19: 292-305.

- CVB. 2005. Handleiding mineralenvoorziening rundvee, schapen, geiten. The Hague, the Netherlands.
- CVB. 2012. CVB farm animal feeding advices 2012. CVB volume 50, the Hague, the Netherlands.
- CVB. 2016. CVB Table Booklet Feeding of Ruminants 2016: Nutrient requirements for cattle, sheep and goats and nutritional values of feeding ingredients for ruminants. The Hague, the Netherlands.
- Dowhan, W. 1997. The role of phospholipids in cell function. In: Gross, R. W. (ed.), *Advances in Lipobiology*. JAI. 2: 79-107.
- European Commission. 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. *Off. J. Eur. Comm.* L327: 1-72.
- Goff, J. P. 2000. Pathophysiology of calcium and phosphorus disorders. *Vet. Clin. North Am. Food Anim. Pract.* 16: 319-337.
- Grünberg, W. 2014. Treatment of phosphorus balance disorders. *Vet. Clin. North Am. Food Anim. Pract.* 30: 383-408.
- Grünberg, W., J. A. Mol, and E. Teske. 2015. Red blood cell phosphate concentration and osmotic resistance during dietary phosphate depletion in dairy cow. *J. Vet. Intern. Med.* 29: 395-399.
- Grünberg, W., P. Scherpenisse, I. Cohrs, L. Golbeck, P. Dobbelaar, L. M. van den Brink, and I. D. Wijnberg. 2019. Phosphorus content of muscle tissue and muscle function in dairy cows fed a phosphorus-deficient diet during the transition period. *J. Dairy Sci.* 102: 4072-4093.
- Hall, O. G., H. D. Baxter, and C. S. Hobbs. 1961. Effect of phosphorus in different chemical forms on *in vitro* cellulose digestion by rumen microorganisms. *J. Anim. Sci.* 20: 817-819.
- Hill, S. R., K. F. Knowlton, E. Kebreab, J. France, and M. D. Hanigan. 2008. A model of phosphorus digestion and metabolism in the lactating dairy cow. *J. Dairy Sci.* 91: 2021-2032.
- Host, R. L. 1986. Regulation of calcium and phosphorus homeostasis in dairy cow. *J. Dairy Sci.* 69: 604-616.
- Kebreab, E., N. E. Odongo, B. W. McBride, M. D. Hanigan, and J. France. 2008. Phosphorus utilization and environmental and economic implications of reducing phosphorus pollution from Ontario dairy cows. *J. Dairy Sci.* 91: 241-246.
- Knowlton, K. F., J. S. Radcliffe, C. L. Novak, and D. A. Emmerson. 2004. Animal management to reduce phosphorus losses to the environment. *J. Anim. Sci.* 82: 173-195.
- Little, D. A., N. J. Siemon, and E. W. Moodie. 1978. Effects of varying phosphorus intake and requirement on measures of skeletal mineralization in the ewe. *Aust. J. Exp. Agric. Anim. Husb.* 18: 514-519.
- Martz, F. A., A. T. Belo, M. F. Weiss, and R. L. Belyea. 1999. True absorption of calcium and phosphorus from corn silage fed to nonlactating, pregnant dairy cows. *J. Dairy Sci.* 82: 618-622.
- Puggaard, L., N. B. Kristensen, and J. Sehested. 2010. Effect of decreasing dietary phosphorus supply on net recycling of inorganic phosphate in lactating dairy cows. *J. Dairy Sci.* 94: 1420-1429.

- Puggaard, L., P. Lund, A. Liesegang, and J. Sehested. 2014. Long term effect of reduced dietary phosphorus on feed intake and milk yield in dry and lactating dairy cows. *Livest. Sci.* 159: 18-28.
- Rizzoli, R., H. Fleisch, and J. P. Bonjour. 1977. Role of 1,25-Dihydroxyvitamin D₃ on intestinal phosphate absorption in rats with a normal vitamin D supply. *J. Clin. Invest.* 60: 639-647.
- Satter, L. 2002. What goes in must come out – phosphorus balance on dairy farm. In *Proceeding of the American Association of Bovine Practitioners*. Madison, Wisconsin. 125-130.
- Ternouth, J. H. 1990. Phosphorus and beef production in northern Australia. 3. Phosphorus in cattle: a review. *Trop. Grassl.* 24: 159-169.
- Valk, H., L. B. J. Sebek, and A. C. Beynen. 2002. Influence of phosphorus intake on excretion and blood plasma and saliva concentrations of phosphorus in dairy cows. *J. Dairy Sci.* 85: 2642-2649.
- Valk, H. 2005. Reviews on the mineral provision in ruminants (II): Phosphorus metabolism and requirements in ruminant. CVB documentation Report 34, Central Bureau for Livestock Feeding, Lelystad, the Netherlands.
- Widiyono, I., K. Huber, K. Failing, and G. Breves. 1998. Renal phosphate excretion in goats. *Zentralblatt für Veterinärmedizin Reihe.* 45: 145-153.

Chapter 2

Effects of dietary phosphorus concentration during the transition period on plasma calcium concentrations, feed intake, and milk production in dairy cows

P. Keanthao*, R. M. A. Goselink[†], J. Dijkstra[‡], A. Bannink[†], and J. T. Schonewille*

* Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, the Netherlands

[†] Wageningen Livestock Research, Wageningen University & Research, PO Box 338, 6700 AH Wageningen, the Netherlands

[‡] Animal Nutrition Group, Wageningen University & Research, PO Box 338, 6700 AH Wageningen, the Netherlands

ABSTRACT

Our aim was to evaluate the effects of a low or high dietary phosphorus (P) concentration during the dry period, followed by either a high or low dietary P concentration during the first 8 weeks of lactation, on plasma Ca concentrations, feed intake and lactational performance of dairy cattle. Sixty pregnant multiparous Holstein Friesian dairy cows were assigned to a randomized block design with repeated measurements and dietary treatments arranged in a 2 × 2 factorial fashion. The experimental diets contained 3.6 (Dry-HP) or 2.2 (Dry-LP) g P/kg dry matter (DM) during the dry period, and 3.8 (Lac-HP) or 2.9 (Lac-LP) g P/kg DM during 56 days after calving period. In dry cows, plasma Ca concentrations were 3.3% greater when cows were fed 2.2 instead of 3.6 g P/kg DM. The proportion of cows being hypocalcemic (plasma Ca concentrations < 2 mM) in the first week after calving was lowest with the low P diets both during the dry period and lactation. Plasma Ca concentrations in week 1 to 8 after calving were affected by dietary P level in the dry period and in the lactation period, but no interaction between both was present. Feeding Dry-LP instead of Dry-HP diets resulted in 4.1% greater plasma Ca values, and feeding Lac-LP instead of Lac-HP diets resulted in 4.0% greater plasma Ca values. After calving, plasma Pi concentrations were affected by a 3-way interaction between sampling day after calving, and dietary P levels during the dry period and lactation. From d 1 to d 7 post-partum, cows fed Lac-HP had increased plasma Pi concentrations, and the rate appeared to be greater in cows fed Dry-LP vs. Dry-HP. In contrast, plasma Pi concentrations decreased from d 1 to d 7 post-partum in cows fed Lac-LP, and this decrease was at a higher rate for cows fed Dry-HP vs. Dry-LP. After d 7, plasma Pi concentrations remained rather constant at 1.5-1.6 mM when cows received Lac-HP while with Lac-LP plasma Pi concentrations reached stable levels (i.e. 1.3-1.4 mM) at d 28 after calving. Milk production, DM intake, and milk concentrations of P, Ca, fat, protein and lactose were not affected by any interaction nor the levels of dietary P. It is concluded that the feeding of diets containing 2.2 g P/kg DM during the last 6 weeks of the dry period and 2.9 g P/kg DM during early lactation increased plasma Ca levels when compared with greater dietary P levels. These low P diets may be instrumental in preventing hypocalcemia in periparturient cows and do not compromise DMI and milk production. Current results suggest that P requirements in dairy cows during dry period and early lactation can be fine-tuned towards lower values than recommended by both the NRC (2001) and the Dutch Central Bureau for Livestock Feeding (CVB, 2012). Caution, however, is warranted to extrapolate current findings to entire lactations because long term effects of feeding low P diets containing 2.9 g/kg DM on production and health needs further investigation.

Key words: dairy cow, phosphorus, hypocalcemia, transition period

INTRODUCTION

Low efficiency of phosphorus (P) use by dairy cattle is associated with excess P excretion into the environment (Klop et al., 2013) and detrimental effects of excess P on water quality which is of major environmental concern (Kebreab et al., 2008). Besides, phosphate rock reserves are expected to run out in 50-100 years (Cordell et al., 2009). Therefore, dietary supply of P should

not exceed the minimum amount of P needed to ensure both the production and health of dairy cattle. It is well known that under practical feeding conditions on farms, cows generally ingest an amount of P in excess to their requirement (Puggaard et al., 2014). The recommended level of dietary P in dairy cow nutrition, however, is still a matter of debate.

In contrast to diets for lactating cows, research-based recommendations on the minimum dietary P concentration of dry cow diets are scarce owing to a dearth of studies addressing the issue of dietary P concentration during the periparturient period. Lean et al. (2006) reported that an increase in dietary P concentration from 3 to 4 g/kg DM during the dry period increases the risk of milk fever by 18%. This result is in line with Peterson et al. (2005), who reported lower serum Ca concentrations at parturition when cows were fed a prepartum diet containing 4.4 vs. 2.1 or 3.1 g P/kg DM. Moreover, Cohrs et al. (2018) found that the feeding of a prepartum diet containing 2.8 g P/kg DM (i.e. adequate dietary P) caused lower plasma Ca concentrations in periparturient cows compared to cows fed for 4 weeks with a diet containing 1.5 g P/kg DM (i.e. a P deficient diet). It can thus be suggested that a reduction in dietary P concentration during the dry period prevents hypocalcemia after calving. This suggestion implies that P and Ca metabolism are, at least to a certain extent, linked in dairy cows. The latter notion, however, seems not in line with the idea that P and Ca homeostatic regulations are independent of each other (Cohrs et al., 2018). Furthermore, no studies have investigated the possible consequences of interaction between high or low dietary P levels in the dry period and high or low P levels postpartum on plasma Pi and Ca levels. Further studies are thus warranted to substantiate the aforementioned observations on hypocalcemia and milk fever as well as effects on feed intake and milk production. Currently, the Dutch (CVB, 2012) versus US recommendations (NRC, 2001) on the P concentration of dairy diets are about 9% lower. However, the Dutch values still contain a safety margin on the recommended dietary P levels, thereby, implying that, at least potentially, dietary P levels can be further fine-tuned towards lower values. Thus, we formulated low P diets by moderately lowering (i.e. 30%) the dietary P concentration below the current values recommended by the Dutch CVB in early lactation. Low P diets fed after calving can cause a depression in DMI (Puggaard et al., 2014). It was, therefore, considered opportune to measure, next to DMI and milk production, selected indices on energy metabolism as well.

The objective of the current experiment was to evaluate the effects of a low or high dietary P concentration during the dry period, followed by either a high or low dietary P concentration during the first 8 weeks of lactation, on plasma Ca concentrations, feed intake and lactational performance of dairy cattle. We hypothesized that the feeding of low vs. high P diets during both the dry period and thereafter, cause greater plasma Ca concentrations. Furthermore, we hypothesized that feed intake and milk production would not be reduced by moderately lowering the dietary P concentration below requirements from CVB (2012) in early lactation.

MATERIALS AND METHODS

All experimental protocols and interventions were conducted under the Dutch Law on Animal Experimentation and approved (approval nr: AVD401002016653) by the Central Authority for Scientific Procedures on Animals (CCD, the Hague, the Netherlands).

Animals, Experimental Design, and Management

Sixty multiparous (parity ≥ 3), pregnant Holstein Friesian dairy cows were used in the current study. The animals entered the experiment approximately 6 weeks before the expected calving date. The experiment had a randomized block design with repeated measurements and a 2×2 factorial arrangement of dietary treatments. Prior to the start of the experiment, cows were grouped in 15 blocks of 4 animals with comparable expected calving dates, parity and milk yields during the previous lactation. Within each block, cows were randomly assigned to dry cow diets containing either 3.6 (**Dry-HP**) or 2.2 (**Dry-LP**) g P/kg DM and, within dry cow treatments, to post-calving diets containing either 3.8 (**Lac-HP**) or 2.9 (**Lac-LP**) g P/kg DM. Cows were housed in 2 groups in a free-stall barn, i.e. a dry-cow group and a postpartum lactation group. The cubicles were covered with rubber cattle mats and with wood shavings as bedding, and were cleaned on a daily basis. Throughout the experiment, fresh drinking water was provided ad libitum. Lactating cows were milked twice daily with a milking interval of 11 to 13 hours. Animal health was monitored by trained animal caretakers according to standard farm protocols. Symptoms of clinical hypocalcemia around calving (i.e. ‘recumbency/not able to stand’, ‘cold ears’ and ‘low rumen fill with no contractions’) were noted by animal caretakers, and when cows showed at least two of these symptoms cows were treated intravenously with a CaMg solution (450 ml with 1.65% Ca and 0.95% Mg; Dechra, Bladel, the Netherlands). The cows left the trial after 56 days of lactation.

Feeding and Experimental Diets

Throughout the experiment, the cows received their allocated, freshly mixed, diets at regular intervals (3 to 5 times per day) with the use of an automatic feeding system (Triomatic HP 2 300, Trioliet, Oldenzaal, the Netherlands). This basal diet was composed of corn silage, grass silage, wheat straw (dry cow diet only), soybean meal (lactating cows only), and a premix; the premix had either a low or a high P concentration. The basal diet ingredients were stored in bunkers (used for roughages) and silos (used for soybean meal and premix). On a weekly basis and based on the DM concentration of each individual basal diet component, the required amount of diet ingredients was calculated to prepare the experimental diets. The dietary components were taken automatically from their bunker or silo, weighted, and transported into the Trioliet feed mixing robot. Within the Trioliet, the amount of each dietary component was weighted again so the weight actually realized with mixing were recorded. Diets were mixed in the mixing robot for on average 6 min. The mixed, basal diets were supplied in weighing troughs with individual transponder-controlled access gates (Roughage Intake Control system (RIC), Hokofarm Group, Marknesse, the Netherlands). For every visit of a cow to a RIC bin, the start and end time of the visit as well as the start and end weight of the RIC bin were recorded. Weekly, the RIC bins were calibrated with a standard weight. A total of 32 RIC bins were available, and cows had access to all RIC-bins that offered their designated diet. Every morning around 0600 h, feed refusals were removed and individual feed intake was recorded daily. Next to the mixed basal diet, pelleted concentrates were fed individually with the use of transponder-controlled concentrate feeders (Hokofarm Group, Marknesse, the Netherlands). Dry cows received 1 kg/d (as fed) of compound concentrate. One day after calving, cows

received 3.0 kg/d of compound concentrate gradually increasing up to 9.3 kg/d at d21 postpartum. The P concentrations of the premix and concentrates were manipulated with the use of disodium phosphate (Na_2HPO_4) and sodium bicarbonate (NaHCO_3), thereby, aiming to prevent potential confounding effects due to differences in Na intake between the low- and high P diets. The ingredient and analyzed composition of the experimental diets is shown in Table 1.

Data Collection and Sampling

Body weights were monitored weekly before calving and daily after calving using an appropriate weight scale with automatic registration. For the determination of DM concentration of the basal mixed diet, corn silage and grass silage were sampled daily while wheat straw, soybean meal and the premixes were sampled weekly. Dry matter concentration was determined by calculating the weight difference before and after oven drying at 104 °C during 36 h. All basal diet feed ingredients and concentrates were sampled weekly and stored at -20 °C. For grass and corn silage, samples of 5–6 consecutive weeks were pooled into 1 composite sample; for premixes, concentrates and wheat straw, all samples were pooled per 16 consecutive weeks (resulting in 2 samples per feedstuff). Once weekly, during four consecutive milking events, milk was sampled and pooled in 2 tubes (one tube for morning milkings, the other tube for afternoon milkings) containing sodium azide for preservation and stored no longer than 1 d at 4 °C, and analyzed for fat, protein, and lactose. Two separate milkings (one morning and one afternoon) were obtained weekly and stored frozen (-20 °C) and (after pooling per cow and week) analyzed for P and Ca. In addition, before calving blood was sampled on a fixed day of the week (Thursday) in week-4, -2 and -1 relative to calving. During lactation, blood samples were obtained immediately after calving (d0), and on d1, d2, d3, d7, d14, d21, d28, and d56 after calving. Blood was collected in serum separator tubes and tubes were centrifuged for 15 min at $3,000 \times g$ within 1 h after collection. Blood plasma was transferred to 3 plasma tubes and stored at -20 °C until analysis of Ca, inorganic phosphate (Pi), non-esterified fatty acids (NEFA) and BHBA.

Chemical Analysis

Feed samples were analyzed by wet chemistry at the MasterLab Laboratory (MasterLab, Boxmeer, the Netherlands). The DM concentration was determined using forced-air oven drying (105 °C) (ISO, 1999b), crude fat concentration was determined gravimetrically as the ether extract (ISO, 1999a) and crude ash concentration after incineration at 550 °C (ISO, 2002). Concentrations of NDF and ADF in samples were determined according to Van Soest et al. (1991) after pre-treatment with α -amylase and expressed without residual ash. Acid detergent lignin was analyzed in feed samples using the method of Robertson and Van Soest (1981) using sulfuric acid and expressed without residual ash. Crude protein was calculated as $6.25 \times N$, where N was determined using the Kjeldahl method with CuSO_4 as a catalyst (ISO, 2005). The sugar concentrations were determined as described by Van Vuuren et al. (1993). Starch was released by heating in a boiling water bath in the presence of 2 M HCl concentration and subsequently starch concentration was determined using the amyloglucosidase method (ISO,

2004). Calcium, sodium, magnesium, potassium, sulphur, chlorine and P concentrations in feed were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Eurofins Agro, Wageningen, the Netherlands). The NE_L, intestinal digestible protein (IDP) and rumen degradable protein balance (RDPB) were obtained by near-infrared spectroscopy for corn and grass silage (Eurofins Agro, Wageningen, the Netherlands). For wheat straw, soybean meal and concentrates, NE_L, IDP, and RDPB values were calculated based on table values of ingredients (CVB, 2012).

All plasma samples were analyzed for Pi concentration (with ammonium molybdate) and Ca concentration (with Arsenazo III) using an automatic analyzer (ABX Pentra 400, Horiba, Europe GmbH, Langenhagen, Germany). Non-esterified fatty acids and BHBA concentrations were determined with the use of an automatic analyzer (Cobas Mira Plus System from Roche Diagnostica Ltd, Basel, Switzerland) using commercial test kits (NEFA: HR(2) R1+R2 Set, WAKO Chemicals GmbH, Neuss, Germany; BHBA: RANBUT, RB 1008, Randox Laboratories GmbH, Wülfrath, Germany).

Pooled milk samples (2 morning and 2 afternoon milkings weekly) were analyzed for fat, protein, and lactose by mid-infrared spectrometry (ISO, 2013; Qlip, Zutphen, the Netherlands). Pooled milk samples (1 morning and 1 afternoon milking weekly) were analyzed by ICP-MS for P and Ca concentration (Qlip, Zutphen, the Netherlands).

Calculations and Statistical Analysis

Prior to statistical analysis, the daily values on DMI and milk yield were averaged per week. Fat and protein corrected milk (FPCM) was calculated as follows (CVB, 2012):

$$\text{FPCM (kg/d)} = \text{MY (kg/d)} \times [0.337 + 0.116 \times \text{MF (\%)} + 0.06 \times \text{MP (\%)}],$$

where MY = milk yield, MF = milk fat concentration, and MP = milk protein concentration. The energy balance (EB) was defined as the difference between net energy intake and net energy requirements for maintenance and milk production and EB was calculated accordingly (Heuer et al., 2001). After calving, due to an allocation error, one cow in the Dry-HP, Lac-LP group received the high P premix in her basal mixed diet, and this cow was excluded from all statistical analyses in the postpartum period. Data was analyzed by repeated measures ANOVA (VSN International, 2018) for the prepartum period and the postpartum period separately. Plasma Ca, Pi, DMI, MY, FPCM, milk concentrations, BW, EB, NEFA, and BHBA concentrations were analyzed with repeated measures that accounted for unequal time intervals of blood sampling. The model for variables in the dry period contained main effects of block (block 1-15), dry period treatment (Dry-LP, 2.2 g P/kg DM; Dry-HP, 3.6 g P/kg DM), week (week 4, 2 and 1 before parturition except BW and DMI which also included week 6, 5, and 3 before parturition), and the interaction effect dry period treatment × week. The model for variables in the lactation period contained main effects of block, dry period treatment, lactation period treatment (Lac-LP, 2.9 g P/kg DM; Lac-HP, 3.8 g P/kg DM), week (week 1 to 8 after parturition), and the interaction effects lactation period treatment × week, dry period treatment × week, lactation period treatment × dry period treatment, and lactation period treatment × dry period treatment × week. For plasma variables in the lactation period, the effect of week was replaced with effect of sampling day after calving (d0, 1, 2, 3, 7, 14, 21, 28, and 56). A chi-square test was used to test for differences in the incidences of clinical and subclinical

hypocalcemia between dietary treatments. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 \leq P \leq 0.10$.

Table 1. Ingredient and chemical composition of the experimental diets¹. The analyzed and calculated composition values of diets are based on the actual, mean DM intakes during the 6-week dry period and the subsequent 8-week lactation period.

Item	Dry period		Lactation period ²	
	Dry-HP	Dry-LP	Lac-HP	Lac-LP
<i>Ingredient composition (% DM)</i>				
Corn silage	36.8	36.9	44.6	44.9
Wilted grass silage	22.5	22.4	13.5	13.9
Wheat straw	29.5	29.7	-	-
Soybean meal	-	-	3.4	4.2
Concentrates (incl. premixes)				
Low P concentration ^{3,5}	-	11.0	-	37.0
High P concentration ^{4,6}	11.2	-	38.5	-
<i>Analyzed composition (g/kg DM)</i>				
OM	924	926	931	930
CP	106	105	149	150
EE	27	27	33	32
NDF	521	525	366	374
ADF	298	301	214	214
ADL	35	35	23	25
Starch	136	136	206	206
Sugar	21	21	39	41
Ca	3.6	3.6	6.1	6.2
P	3.6	2.2	3.8	2.9
Mg	2.4	2.2	3.6	3.6
Na	1.9	1.6	1.7	1.6
K	17.4	17.5	16.2	16.3
S	1.9	1.9	2.2	2.2
Cl	3.6	3.5	3.3	3.7
DCAD (mEq/kg DM) ⁷	307	299	263	248
<i>Calculated values (unit/kg DM)⁸</i>				
NE _L (MJ)	5.6	5.6	6.8	6.8
IDP (g)	57	57	92	93
RDPB (g)	-6	-7	7	7

¹ Dry-HP, Dry-LP, Lac-HP, and Lac-LP = experimental diets fed during the dry period (Dry) or the lactation period (Lac) high in phosphorus (HP) or low in phosphorus (LP) containing either 3.6, 2.2, 3.8 or 2.9 g P/kg DM, respectively.

² Lactation concentrate was gradually increased from 1.0 kg/d at d 1 post calving to 9.3 kg/d at d 21 post calving.

³ The low P concentration concentrate in the dry period consisted of (% DM): sugar beet pulp = 28.1, potato protein = 25.8, soybean meal = 21.1, molasses = 10.0, urea = 5.4, vitamins and minerals = 3.0, sodium bicarbonate = 2.5, sugar = 1.9, magnesium oxide = 1.1, palm oil = 1.1.

- ⁴ The high P concentration concentrate in the dry period consisted of (% DM): sugar beet pulp = 26.5, potato protein = 26.3, soybean meal = 19.9, molasses = 9.6, urea = 5.5, disodium phosphate = 5.3, vitamins and minerals = 3.0, sugar = 1.8, magnesium oxide = 1.1, palm oil = 1.0.
- ⁵ The low P concentration concentrate in the lactation period consisted of (% DM): sugar beet pulp = 24.8, soyhulls = 23.1, corn = 14.3, soybean meal = 11.7, wheat = 6.6, potato protein = 5.8, molasses = 5.3, palm kernel meal = 2.8, urea = 1.6, calcium carbonate = 1.4, magnesium oxide = 0.8, palm oil = 0.7, vitamins and minerals = 0.4, sodium bicarbonate = 0.4, sodium chloride = 0.2, disodium phosphate = 0.1.
- ⁶ The high P concentration concentrate in the lactation period consisted of (% DM): sugar beet pulp = 24.7, soyhulls = 22.9, corn = 14.2, soybean meal = 11.7, wheat = 6.6, potato protein = 5.9, molasses = 5.2, palm kernel meal = 2.8, urea = 1.6, calcium carbonate = 1.4, disodium phosphate = 1.1, magnesium oxide = 0.8, palm oil = 0.7, vitamins and minerals = 0.4, sodium chloride = 0.1.
- ⁷ DCAD = Dietary Cation-Anion Difference calculated as $(\text{Na}^+ + \text{K}^+) - (\text{Cl}^- + \text{S}^{2-})$ where Na, K, Cl and S are expressed as mEq/kg DM.
- ⁸ The NE_L values were calculated according to the Dutch NE-system (Van Es, 1978). IDP = intestinal digestible protein and RDPB = rumen-degradable protein balance, the latter values are calculated according to the Dutch DVE/OEB-system (Tamminga et al., 1994).

RESULTS

Dry Period

During the dry period, both BW of the cows and DMI were neither affected by a week \times dietary P level interaction ($P \geq 0.11$) nor by the level of dietary P ($P \geq 0.36$) but in time BW increased by 3.0 % while DMI decreased by 17.8% ($P < 0.01$) (Table 2). Plasma Pi values were not affected by dietary P level, week before parturition, or their interaction ($P \geq 0.15$). Plasma Ca concentrations were not affected by a dietary P level \times week interaction or by week ($P \geq 0.36$), but in contrast to plasma Pi, plasma Ca concentrations were affected by dietary P supply and were 3.3% greater ($P = 0.05$) when cows were fed 2.2 compared with 3.6 g P/kg DM. Plasma NEFA concentrations were neither affected by week \times dietary P level interaction ($P = 0.52$) nor by dietary P level ($P = 0.37$), but 1 week before calving, NEFA concentrations in plasma were 2.4 times greater compared to the values observed 4 weeks before calving ($P < 0.01$). Plasma BHBA concentrations tended to be affected by dietary P level \times week before parturition ($P = 0.07$), indicating increased BHBA concentrations with time in the high P group, but decreased BHBA concentrations with time in the low P group.

Periparturient Hypocalcemia

No distinct differences between treatment groups were found ($P = 0.81$) in the number of cows treated with intravenous Ca administration (Table 3). However, the number of cows with plasma Ca concentrations < 2.0 mM during the first week after calving was lowest ($P = 0.02$) when the cows were fed low P diets during both the dry period (Dry-LP) and after calving (Lac-LP). The number of cows having plasma Ca concentrations < 2.0 mM during week 2-8 was not affected by the level of dietary P ($P = 0.20$).

Post Calving Plasma Pi, Ca, NEFA, and BHBA

A sampling day \times dry period P level \times lactation period P level interaction affected post-partum plasma Pi concentration ($P < 0.01$). From d1 to d7 post-partum, cows receiving the high P diet had increased plasma Pi concentrations, and the rate of increase from d1 to d7 greater in cows fed Dry-LP during the dry period compared with those having received the high P diet during the dry period (Figure 1). In contrast, plasma Pi concentrations decreased from d1 to d7 post-partum in cows receiving the low P diet, but this decrease was more pronounced for cows having received the high P diet during the dry period compared with those having received the low P diet during the dry period. After d7, plasma Pi concentrations remained rather constant at 1.5-1.6 mM for cows receiving the high P diet irrespective of dietary P level received during the dry period. Similarly, levels of plasma Pi increased in cows fed the low P diet from d7 onwards and reached stable levels of 1.3-1.4 mM at d28. For plasma Ca, BHBA and NEFA concentrations, none of the 2-way or 3-way interactions were significant ($P \geq 0.16$) (Table 4). Plasma Ca and BHBA concentrations generally increased, and plasma NEFA concentrations decreased, with time postpartum ($P \leq 0.01$). The cows fed high P diets during the dry period had 4.1% lower plasma Ca concentrations after calving ($P < 0.01$). After calving, feeding the low P diet resulted on average in 4.0% greater plasma Ca concentrations ($P = 0.01$). Post-partum plasma concentrations of BHBA and NEFA were neither affected by dietary P level in the dry period nor in the lactation period ($P \geq 0.26$).

Post Calving DMI, Milk Production, Energy Balance, and Body Weight

Total DMI (basal diet plus concentrates) was not affected by any interaction ($P \geq 0.58$), except for a tendency for a week \times dietary P level in lactation period interaction ($P = 0.06$), due to differences in DMI during the first 3 weeks (Table 5). Cows fed the low P diet tended to have a lower rate of increase in DMI in wk2 and 3 compared with wk1, than cows fed the high P diet (i.e. Lac-HP). In week 8, DMI did not differ and was on average 55% greater than in wk1. The level of dietary P in the dry cow diet did not affect DMI ($P = 0.19$) or FPCM ($P = 0.51$). The rate of increase in FPCM yield was greater in cows fed Lac-HP versus Lac-LP ($P = 0.04$) but this effect was mainly restricted to the first 2 weeks after calving. Energy balance and BW were not affected by any of the interactions or by dietary P level ($P \geq 0.09$). Energy balance increased and BW decreased from week 1 to week 8 postpartum ($P < 0.01$).

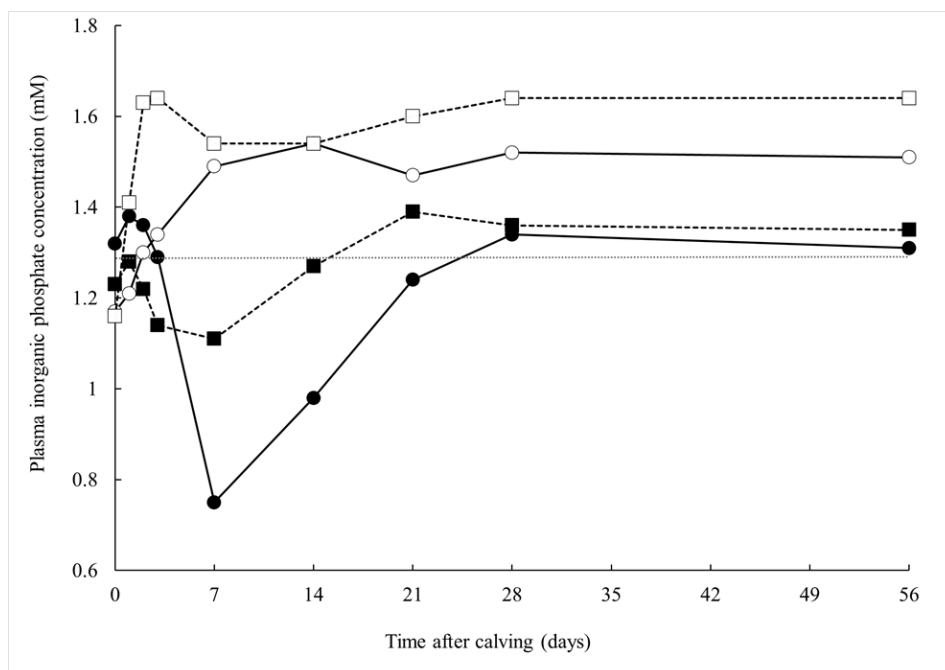


Figure 1. Time courses on plasma inorganic phosphate (Pi) concentrations after calving. The cows were either fed a low P (LP) or a high P (HP) diet during the dry period (Dry) and after calving (Lac). High P diets contained 3.6 g P/kg DM during the dry period and 3.8 g P/kg DM after calving while low P diets contained 2.2 g P/kg DM during the dry period and 2.9 g P/kg DM after calving. Symbols: solid line ●, Dry-HP/Lac-LP; solid line ○, Dry-HP/Lac-HP; dotted line ■, Dry-LP/Lac-LP; dotted line □, Dry-LP/Lac-HP, dotted grey line without markers represents the threshold for hypophosphatemia, i.e. 1.3 mM. Standard error of the difference is 0.104 mM. *P* values: Sampling day × Dry × Lac = < 0.01; Sampling day × Dry = 0.31; Sampling day × Lac = < 0.01; Dry × Lac = 0.20; Sampling day = < 0.01; Dry-HP vs Dry-LP = 0.03; Lac-HP vs Lac-LP = < 0.01.

Milk Constituents

Except for the lactose concentration of milk, concentrations of P, Ca, fat, and protein in milk (Table 6) were not affected by any interaction ($P \geq 0.42$). The lactose concentration of milk tended to be affected by week × dietary P level ($P = 0.07$). Milk lactose concentration increased with lactation week, mainly from wk1 to wk2, and tended to be somewhat greater in cows fed Lac-HP, whereas milk lactose concentration tended to be slightly less in later weeks (i.e. wk4 to 8) in cows fed Lac-HP. Dietary P level in the dry period or in the lactation period did not affect milk fat and milk protein concentration ($P > 0.17$). Both milk fat and milk protein concentration decreased with week of lactation, in particular in the first 3 weeks ($P < 0.01$). Dietary P level in the dry period or in the lactation period did not affect milk P concentration ($P > 0.62$). On average, milk P concentrations decreased 25% (from 1.30 to 0.97 g/kg milk) during the first 4 weeks of lactation ($P < 0.01$) and remained essentially unchanged thereafter. Similarly, milk Ca concentrations dropped 26% (from 1.43 to 1.21 g/kg of milk) during the first 4 weeks of lactation and remained stable thereafter ($P < 0.01$). The Ca concentration of milk tended to be greater (i.e. 2.6%; $P = 0.09$) for Lac-LP compared to Lac-HP, whereas dietary P concentration in the dry period did not affect milk Ca concentration ($P = 0.59$).

Table 2. Body weight, dry matter intake (DMI) and plasma inorganic phosphate, calcium, non-esterified fatty acids and β -hydroxybutyric acid concentrations in cows fed experimental diets differing in dietary phosphorus concentrations¹ during the dry period.

Experimental diet	Weeks before parturition						SED	<i>P</i> -value		
	6	5	4	3	2	1		Week (W)	Diet (D)	D×W
	Body weight, kg									
Dry-HP	783	794	802	814	814	833	16.1	< 0.01	0.36	0.11
Dry-LP	770	777	792	797	810	809				
	DMI, kg/d									
Dry-HP	14.2	14.2	14.8	14.3	13.5	12.1	0.55	< 0.01	0.78	0.61
Dry-LP	14.6	14.2	14.4	14.0	13.2	11.9				
	Inorganic phosphate, mM									
Dry-HP	-	-	1.75	-	1.74	1.75	0.052	0.60	0.15	0.47
Dry-LP	-	-	1.65	-	1.70	1.69				
	Calcium, mM									
Dry-HP	-	-	2.34	-	2.32	2.35	0.040	0.82	0.05	0.36
Dry-LP	-	-	2.42	-	2.42	2.40				
	Non-esterified fatty acids, mM									
Dry-HP	-	-	0.09	-	0.12	0.22	0.028	< 0.01	0.37	0.52
Dry-LP	-	-	0.08	-	0.11	0.18				
	β-hydroxybutyric acid, mM									
Dry-HP	-	-	0.49	-	0.52	0.53	0.038	0.67	0.40	0.07
Dry-LP	-	-	0.57	-	0.53	0.50				

¹ Dry-HP and Dry-LP = experimental diets fed during the dry period (Dry) high in phosphorus concentration (HP) or low in phosphorus concentration (LP) containing either 3.6 or 2.2 g P/kg DM, respectively. Inorganic phosphate, Ca, non-esterified fatty acids and β -hydroxybutyric acid were sampled during 4, 2, 1 weeks before calving. SED = Standard error of difference.

Table 3. Numbers of cows associated with hypocalcemia when fed experimental diets differing in dietary P concentrations¹ during both the dry period and lactation.

Dry period	Dry-HP		Dry-LP		<i>P</i> -value
Lactation	Lac-HP	Lac-LP	Lac-HP	Lac-LP	
No. of cases/No. of total cows					
CaMg i.v. treatment	5/15	4/14	4/15	2/15	0.81
Plasma Ca < 2.0 mM first week pp	14/15	12/14	14/15	5/15	0.02
Plasma Ca < 2.0 mM during week 2-8	2/15	2/14	0/15	4/15	0.20

¹ Dry-HP, Dry-LP, Lac-HP, and Lac-LP = Experimental diets fed during the dry period (Dry) or lactation period (Lac) with either a high phosphorus (HP) or a low phosphorus (LP) concentration. The diets contained either 3.6 (HP) or 2.2 (LP) g P/kg DM during the dry period and 3.8 (HP) or 2.9 (LP) g P/kg DM during lactation. CaMg i.v. = Intravenous treatment with 7.44 and 4.28 g of Ca and Mg, respectively. Week pp = Week post-partum.

Table 4. Plasma calcium, non-esterified fatty acids and β -hydroxybutyric acid concentrations in cows fed experimental diets differing in dietary P concentrations¹ during both the dry period (Dry) and lactation period (Lac). All values are expressed as mM.

Experimental diets		Sampling day after calving									SED	<i>P</i> -values ²				
		0	1	2	3	7	14	21	28	56		Sampling day (S)	Dry	Lac	Dry × Lac	S × Lac
		Calcium														
Dry-HP	Lac-HP	1.81	1.74	2.02	2.20	2.30	2.34	2.33	2.33	2.29	0.091	< 0.01	< 0.01	0.01	0.87	0.32
	Lac-LP	1.85	1.89	2.12	2.38	2.34	2.42	2.40	2.28	2.47						
Dry-LP	Lac-HP	1.93	1.88	2.07	2.39	2.28	2.40	2.45	2.40	2.44						
	Lac-LP	2.05	2.16	2.25	2.37	2.39	2.38	2.44	2.44	2.44						
		Non-esterified fatty acids														
Dry-HP	Lac-HP	0.99	0.74	0.67	0.69	0.56	0.39	0.38	0.32	0.17	0.110	< 0.01	0.27	0.61	0.74	0.53
	Lac-LP	0.94	0.93	0.67	0.65	0.59	0.44	0.37	0.25	0.14						
Dry-LP	Lac-HP	0.92	0.67	0.66	0.59	0.47	0.28	0.29	0.25	0.13						
	Lac-LP	0.78	0.66	0.71	0.65	0.62	0.39	0.38	0.26	0.21						
		β-hydroxybutyric acid														
Dry-HP	Lac-HP	0.62	0.80	0.88	0.83	0.82	0.71	0.86	0.99	0.80	0.150	< 0.01	0.77	0.26	0.55	0.16
	Lac-LP	0.67	0.87	1.07	0.79	0.90	0.68	0.69	0.70	0.71						
Dry-LP	Lac-HP	0.54	0.83	0.89	0.80	0.80	0.93	0.82	0.90	0.99						
	Lac-LP	0.53	0.65	0.73	0.82	1.01	0.66	0.71	0.66	0.81						

¹ Dry-HP, Dry-LP, Lac-HP, and Lac-LP = experimental diets fed during the dry period (Dry) period or lactation period (Lac) with either a high phosphorus (HP) or a low phosphorus (LP) concentration. The diets contained either 3.6 (HP) or 2.2 (LP) g P/kg dry matter (DM) during the dry period and 3.8 (HP) or 2.9 (LP) g P/kg DM during lactation.

² No significant Dry \times Sampling day and Lac \times Dry \times Sampling day effects were detected for all parameters ($P \geq 0.38$).
SED = Standard error of difference.

Table 5. Post-calving feed intake, milk production, body weights and energy balances in cows fed experimental diets differing in dietary P concentrations¹ during both the dry period (Dry) and lactation (Lac).

Experimental diets		Week of lactation								SED	<i>P</i> -values				
		1	2	3	4	5	6	7	8		Week	Dry	Lac	Dry × Lac	Week × Lac
		Total DMI (kg/day)								0.97	< 0.01	0.19	0.71	0.86	0.06
Dry-HP	Lac-HP	14.4	17.6	19.0	19.0	21.1	22.3	22.3	22.7						
	Lac-LP	14.3	15.6	17.8	19.7	20.9	21.3	22.1	22.4						
Dry-LP	Lac-HP	14.9	17.8	20.1	20.0	21.4	21.6	22.1	23.0						
	Lac-LP	15.2	16.9	18.9	20.3	22.4	22.5	23.1	23.0						
		Milk yield (kg/day)								2.37	< 0.01	0.32	0.86	0.94	0.13
Dry-HP	Lac-HP	27.8	38.0	41.5	41.6	43.1	43.4	42.2	42.7						
	Lac-LP	29.9	37.3	39.5	42.5	43.1	43.5	43.4	42.0						
Dry-LP	Lac-HP	28.5	38.3	42.2	44.3	44.8	44.2	44.3	44.7						
	Lac-LP	30.8	38.3	41.6	44.4	45.7	45.2	45.0	43.5						
		FPCM yield (kg/day)								2.36	< 0.01	0.51	0.49	0.85	0.04
Dry-HP	Lac-HP	32.7	41.1	44.1	42.3	42.0	42.0	40.0	41.2						
	Lac-LP	36.4	40.1	40.9	42.7	42.4	42.8	42.5	41.3						
Dry-LP	Lac-HP	33.3	40.5	42.6	43.6	42.8	42.1	41.6	42.2						
	Lac-LP	37.1	41.6	43.3	44.6	44.8	43.9	43.1	43.1						
		Energy balance (kJ/kg ^{0.75} /day)								54.2	< 0.01	0.74	0.27	0.73	0.11
Dry-HP	Lac-HP	-358	-399	-403	-350	-238	-181	-126	-141						
	Lac-LP	-446	-479	-391	-335	-245	-242	-207	-152						
Dry-LP	Lac-HP	-358	-374	-305	-329	-242	-211	-174	-155						
	Lac-LP	-439	-455	-397	-336	-242	-202	-167	-164						
		Body weight (kg)								19.1	< 0.01	0.56	0.12	0.22	0.09
Dry-HP	Lac-HP	744	728	716	706	703	707	707	709						
	Lac-LP	712	684	672	672	669	673	674	677						
Dry-LP	Lac-HP	718	707	695	687	681	679	681	686						
	Lac-LP	711	690	681	679	683	682	685	684						

¹ Dry-HP, Dry-LP, Lac-HP, and Lac-LP = Experimental diets fed during the dry period (Dry) period or lactation period (Lac) with either a high phosphorus (HP) or a low phosphorus (LP) concentration. The diets contained either 3.6 (HP) or 2.2 (LP) g P/kg DM during the dry period and 3.8 (HP) or 2.9 (LP) g P/kg DM during lactation. FPCM = Fat and Protein Corrected Milk. No significant Dry × Week and Lac × Dry × Week effects were detected for all parameters ($P \geq 0.20$). SED = Standard error of difference.

Table 6. Concentrations of phosphorus, calcium, protein, fat, and lactose in milk from cows fed experimental diets differing in dietary P concentrations¹ during both the dry period (Dry) and lactation (Lac). All values are expressed as g/kg.

Experimental diets		Week of lactation								SED	<i>P</i> -values				
		1	2	3	4	5	6	7	8		Week	Dry	Lac	Dry × Lac	Week × Lac
		Phosphorus													
Dry-HP	Lac-HP	1.30	1.08	1.02	0.98	0.96	0.95	0.98	0.96	0.033	< 0.01	0.62	0.91	0.40	0.18
	Lac-LP	1.26	1.06	1.00	0.98	0.97	0.96	0.96	0.97						
Dry-LP	Lac-HP	1.35	1.08	0.98	0.96	0.95	0.92	0.94	0.93						
	Lac-LP	1.29	1.09	1.01	0.97	0.95	0.94	0.94	0.97						
		Calcium													
Dry-HP	Lac-HP	1.39	1.31	1.24	1.19	1.18	1.17	1.17	1.17	0.033	< 0.01	0.59	0.09	0.31	0.53
	Lac-LP	1.45	1.34	1.28	1.24	1.23	1.21	1.22	1.23						
Dry-LP	Lac-HP	1.46	1.33	1.25	1.18	1.17	1.16	1.16	1.14						
	Lac-LP	1.42	1.35	1.26	1.21	1.19	1.18	1.19	1.19						
		Protein													
Dry-HP	Lac-HP	42.0	35.0	32.5	31.4	31.1	30.6	31.0	31.0	0.95	< 0.01	0.67	0.36	0.68	0.30
	Lac-LP	39.7	35.1	32.3	31.8	31.4	31.2	31.9	31.6						
Dry-LP	Lac-HP	40.3	34.5	32.0	31.0	30.6	29.9	30.5	30.7						
	Lac-LP	41.0	35.4	33.2	31.8	31.6	31.0	31.0	31.4						
		Fat													
Dry-HP	Lac-HP	51.4	46.0	45.2	43.1	39.1	38.7	36.8	38.3	2.05	< 0.01	0.23	0.17	0.42	0.12
	Lac-LP	54.2	46.2	45.2	42.3	39.7	39.9	38.6	39.6						
Dry-LP	Lac-HP	49.2	44.0	41.5	39.6	37.7	37.8	36.3	36.8						
	Lac-LP	55.1	47.7	43.7	40.6	39.4	36.8	37.4	39.4						
		Lactose													
Dry-HP	Lac-HP	43.2	45.3	46.4	45.9	46.1	45.8	45.8	46.0	0.53	< 0.01	0.92	0.62	0.71	0.07
	Lac-LP	42.8	45.2	45.8	46.3	46.3	46.3	46.5	46.1						
Dry-LP	Lac-HP	43.1	45.3	45.9	46.3	46.1	46.0	45.9	45.9						
	Lac-LP	42.7	44.9	45.9	46.4	46.4	46.7	46.4	46.6						

¹ Dry-HP, Dry-LP, Lac-HP, and Lac-LP = Experimental diets fed during the dry period (Dry) period or lactation period (Lac) with either a high phosphorus (HP) or a low phosphorus (LP) concentration. The diets contained either 3.6 (HP) or 2.2 (LP) g P/kg DM during the dry period and 3.8 (HP) or 2.9 (LP) g P/kg DM during lactation. No significant Dry × Week and Lac × Dry × Week effects were detected for all parameters ($P \geq 0.31$). SED = Standard error of difference.

DISCUSSION

P Intake, Requirement, and Plasma Pi Concentrations

The absolute intakes of P (g/d) mirrored the various dietary P concentrations of the experimental diets in the current study (Figure 2). During the dry period, when the high P diet was fed the mean P intake was greater than the P requirement recommended by either the NRC (2001) or CVB (2012) system, while P intake was approximately equal to P requirements when the low P diet was fed. These findings are in line with the observation that hypophosphatemia (i.e. plasma Pi < 1.3 mM; Goff, 1999) did not occur during the dry period. Plasma Pi concentrations in the dry period were only numerically lower (4%) with LP compared with HP and values are similar to those reported by Peterson et al. (2005) when cows were fed 2.1 g P/kg DM. During the first week of lactation, P intakes were lower than those recommended by the NRC (2001) and CVB (2012), irrespective of the P concentration of experimental diets. This observation is most likely explained by the low DMI during the first week of lactation. Phosphorus intakes during weeks 3 until 8 of lactation were similar to the P requirements in both the NRC (2001) and CVB (2012) system when Lac-HP was fed. In contrast to the high P diet, with the low P diet P intakes during week 3 until 8 were ~ 36% (NRC, 2001) or ~ 21% (CVB, 2012) lower than requirements. This is largely in line with the current observations on plasma Pi concentrations (Figure 1). Hypophosphatemia did not occur in cows receiving the Lac-HP diet, except for d0 and 1 after calving. Hypophosphatemia did occur during the first 2 to 3 weeks after calving when Lac-LP was fed (except for the first 3 days in cows having received the high P level in the dry period), and plasma Pi values were restored to values ~ 1.3 mM from 4 weeks after calving onwards. Plasma Pi concentrations shortly after parturition were considerably lower (1.27 mM at d0 and d1 post-partum) than in the week before calving (1.72 mM). This sharp drop in plasma Pi concentration is a common phenomenon in dairy cattle. The high P losses with milk immediately after calving may have contributed to this drop in plasma Pi concentration, but it is unlikely to be the sole reason. A similar drop around parturition also occurs in mastectomized cows (Goff et al., 2002). The decrease in plasma Pi concentration may be related to the reduction in feed intake around calving, an increase in plasma corticosteroids, and more generally a redistribution of intracellular and extracellular P, because changes in the balance between intracellular and extracellular P may occur suddenly (Grünberg, 2014).

After calving, plasma Pi concentrations were affected by a 3-way week × dry period P level × lactation period P level interaction. This 3-way interaction is not easy to explain because the current data do not provide clues on the regulation of P absorption (Goff, 2004) and P mobilization from bone (Wu et al., 2000; Moreira et al., 2009; Puggaard et al., 2014). It can be speculated that the observed transient hypophosphatemia in cows fed low P diets after calving has triggered mobilization of P from the skeleton (Puggaard et al., 2014). Mobilization of P from bones may supply significant amounts of P (500 to 600 g) during the first weeks of lactation (Wu et al., 2000). Furthermore, it cannot be excluded that the efficiency of P absorption was affected by the level of dietary P prepartum. It can be suggested that the feeding of high P versus low P diets during the dry period caused a low efficiency of P absorption before calving (Shirazi-Beechey et al., 1996). This notion is in line with the observed rapid increase in plasma Pi concentrations when cows switched from Dry-LP to Lac-HP but the initial increase

in plasma Pi concentrations when cows switched from Dry-HP to Lac-LP is not in line with the aforementioned reasoning on the inverse relationship between P intake and efficiency of P absorption. Caution, however, is warranted in interpreting plasma Pi concentrations. Although plasma Pi concentration is widely used to diagnose P status of dairy cows, it is an unreliable parameter to diagnose P balance disorders (Grünberg, 2014). Thus, future studies are needed to evaluate if current P recommendations as set by NRC (2001) and CVB (2012) and others may be further lowered.

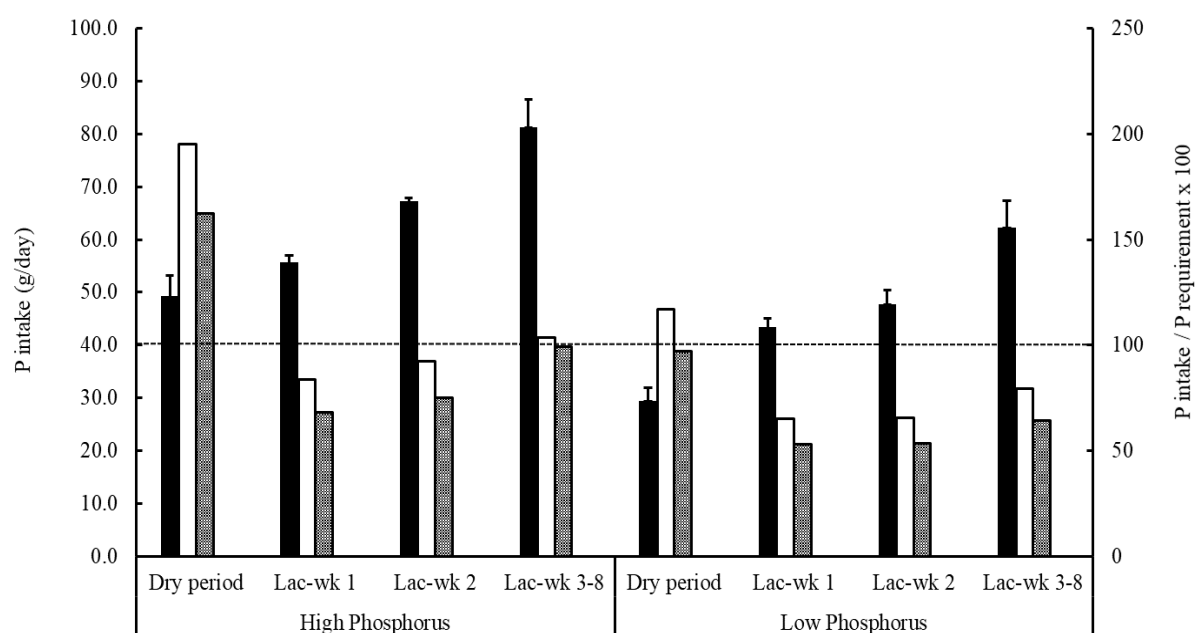


Figure 2. Mean total P intakes expressed as g/day (■, left y-axis) and as an index relative to recommended P intake (right y-axis, CVB (□) and NRC (▨)) during the dry period, the first week of lactation (Lac-wk 1), the second week of lactation (Lac-wk 2) and weeks 3 until 8 of lactation (Lac-wk 3-8). The cows were either fed a low P (LP) or a high P (HP) diet during the dry period (Dry) and after calving (Lac). High P diets contained 3.6 g P/kg DM during the dry period and 3.8 g P/kg DM after calving while low P diets contained 2.2 g P/kg DM during the dry period and 2.9 g P/kg DM after calving. The P intakes during lactation (expressed either as absolute values or as an index relative to recommended P intakes) are the mean values of the 2 groups (i.e. Dry-HP and Dry-LP) fed either HP or LP during the lactation period. Recommended P intakes are calculated using the actual DMI and milk yield production (Table 5) and the actual P concentration of milk (Table 6). The dotted black line represents P intake being equal with P requirement. The error bar represents the standard deviation associated with the mean P intake.

Periparturient Hypocalcemia

The current results on plasma Ca concentrations clearly indicate that the feeding of low P diets (i.e. 2.2 g P/kg DM; P intake roughly equal to CVB (2012) and NRC (2001) requirements) during the dry period improves plasma Ca concentrations postpartum (week 1-8) compared with feeding high P diets (3.6 g P/kg DM; above requirements). These results are in line with Lean et al. (2006). In our study, similar to the impact of low P diets fed during the dry period on plasma Ca levels in week1 to 8 after calving, low P diets fed during the lactation period

increased plasma Ca levels. Puggaard et al. (2014) also reported elevated plasma Ca levels in week 2 to 5 postpartum upon feeding diets containing 2.3 g P/kg DM compared with 3.4 g P/kg DM. We did not find interactions between sampling day, dietary P level during the dry period, and dietary P level during the lactation period on plasma Ca concentrations. This indicates that the impact on plasma Ca levels of low vs high dietary P levels in the lactation period did not depend on the dietary P level in the dry period. However, the number of hypocalcemic cows (having plasma Ca levels less than 2 mM) in the first week postpartum was smaller when the low dietary P concentration in the dry period was continued in the lactation period. The current data on periparturient plasma Ca concentration warrant the feeding of low P diets to dairy cows, but they do not identify underlying mechanisms explaining our observations. Phosphorus supply may have interfered with vitamin D₃ metabolism (Köhler et al., 2020) and thus Ca metabolism. In rats, hyperphosphatemia (serum P > 3 mM) reduced the activity of renal 25-hydroxyvitamin D1 alpha-hydroxylase leading to a reduced production of 1,25(OH)₂ vitamin D₃, thereby, causing hypocalcemia (Tallon et al., 1996; Silver et al., 1999; Masuyama et al., 2000). Vitamin D₃ metabolism may likewise be affected in dairy cows, thereby, explaining the effect of low P diets on Ca metabolism (Julien et al., 1977; Kichura et al., 1982; Barton et al., 1987). Indeed, in periparturient P-deprived cows, a typical increase in plasma 1,25-dihydroxyvitamin D concentration and bone resorption occurred despite a less pronounced rise in plasma parathyroid hormone concentrations (Cohrs et al., 2018). These authors suggested either greatly increased sensitivity to parathyroid hormone or bone mobilization independent of parathyroid hormone occurred at low dietary P levels.

DMI, Milk Production, NEFA, and BHBA

The current observations on DMI and milk production are corroborated by various studies (Valk and Sebek, 1999; Wu and Satter, 2000; Peterson et al., 2005; Puggaard et al., 2014) who also reported that post-partum diets containing at least 2.8 g P/kg DM do not compromise DMI and milk yield. Furthermore, the current results on EB, plasma NEFA, plasma BHBA, and BW are in line with our observations on DMI and MY. The lack of effect of the current low P diets on FPCM yield, and the various indices related to energy status of the cows, is most likely explained by the fact that DMI was hardly affected when the low P diet was fed. It is well known that fiber digestion is an important determinant of DMI (Oba and Allen, 1999) and various studies have shown that rumen fiber digestion is sensitive to the P supply of the rumen microbes (Hall et al., 1961; Komisarczuk-Bony and Durand, 1991; Puggaard et al., 2011). Komisarczuk-Bony and Durand (1991) reported that a minimum P concentration of 0.5 mM in rumen fluid is required for optimum fiber digestion under *in vitro* conditions. This observation is in line with Rodehutsord et al. (1994) who reported that the feeding of a diet containing 1.4 g P/kg DM to goats was associated with rumen P concentrations ~ 5 mM without an adverse effect on DMI. In contrast, Puggaard et al. (2011) did not observe decreased DMI of dairy cattle upon feeding diets with 2.4 g P/kg DM, whereas Puggaard et al. (2014) reported that a diet containing 2.3 g P/kg DM did compromise DMI compared with 3.4 g P/kg DM. However, these authors did not measure rumen P concentrations which hinders further speculation on cause and effect on their observations on DMI. Overall, at the milk production levels and lactation stage

in the current study, our results indicate that a dietary P concentration of 2.9 g P/kg DM is sufficient to maintain DMI and FPCM production in dairy cows.

In the current study, P and Ca concentrations of milk did decrease with week of lactation. These minerals have been shown to be positively related with the concentration of milk protein and (for milk P only) to a lesser extent with the concentration of milk lactose (Bijl et al., 2013; Klop et al., 2014). The significant and large decrease in milk protein concentration with week of lactation, and the quantitatively less pronounced increase in lactose concentration, may help to explain the decrease in Ca and P concentrations in milk with lactation week. In the NRC (2001) and CVB (2012) requirement systems for dairy cows, a fixed concentration of 0.9 or 1.0 g P/kg milk is assumed to calculate net P requirements. The large variation in milk P concentration (between 0.92 and 1.35 g/kg) observed during the first 8 weeks postpartum indicates P requirements of dairy cattle in specific weeks of lactation may be under- or overestimated in current P requirement systems.

CONCLUSIONS

Reduction in dietary P concentration from 3.6 (exceeding P requirements) to 2.2 g/kg DM (equal to P requirement) during the dry period increased plasma Ca concentrations during the first 8 weeks after calving without affecting DMI or milk production. Likewise, a reduction in dietary P concentration from 3.8 g/kg DM (equal to P requirements in week 3 to 8 postpartum) to 2.9 g/kg DM (below P requirements) after calving also improved plasma Ca levels without compromising DMI and milk production. Our findings indicate that feeding diets low in P concentration in late gestation or early lactation has a positive influence on periparturient Ca homeostasis and may help to prevent (sub-) clinical hypocalcemia during the first week postpartum. Current NRC (2001) and CVB (2012) estimates of the dietary P requirements in early lactating dairy cows may be too high. The present study was short term and, therefore, long term impact of this dietary P strategy needs to be investigated before practical recommendations can be made.

ACKNOWLEDGEMENTS

The authors acknowledge the staff of Dairy Campus (Leeuwarden, the Netherlands) for their contribution to this experiment. This research was commissioned and funded by the Ministry of Agriculture, Nature and Food Quality (the Hague, the Netherlands) within the framework of Policy Support Research theme 'Feed4Foodure' (BO-31.03-005-001; TKI-AF12039), and by the Vereniging Diervoederonderzoek Nederland (Rijswijk, the Netherlands).

REFERENCES

- Barton, B. A., N. A. Jorgensen, and H. F. DeLuca. 1987. Impact of prepartum dietary phosphorus intake on calcium homeostasis at parturition. *J. Dairy Sci.* 70: 1186-1191.
- Bijl, E., H. J. F. van Valenberg, T. Huppertz, and A. C. M. van Hooijdonk. 2013. Protein, casein, and micellar salts in milk: Current content and historical perspectives. *J. Dairy Sci.* 96: 5455-5464.

- Cohrs, I., M. R. Wilkens, and W. Grünberg. 2018. Short communication: Effect of dietary phosphorus deprivation in late gestation and early lactation on the calcium homeostasis of periparturient dairy cows. *J. Dairy Sci.* 101: 9591-9598.
- Cordell, D., J. O. Drangert, and S. White. 2009. The story of phosphorus: Global food security and food for thought. *Global Environ. Change* 19: 292-305.
- CVB. 2012. CVB farm animal feeding advices 2012. CVB volume 50, the Hague, the Netherlands.
- Goff, J. P. 1999. Treatment of calcium, phosphorus, and magnesium balance disorders. *Vet. Clin. North Am. Food Anim. Pract.* 15: 619-639.
- Goff, J. P., K. Kimura, and R. L. Horst. 2002. Effect of mastectomy on milk fever, energy, and vitamins A, E, and β -carotene status at parturition. *J. Dairy Sci.* 85: 1427-1436.
- Goff, J. P. 2004. Macro mineral disorders of transition cow. *Vet. Clin. Food Anim.* 20: 471-494.
- Grünberg, W. 2014. Treatment of phosphorus balance disorders. *Vet. Clin. North Am. Food Anim. Pract.* 30: 383-408.
- Hall, O. G., H. D. Baxter, and C. S. Hobbs. 1961. Effect of phosphorus in different chemical forms on in vitro cellulose digestion by rumen microorganisms. *J. Anim. Sci.* 20: 817-819.
- Heuer, C., W. M. Van Straalen, Y. H. Schukken, A. Dirkzwager, and J. P. T. M. Noordhuizen. 2001. Prediction of energy balance in high yielding dairy cows with test-day information. *J. Dairy Sci.* 84: 471-481.
- ISO. 1999a. Animal feeding stuffs. Determination of fat content. ISO 6492. International Organization for Standardization (ISO), Geneva, Switzerland.
- ISO. 1999b. Animal feeding stuffs. Determination of moisture and other volatile matter content. ISO 6496. ISO, Geneva, Switzerland.
- ISO. 2002. Animal feeding stuffs. Determination of crude ash. ISO 5984. ISO, Geneva, Switzerland.
- ISO. 2004. Animal feeding stuffs. Enzymatic determination of total starch content. ISO 15914. ISO, Geneva, Switzerland.
- ISO. 2005. Animal feeding stuffs. Determination of nitrogen content and calculation of crude protein content-Part 1: Kjeldahl method. ISO 5983-1. ISO, Geneva, Switzerland.
- ISO. 2013. Whole milk. Determination of milk fat, protein and lactose content - Guidance on the operation of mid-infrared instruments. ISO 9622. ISO, Geneva, Switzerland.
- Julien, W. E., H. R. Conrad, J. W. Hibbs, and W. L. Crist. 1977. Milk fever in dairy cows. viii. Effect of injected vitamin D₃ and calcium and phosphorus intake on incidence. *J. Dairy Sci.* 60: 431-436.
- Kebreab, E., N. E. Odongo, B. W. McBride, M. D. Hanigan, and J. France. 2008. Phosphorus utilization and environmental and economic implications of reducing phosphorus pollution from Ontario dairy cows. *J. Dairy Sci.* 91: 241-246.
- Kichura, T. S., R. Host, D. C. Beitz, and E. T. Littledike. 1982. Relationships between prepartal dietary calcium and phosphorus, vitamin D metabolism, and parturient paresis in dairy cows. *J. Nutr.* 112: 480-487.
- Klop, G., J. L. Ellis, A. Bannink, E. Kebreab, J. France, and J. Dijkstra. 2013. Meta-analysis of factors that affect the utilization efficiency of phosphorus in lactating dairy cows. *J. Dairy Sci.* 96: 3936-3949.

- Klop, G., J. L. Ellis, M. C. Blok, G. G. Brandsma, A. Bannink, and J. Dijkstra. 2014. Variation in phosphorus content of milk from dairy cattle as affected by differences in milk composition. *J. Agric. Sci.* 152: 860-869.
- Köhler, M. O., W. Grünberg, N. Schnepel, S. A. Muscher-Banse, A. Rajaeerad, J. Hummel, G. Breves, and M. R. Wilkens. 2020. Dietary phosphorus restriction affects bone metabolism, vitamin D metabolism and rumen fermentation traits in sheep. *J. Anim. Physiol. Anim. Nutr.* 105: 35-50.
- Komisarczuk-Bony, S., and M. Durand. 1991. Effects of minerals on microbial metabolism. Ed. J. P. Jouany. Institut National de la Recherche Agronomique (INRA), Paris. *Rumen Microbial Metabolism and Ruminant Digestion*. 179-198.
- Lean, I. J., P. J. DeGaris, D. M. McNeil, E. Block. 2006. Hypocalcemia in dairy cows: meta-analysis and dietary cation anion difference theory revisited. *J. Dairy Sci.* 89: 669-684.
- Masuyama, R., M. Uehara, and K. Suzuki. 2000. High P diet induces acute secretion of parathyroid hormone without alteration of serum calcium levels in rats. *Biosci. Biotechnol. Biochem.* 64: 2316-2319.
- Moreira, V. R., L. K. Zeringue, C. C. Williams, C. Leonardi, and M. E. McCormick. 2009. Influence of calcium and phosphorus feeding on marker of bone metabolism in transition cows. *J. Dairy Sci.* 92: 5189-5198.
- NRC. 2001. Nutrient requirements of Dairy Cattle, 7th revised edition. National Academy of Science, Washington, DC.
- Oba, M., and M. S. Allen. 1999. Evaluation of the importance of the digestibility of neutral detergent fiber from forage: effects on dry matter intake and milk yield of dairy cows. *J. Dairy Sci.* 82: 589-596.
- Peterson, A. B., M. W. Orth, J. P. Goff, and D. K. Beede. 2005. Periparturient responses of multiparous Holstein cows fed different dietary phosphorus concentrations prepartum. *J. Dairy Sci.* 88: 3582-3594.
- Puggaard, L., N. B. Kristensen, and J. Sehested. 2011. Effect of decreasing dietary phosphorus supply on net recycling of inorganic phosphate in lactating dairy cows. *J. Dairy Sci.* 94: 1420-1429.
- Puggaard, L., P. Lund, A. Liesegang, and J. Sehested. 2014. Long term effect of reduced dietary phosphorus on feed intake and milk yield in dry and lactating dairy cows. *Livest. Sci.* 159: 18-28.
- Robertson, J. B., and P. J. Van Soest. 1981. The detergent system of analysis and its application to human foods. In: *The analysis of dietary fiber in food* (Eds. James W. P. T. and Theander O.), Marcel Dekker, New York, pp. 123-158.
- Rodehutsord, M., A. Pauen, P. Windhausen, R. Brintrup, and E. Pfeffer. 1994. Effects of drastic changes in P intake on P concentrations in blood and rumen fluid of lactating ruminants. *J. Vet. Med. A.* 41: 611-619.
- Shirazi-Beechey, S. P., J. I. Penny, J. Dyer, I. S. Wood, P. S. Tarpey, D. Scott, and W. Buchan. 1996. Epithelial phosphate transport in ruminants, mechanisms and regulation. *Kidney Int.* 49: 992-996.
- Silver, T., C. Yalcindag, A. Sela-Brown, R. Kilav, and T. Naveh-Many. 1999. Regulation of the parathyroid hormone gene by vitamin D, calcium and phosphate. *Kidney Int.* 56: S2-S7.

- Tallon, S., I. Berdud, A. Hernandez, M. T. Concepcion, Y. Almaden, A. Torres, A. Martin-Malo, A. J. Felsenfeld, P. Aljama, and M. Rodriguez. 1996. Relative effects of PTH and dietary phosphorus on calcitriol production in normal and azotemic rats. *Kidney Int.* 49: 1441-1446.
- Tamminga, S., W. M. Van Straalen, A. P. J. Subnel, R. G. M. Meijer, A. Steg, C. J. G. Wever, and M. C. Blok. 1994. The Dutch protein evaluation system: the DVE/OEB-system. *Livest. Prod. Sci.* 40: 139-155.
- Valk, H., and L. B. J. Sebek. 1999. Influence of long-term feeding of limited amounts of phosphorus on dry matter intake, milk production, and body weight of dairy cows. *J. Dairy Sci.* 82: 2157-2163.
- Van Es, A. J. H. 1978. Feed evaluation for ruminants. I. The systems in use from May 1977-onwards in the Netherlands. *Livest. Prod. Sci.* 5: 331-345.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74: 3583-3597.
- Van Vuuren, A. M., C. J. Van der Koelen, H. Valk, and H. De Visser. 1993. Effects of partial replacement of ryegrass by low protein feeds on rumen fermentation and nitrogen loss by dairy cows. *J. Dairy Sci.* 76: 2982-2993.
- VSN International. 2018. Genstat for Windows 19th Edition. VSN International, Hemel Hempstead, UK.
- Wu, Z., and L. D. Satter. 2000. Milk production and reproductive performance of dairy cows fed concentrations of phosphorus for two years. *J. Dairy Sci.* 83: 1052-1063.

Chapter 3

Effects of low phosphorus diets on phosphorus balance and plasma concentrations of PTH, 25(OH)D₃, and CTX during the transition period in dairy cows

P. Keanthao*, R. M. A. Goselink[†], J. Dijkstra[‡], and J. T. Schonewille*

* Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, the Netherlands

[†] Wageningen Livestock Research, Wageningen University & Research, PO Box 338, 6700 AH Wageningen, the Netherlands

[‡] Animal Nutrition Group, Wageningen University & Research, PO Box 338, 6700 AH Wageningen, the Netherlands

To be submitted

Journal of Dairy Science

ABSTRACT

Our aim was to determine effects of low P diets on plasma hormone levels and bone resorption during the final 4 weeks prepartum and first 8 weeks of lactation. Sixty pregnant multiparous Holstein Friesian dairy cows were assigned to a randomized block design with repeated measurements and dietary treatments arranged according to a 2 × 2 factorial design. The experimental diets contained 3.6 (Dry-HP) or 2.2 (Dry-LP) g P/kg dry matter (DM) during the dry period, and 3.8 (Lac-HP) or 2.9 (Lac-LP) g P/kg DM during 56 days after calving. Both P intake and fecal P excretion decreased in the dry period to increase again in subsequent lactation. Cows fed high dietary P excreted more P in the feces than cows fed low dietary P pre- and postpartum. Cows in both Dry-HP and Dry-LP were in positive P balance in the dry period. Cows in both Lac-LP and Lac-HP were in negative P balance after calving, and the negative P balance after calving was more pronounced with Lac-LP than with Lac-HP. Plasma concentrations of PTH and 25(OH)D₃, and apparent total tract OM and NDF digestibility, were neither affected by any 2-way or 3-way interaction between time of sampling and dietary treatments nor by the P concentration of the experimental diets during pre- and post-partum period. Before calving, plasma CTX concentration decreased with Dry-HP and increased with Dry-LP. After calving, plasma CTX concentrations increased but this increase was more pronounced when Lac-LP instead of Lac-HP was fed. The results suggest that when feeding diets containing low P (2.9 g/kg DM) postpartum, cows excreted less P in the feces than at sufficient dietary P (3.8 g P/kg DM) without negative impact on OM and NDF digestibility. Moreover, feeding postpartum diets containing low P compared with sufficient P enlarged bone resorption and increased plasma CTX concentrations, without increasing plasma concentrations of PTH and 25(OH)D₃, indicating a more prominent role of bone resorption than of increased P absorption to meet P demands in the first 8 weeks postpartum. The present trial focused on the transition period only, and long-term effects of low dietary P on plasma PTH and 25(OH)D₃ and on bone P dynamics in mid and late lactation need to be further investigated.

Key words: transition cow, low phosphorus, blood plasma, bone resorption, carboxy-terminal collagen crosslinks

INTRODUCTION

The efficiency by which dietary phosphorus (P) is secreted with milk (i.e. in % of P intake) varies greatly between farms (Akert et al., 2020; Harrison et al., 2021). Milk P efficiency is positively related to feed efficiency and negatively related to dietary P content (Klop et al., 2013). In view of the detrimental environmental impact of excess P intake by dairy cows (Kebreab et al., 2008), as well as concerns on the exhaustion of natural rock phosphate resources, fine tuning of the current recommendations on dietary P intake towards lower values can be considered opportune. For obvious reasons, the cow's health and production performance are important principles to determine the minimum P intake. Feeding rations with high P content remains common practice, as restricted P supply in late gestation and early

lactation is thought to present a risk for health and productivity of dairy cows (Wächter et al., 2022).

Reduction of the dietary P concentration from 4.4 to 2.1 g/kg dry matter (DM) prepartum has been shown to cause a less pronounced decrease in plasma calcium (Ca) concentration at parturition (Peterson et al., 2005). Cohrs et al. (2018) and Wächter et al. (2022) reported that the feeding of prepartum diets containing 1.5 versus 2.8 g P/kg DM or 1.6 versus 3.0 g P/kg of DM, respectively, caused greater plasma Ca concentrations in periparturient cows. The low dietary P concentrations, and corresponding P intakes, used in these aforementioned studies were lower than recommended by the NRC (2001) and CVB (2012). Recently, Keanthao et al. (2021) showed that feeding diets containing 2.2 versus 3.6 g P/kg DM during the last 6 weeks of the dry period and 2.9 versus 3.8 g P/kg DM from calving up until 56 days in milk (DIM) was instrumental to prevent hypocalcemia in periparturient cows and did neither compromise dry matter intake (DMI) nor milk production. The lower dietary P concentrations were below current recommendations, and it was, therefore, suggested that the current recommendations on P supply of dairy cows can be considered too high. Caution is warranted though, because hypophosphatemia may occur upon feeding P intake levels below current recommendations of postpartum dairy cattle (Cohrs et al., 2018; Keanthao et al., 2021). These observations fuel the notion that the low P diets caused a negative P balance with possibly negative impact on health and production. After feeding low P diets in the prepartum period, Wächter et al. (2022) used postpartum diets with high dietary P content (4.6 g/kg DM) and did not find negative effects of prepartum low P diet on DMI and milk production during lactation. Keanthao et al. (2021) fed low or moderate P diets postpartum upon feeding low P diets prepartum, and those low dietary P intakes postpartum compared with high P intakes in Wächter et al. (2022) may not have been sufficient to prevent significant bone mobilization. However, Keanthao et al. (2021) did not report on the P balances of the cows in their experiment. Such information is considered relevant to substantiate the suggestion that current P recommendations are too high. The current, follow up study, therefore, reports on the aforementioned information gap.

In dairy cows, mobilization of P from bone is common during early lactation (Wu et al., 2000). An increase in net P absorption is ultimately required to terminate the initial skeletal demineralization and to subsequently replenish the P stores in bone (Ekelund et al., 2006). Carboxy-terminal collagen crosslinks (CTX) is an indicator of P mobilization from bone (Puggaard et al., 2014) while calcitriol ($1,25(\text{OH})_2\text{D}_3$) has been implicated to stimulate P absorption in ruminants (Liesegang et al., 2006). A second objective of the present study was, therefore, to determine effects of low P diets on plasma hormone levels and bone resorption during 4 weeks prepartum and 8 weeks postpartum. We hypothesized that the feeding of postpartum diets containing low P (2.9 g/kg DM) versus normal P (3.8 g P/kg DM) induced the release of both CTX and 25 hydroxy vitamin D₃ ($25(\text{OH})\text{D}_3$). The measurement of $25(\text{OH})\text{D}_3$ instead of $1,25(\text{OH})_2\text{D}_3$ was considered opportune to evaluate a P induced response on P absorption, if any, because $25(\text{OH})\text{D}_3$ is a precursor of $1,25(\text{OH})_2\text{D}_3$ (Shekariz-Foumani and Khodaie, 2016) and the measurements of $25(\text{OH})\text{D}_3$ concentrations are less laborious (Hollis and Host, 2007). Finally, Keanthao et al. (2021) reported that neither DMI nor milk production were affected when cows were fed diets containing 2.2 versus 3.6 g P/kg DM during the last 6 weeks of the dry period and 2.9 versus 3.8 g P/kg DM from calving up until 56 DIM. These observations suggest that nutrient digestibility was not influenced by the low dietary P

concentrations. In the current follow up study we, therefore, provide data on apparent total tract OM- and NDF digestibility to provide an, at least partial, explanation for the lack of effect of the low dietary P concentration on DMI and milk production.

MATERIALS AND METHODS

All experimental protocols and interventions were conducted under the Dutch Law on Animal Experimentation and approved (approval nr: AVD401002016653) by the Central Authority for Scientific Procedures on Animals (CCD, the Hague, the Netherlands).

Animals, Experimental Design, Feeding, and Experimental Diets

Full details on animals, experimental design, housing, and feeding, are described by Keanthao et al. (2021). Briefly, sixty multiparous (parity ≥ 3), pregnant Holstein Friesian dairy cows were used. The experiment had a randomized block design with repeated measurements and a 2×2 factorial arrangement of dietary treatments (Figure 1). Within each block, cows were randomly assigned to dry cow rations containing either 3.6 (**Dry-HP**) or 2.2 (**Dry-LP**) g P/kg of DM and, within dry cow treatments, to postcalving rations containing either 3.8 (**Lac-HP**) or 2.9 (**Lac-LP**) g P/kg of DM. Throughout the experiment, fresh drinking water was provided ad libitum. Lactating cows were milked twice daily with a milking interval of 11 to 13 h. The cows left the trial after 56 days of lactation.

The cows received their allocated, freshly mixed, basal rations at regular intervals (3 to 5 times/d). The basal ration was composed of corn silage, grass silage, wheat straw (dry cows only), soybean meal (lactating cows only), and a premix containing either a low or a high P concentration. On a weekly basis, and based on the DM concentration of each individual basal ration component, the required amount of ration ingredients was calculated to prepare the experimental rations. Rations were mixed with the use of a Trioliet™ mixing robot for on average 6 min. The mixed basal diets were supplied in weighing troughs with individual transponder-controlled access gates [Roughage Intake Control system (RIC), Hokofarm Group]. For every visit of a cow to a RIC bin, the start and end time of the visit as well as the start and end weight of the RIC bin were recorded. Next to the mixed basal diet, pelleted concentrates were fed individually with the use of transponder-controlled concentrate feeders (Hokofarm Group). Dry cows received 1 kg/d of compound concentrate, with TiO₂ (10 g TiO₂/kg as fed) added as an indigestible marker, fed in individual concentrate dispensers. One day after calving, cows received 3.0 kg/d of compound concentrate gradually increasing up to 9.3 kg/d at d 21 postpartum including 2 kg of compound concentrate containing 5 g TiO₂/kg as an indigestible marker. The dry cow ration provided on average 5.6 MJ NE_L/kg of DM (calculated according to the Dutch NE-system; van Es, 1978), 57 g/kg of DM of intestinal digestible protein (IDP) and -6 g/kg of DM of rumen-degradable protein balance (RDPB, calculated according to the Dutch DVE/OEB-system; Tamminga et al., 1994), and 3.6 g Ca/kg of DM. On average, the lactation ration provided 6.8 MJ NE_L/kg of DM, 92 g IDP/kg of DM, 7 g RDPB/kg of DM, and 6.1 g Ca/kg of DM.

Dry period, last 6 weeks before calving		Directly after calving up until 56 DIM	
Experimental treatments	No. of cows	Experimental treatments	No. of cows
Dry-LP, 2.2 g P /kg DM	30	Lac-LP, 2.9 g P /kg DM	15
		Lac-HP, 3.8 g P /kg DM	15
Dry-HP, 3.6 g P /kg DM	30	Lac-LP, 2.9 g P /kg DM	15
		Lac-HP, 3.8 g P /kg DM	15

Figure 1. Experimental design of the study (Keanthao et al., 2021). The cows were either fed a low P (LP) or a high P (HP) diet during the dry period (Dry) and after calving (Lac).

Data Collection and Sampling

The sampling procedures of feed and milk are described by Keanthao et al. (2021). Except for the wk of calving, feces were sampled at 2 wks intervals, in wk-4, -2, 2, 4, 6 and 8 relative to the (expected) calving date. In each sampling wk, feces were sampled during 3 consecutive days at different time intervals (approximate times of sampling: d1, 0800 and 1400 h; d2, 1000 and 1400 h; d3, 1200 and 1800 h). Then, the 3-d feces collections of each individual cow were pooled and the pooled samples were subsequently stored at -20 °C until analysis for Ti, P, crude ash and NDF.

Before calving, blood samples were collected from the jugular vein on a fixed day (i.e. Thursday) of the week in wk-4, -2 and -1 relative to calving. During lactation, blood was sampled immediately after calving (d0), and after calving on d1, 2, 3, 7, 14, 21, 28, and 56. Blood was collected in serum separator tubes and tubes were centrifuged for 15 min at 3,000 × g and 4 °C within 1 h after collection. Then, blood plasma from each tube was transferred to 3 plasma tubes and stored at -20 °C until analysis for Ca, inorganic phosphate (Pi), parathyroid hormone (PTH), 25(OH)D₃, and CTX.

Chemical Analysis

Chemical analyses of feed and milk was performed as described by Keanthao et al. (2021). All plasma samples were analyzed for their plasma Pi concentration (with ammonium molybdate) and Ca concentration (with Arsenazo III) using an automatic analyzer (ABX Pentra 400, Horiba, Europe GmbH, Langenhagen, Germany). The concentrations of CTX, PTH, and 25(OH)D₃ were determined by means of commercially available ELISA kits, i.e. CTX: Serum crosslaps ELISA (Immundiagnostic Systems (ids) GmbH, Frankfurt am Main, Germany), PTH: Bovine Intact PTH ELISA Kit (Immuntopics Inc., San Clement, CA), and 25(OH)D₃ direct day ELISA (Immundiagnostik AG, Bensheim, Germany).

Feces samples were air dried at 60 °C for 24 h and the DM of the air-dried samples was determined with the use of a forced-air oven (105 °C, 24 h) according to ISO standards (1999). The crude ash concentration of feces was gravimetrically determined after incineration at 550 °C (ISO, 2002). The NDF concentration was analyzed as described by Van Soest et al. (1991) and expressed without residual ash. The concentrations of P were determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Eurofins Agro, Wageningen, the Netherlands). The Ti concentration of feed and feces was analyzed spectroscopically after sulfuric acid digestion in the presence of Cu as described by Nichols et al. (2018).

Calculations and Statistical Analysis

Prior to statistical analysis, the daily values on DMI and milk yield were averaged per week. Feces excretion (kg DM/d) was calculated as Ti intake (g/d) / Ti concentration of feces (g/kg of DM). The apparent digestibility (% of intake) of P, OM and NDF was calculated as [(nutrient intake, g/d – nutrient excretion with feces, g/d) / nutrient intake, g/d] × 100. For dry cows, the P balance was calculated by subtracting the daily P excretion with feces from the daily P intake. The P balance of lactating cows was calculated taking the secretion of P with milk also into account. The amount of P excreted with urine in healthy ruminants is negligible (Grünberg, 2014) and was, therefore, ignored to calculate the P balance of the cows. Due to a treatment allocation error postpartum, one cow in the Dry-HP, Lac-LP group was excluded from all statistical analyses in the postpartum period.

Data related to total tract apparent digestibility, P balance and plasma concentrations were statistically analyzed using ANOVA with repeated measures (Genstat, 19th edition, 2018) for the prepartum and the postpartum periods separately. In the dry period the model contained main effects of block (block 1-15), dry-period treatment (Dry-LP, 2.2 g of P/kg of DM; Dry-HP, 3.6 g of P/kg of DM), week (wk4, 2, and 1 before parturition except digestibility and P balance variables that did not include wk1 before parturition), and the interaction effect dry-period treatment × week. The model for digestibility and P balance variables in the lactation period contained main effects of block, dry-period treatment, lactation-period treatment (Lac-LP, 2.9 g of P/kg of DM; Lac-HP, 3.8 g of P/kg of DM), week (wk2, 4, 6, and 8 after parturition), and the interaction effects lactation-period treatment × week, dry-period treatment × week, lactation-period treatment × dry-period treatment, and lactation-period treatment × dry-period treatment × week. For plasma variables in the lactation period, the effect of week was replaced with effect of sampling day after calving (d0, 1, 2, 3, 7, 14, 21, 28, and 56). Linear regression analysis was used to determine the relationships, if any, between group (i.e. all cows in the same treatment and at the same sampling day relative to calving) mean plasma Pi and Ca concentrations and PTH, 25(OH)D₃, and CTX concentrations. Prior to regression analysis, plasma values on PTH, 25(OH)D₃ (both expressed as ng/L), and CTX (expressed as ng/ml) were logarithmically converted using the natural logarithm. Furthermore, the data from the dry cows were categorized according to their postcalving dietary treatments (Figure 1) to obtain equal weights of the group mean plasma values (i.e. $n = 15/\text{mean}$) before and after calving. Differences were considered significant at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

RESULTS

Dry Period: P Balance, OM and NDF Digestibility

Except for P intake, data related to the P balance and the apparent digestibility of OM and NDF were not affected by the interaction between diet and wk ($P \geq 0.12$) during the dry period (Table 1). During the dry period, P intake decreased in time irrespective the dietary P concentration, but the decrease was slightly greater ($P = 0.04$) in the cows fed Dry-HP compared with Dry-LP. Fecal P excretion was 38.8% lower ($P < 0.01$) when Dry-LP instead of Dry-HP was fed and 14.4% lower ($P < 0.01$) at 2 versus 4 wk before calving. Phosphorus balance, absolute (g/d) and relative (% of P intake) apparent P absorption were neither affected by diet nor by wk ($P \geq 0.12$).

The apparent total tract OM digestibility was neither affected by the level of dietary P nor by wk ($P \geq 0.28$) while apparent total tract NDF digestibility tended to be lower ($P = 0.07$) at 2 wk before calving without any differences between the two dietary treatments ($P = 0.50$).

Table 1. Phosphorus balance, apparent P absorption and apparent total tract digestibility of organic matter (OM) and neutral detergent fiber (NDF) in pregnant cows fed experimental rations differing in dietary P concentrations during the dry period¹.

Item	Week relative to calving		SED	P-value	Experimental diet ²		SED	P-value
	-4	-2			Dry-HP	Dry-LP		
P intake (g/d)	43.0	38.9	0.82	< 0.01	51.2	30.4	1.60	< 0.01
P feces (g/d)	38.9	33.3	1.41	< 0.01	44.8	27.4	2.86	< 0.01
Apparent P absorption								
Absolute (g/d)	4.1	5.5	1.41	0.31	6.6	2.9	2.59	0.16
Relative (% of intake)	8.5	14.2	3.77	0.12	12.5	10.3	6.00	0.72
P balance (g/d)	4.1	5.5	1.41	0.31	6.6	2.9	2.59	0.16
Digestibility (% of intake)								
OM	57.2	55.3	1.56	0.28	57.3	55.1	2.95	0.39
NDF	53.6	49.9	1.91	0.07	52.8	50.8	2.97	0.50

¹ Except for P-intake, $P_{\text{diet} \times \text{week}} \geq 0.12$. $P_{\text{diet} \times \text{week}}$ for P intake = 0.04. Week 4 ap and Dry-HP = 53.9^a; week4 ap and Dry-LP = 31.4^b; week2 prepartum and Dry-HP = 48.4^a; week2 prepartum and Dry-LP = 29.4^b. ² Dry-HP and Dry-LP = experimental diets fed during the dry period (Dry) either high (HP) or low (LP) in phosphorus concentration, i.e. 3.6 or 2.2 g P/kg DM, respectively. SED = Standard error of difference.

Lactation: P balance, OM and NDF Digestibility

After calving, P balance data and the apparent digestibilities of OM and NDF (Table 2) were not affected by any 2-way or 3-way interaction ($P \geq 0.10$). The intake of P increased in the course of time ($P < 0.01$) and across time, the mean P intake was 23.0% lower ($P < 0.01$) when Lac-LP was fed compared with Lac-HP. Fecal P excretion also increased with time ($P < 0.01$) and was found to be lower when feeding the Lac-LP diet ($P < 0.01$). Absolute apparent P

absorption (g/d) tended to increase with week after parturition ($P = 0.09$) and tended to be greater for Lac-HP than Lac-LP ($P = 0.07$), whereas the apparent efficiency of P absorption (% of P intake) was not influenced by the P concentration of the diet ($P = 0.11$). In contrast, the relative P absorption decreased in the course of time ($P < 0.01$). The P secretion with milk was neither affected by time nor by P intake ($P \geq 0.89$). The P balance was negative after calving but values tended to increase with time ($P = 0.08$). The P balance was 66% lower when Lac-LP compared with Lac-HP was fed.

Both apparent total tract OM and NDF digestibility (Table 2) were not affected by the P concentration of the lactation diets ($P \geq 0.62$). In contrast, OM digestibility tended to decrease in time ($P = 0.07$) while NDF digestibility clearly decreased in time ($P = 0.01$).

Plasma PTH, 25(OH)D₃, and CTX in Response to Dietary Treatments

Throughout the experiment, both plasma PTH and 25(OH)D₃ concentrations were neither affected by any 2-way or 3-way interaction between time of sampling and dietary treatments ($P \geq 0.14$) nor the P concentration of the experimental diets ($P \geq 0.49$). Plasma PTH concentrations (Figure 2, panel A) were affected by the day of sampling, both during the dry period and thereafter ($P \leq 0.01$). From d-28 to d-7 relative to calving, plasma PTH values rose 18.5% and values peaked at the day of calving. After calving, plasma PTH values declined to nadir PTH concentrations (i.e. ~ 70% lower compared to peak values) at d21 after calving which was followed by a gradual increase again until the end of the experiment at d56 after calving. During the dry period, plasma 25(OH)D₃ concentrations (Figure 2, panel B) were not affected the day of sampling ($P = 0.24$). In contrast, plasma 25(OH)D₃ concentrations tended to be influenced by sampling day ($P = 0.06$) after calving. The greatest plasma 25(OH)D₃ concentrations were found shortly after calving and concentrations numerically decreased in the course of lactation.

Plasma CTX concentrations (Figure 2, panel C) during the dry period were affected by sampling day \times dietary P concentration ($P = 0.01$), where from wk4 to wk1 before calving the CTX concentration decreased and increased with Dry-HP and Dry-LP, respectively. After calving, plasma CTX concentrations were influenced by an interaction between sampling day and dietary P concentration ($P < 0.01$) but not by any other 2-way or 3-way interaction between time of sampling and dietary treatments ($P \geq 0.12$). After calving, plasma CTX concentrations increased in the course of time, but the increase in plasma CTX values was more pronounced when Lac-LP instead of Lac-HP was fed ($P < 0.01$).

Table 2. Phosphorus balance, apparent P absorption and apparent total tract digestibility of organic matter (OM) and neutral detergent fiber (NDF) in lactating cows fed experimental rations differing in dietary P concentrations¹.

Item	Week relative to calving				SED	P-value	Experimental diet ²		SED	P-value
	2	4	6	8			Lac-HP	Lac-LP		
P intake (g/d)	59.0 ^a	67.1 ^b	73.4 ^c	76.1 ^c	1.33	< 0.01	77.8	59.9	2.13	< 0.01
P feces (g/d)	31.8 ^a	38.2 ^b	44.7 ^c	46.2 ^c	1.81	< 0.01	47.0	33.5	2.36	< 0.01
Apparent P absorption										
Absolute (g/d)	28.5	30.2	29.1	32.1	1.75	0.09	32.3	27.7	2.19	0.07
Relative (% of intake)	48.2 ^b	45.0 ^a	39.6 ^a	42.4 ^a	2.15	< 0.01	41.5	46.0	2.76	0.11
P milk (g/d)	41.3	41.8	41.5	41.3	0.72	0.89	42.0	40.9	1.45	0.91
P balance (g/d)	-12.7	-11.6	-12.4	-9.2	1.65	0.08	-8.6	-14.3	1.83	0.01
Digestibility (% of intake)										
OM	65.8	66.1	63.5	63.8	1.29	0.07	65.5	63.8	1.52	0.62
NDF	56.3	57.4	52.9	53.1	1.83	0.01	55.3	54.8	2.24	0.90

^{a-c} Different superscripts indicate a significant differences ($P < 0.05$).

¹ Variables were not affected ($P \geq 0.10$) by the dietary P concentration before calving (Dry), Dry \times Lac, Lac \times Week, and Dry \times Lac \times Week.

² Lac-HP and Lac-LP = experimental diets fed during lactation (Lac) containing either a high (HP) or a low (LP) dietary P concentration, i.e. 3.8 or 2.9 g P/kg DM, respectively. SED = Standard error of difference.

Relationships between Ca, Pi, PTH, CTX, and 25(OH)D₃ Concentrations in Plasma

Plasma Pi and Ca concentrations in response to the dietary treatments were previously reported by Keanthao et al. (2021). Plasma Pi concentration tended to be negatively related with plasma PTH ($P = 0.08$) but the variation in plasma Pi concentrations accounted for only 5.1% of the variation in plasma PTH values (Figure 3, panel A). The variation in plasma Pi concentrations explained 18.2% of the observed variation in plasma CTX concentrations and concentrations were negatively ($P < 0.01$) associated (Figure 3, panel B). Likewise, plasma Ca concentrations were negatively associated ($P < 0.01$) with plasma PTH (Figure 3, panel C) and the variation in plasma Ca concentrations accounted for 35.3% of the variation in plasma PTH values. The plasma CTX values were found to be unrelated ($R^2_{\text{adj}} = 3.5\%$, $P = 0.12$) with the plasma Ca concentrations (Figure 3, panel D). Both the variation in plasma Pi and plasma Ca concentrations were found to be unrelated with the variation in plasma 25(OH)D₃ (data not shown, $R^2_{\text{adj}} \leq 0.8\%$, $P \geq 0.25$).

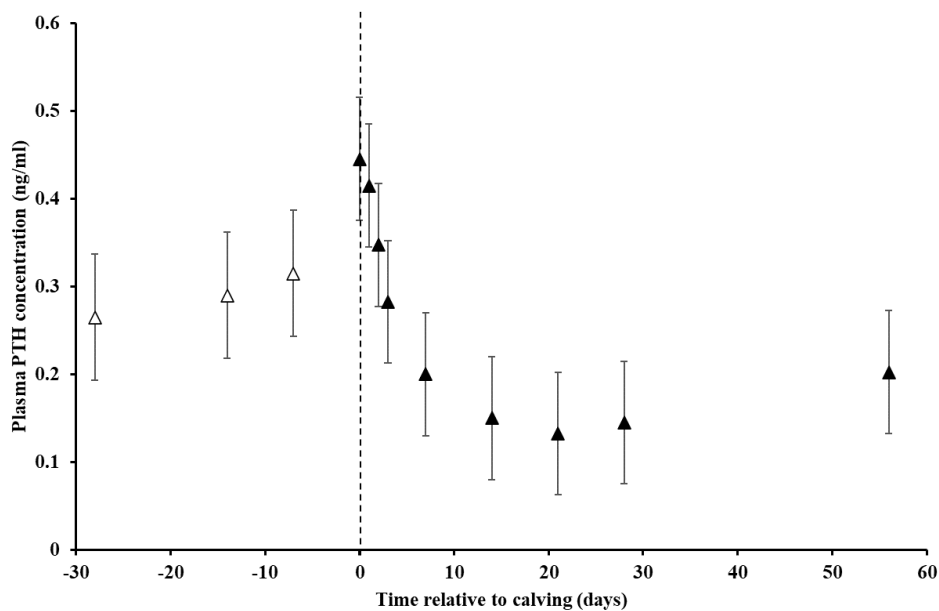


Figure 2. Plasma PTH concentration from 4 weeks prepartum until 56 days in milk (error bar = SED). The cows were either fed a low P (LP) or a high P (HP) diet during the dry period (Dry) and after calving (Lac). High P diets contained 3.6 g P/kg DM during the dry period and 3.8 g P/kg DM after calving while low P diets contained 2.2 g P/kg DM during the dry period and 2.9 g P/kg DM after calving. The dotted line represents the day of calving. Symbols \triangle , PTH concentrations during the dry period; \blacktriangle , PTH concentrations during lactation. P values related to PTH concentrations during the dry period: Sampling day \times Dry = 0.57; Dry-HP vs. Dry-LP = 0.49, Sampling day = 0.01.

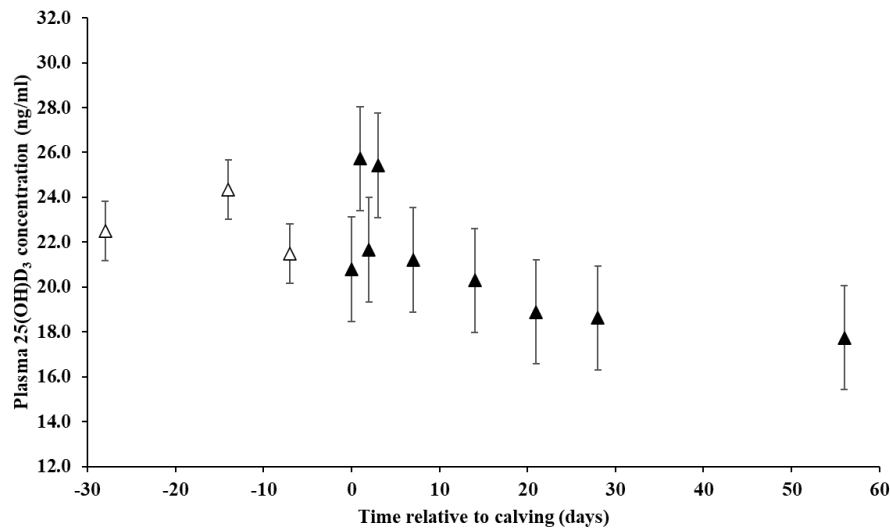


Figure 3. Plasma 25(OH)D₃ concentration from 4 weeks prepartum until 56 days in milk (error bar = SED). The cows were either fed a low P (LP) or a high P (HP) diet during the dry period (Dry) and after calving (Lac). High P diets contained 3.6 g P/kg DM during the dry period and 3.8 g P/kg DM after calving while low P diets contained 2.2 g P/kg DM during the dry period and 2.9 g P/kg DM after calving. The dotted line represents the day of calving. Symbols \triangle , 25(OH)D₃ concentrations during the dry period; \blacktriangle , 25(OH)D₃ concentrations during lactation. *P* values related to 25(OH)D₃ concentrations during lactation: Sampling day \times Dry \times Lac = 0.38, Sampling day \times Dry = 0.57, Sampling day \times Lac = 0.43, Dry \times Lac = 0.09, Lac-HP vs. Lac-LP = 0.62, Sampling day = 0.06.

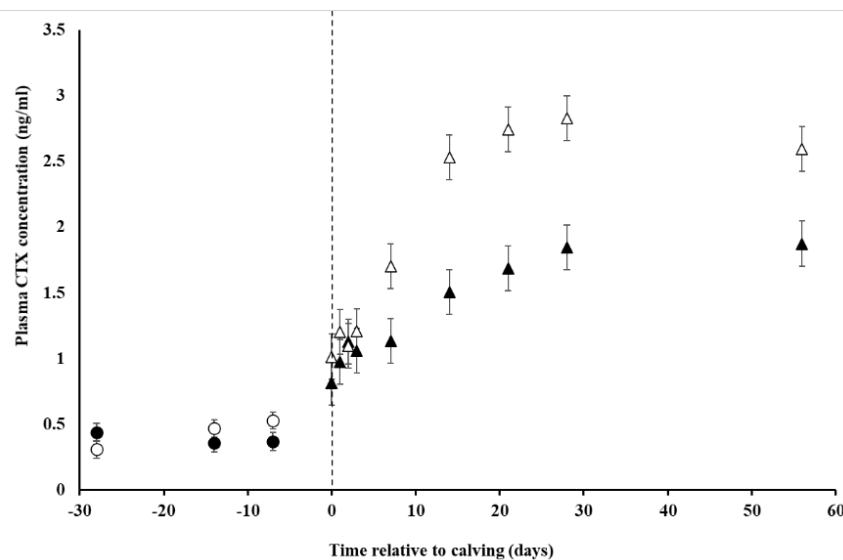


Figure 4. Plasma carboxy-terminal collagen crosslinks (CTX) (panel C) concentrations from 4 weeks prepartum until 56 days in milk (error bar = SED). The cows were either fed a low P (LP) or a high P (HP) diet during the dry period (Dry) and after calving (Lac). High P diets contained 3.6 g P/kg DM during the dry period and 3.8 g P/kg DM after calving while low P diets contained 2.2 g P/kg DM during the dry period and 2.9 g P/kg DM after calving. The dotted line represents the day of calving. Symbols \bullet , Dry HP; \circ , Dry LP; \triangle , Lac-LP; \blacktriangle , Lac-HP. *P* values related to CTX during lactation: Sampling day \times Dry \times Lac = 0.40, Sampling day \times Dry = 0.12, Sampling day \times Lac < 0.01, Dry \times Lac = 0.55, Lac-HP vs. Lac-LP = 0.01, Sampling day < 0.01.

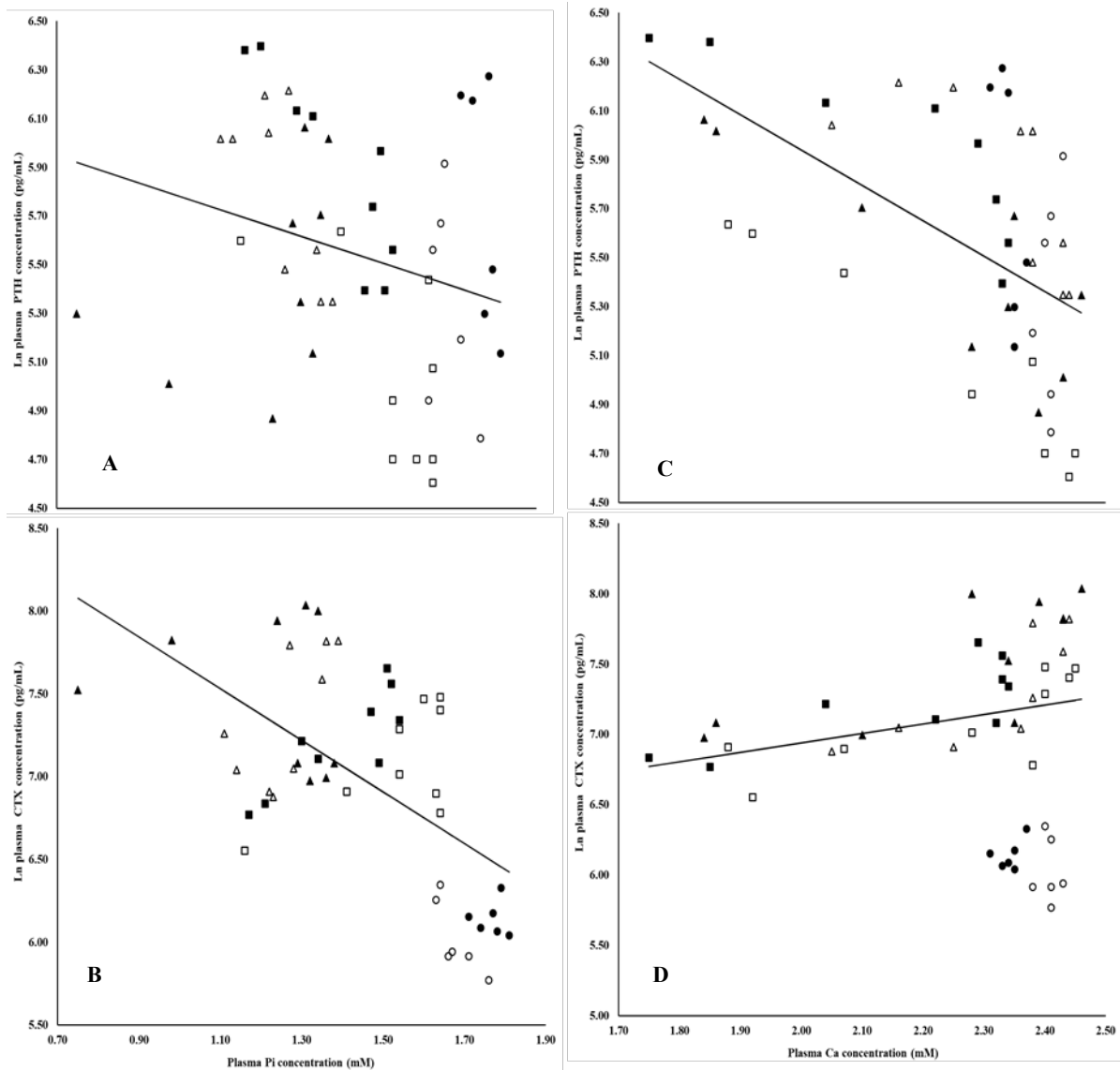


Figure 5. Relationships between plasma inorganic P (Pi) concentrations (mM) and plasma concentrations of parathyroid hormone (PTH) (panel A) and carboxy-terminal collagen crosslinks (CTX) (panel B). Panels C and D show the relationships between plasma Ca concentrations (mM) and plasma concentrations of PTH and CTX, respectively. In each panel, the values on the y-axis represent natural logarithmically converted concentrations, expressed as pg/ml, of either the plasma PTH- or plasma CTX concentrations. The linear regression formulas are: Panel A, $y = 6.39 - 0.59x$ ($n = 48$, $R^2_{\text{adj}} = 5.1\%$, $P = 0.08$); Panel B, $y = 8.72 - 1.14x$ ($n = 48$, $R^2_{\text{adj}} = 18.2\%$, $P < 0.01$); Panel C, $y = 8.83 - 1.44x$ ($n = 48$, $R^2_{\text{adj}} = 35.3\%$, $P < 0.01$); Panel D, $y = 5.59 - 0.67x$ ($n = 48$, $R^2_{\text{adj}} = 3.5\%$, $P = 0.12$). The cows were either fed a low P (LP) or a high P (HP) diet during the dry period (Dry) and after calving (Lac). High P diets contained 3.6 g P/kg DM during the dry period and 3.8 g P/kg DM after calving while low P diets contained 2.2 g P/kg DM during the dry period and 2.9 g P/kg DM after calving. Symbols: ●, Dry-HP; ○, Dry-LP; ■, Dry-HP/Lac-HP; □, Dry-LP/Lac-HP; ▲, Dry-HP/Lac-LP; △, Dry-LP/Lac-LP.

DISCUSSION

P Balance

In the dry period, across time (wks-4 and -2 before calving) and dietary treatments, the overall mean P retention was found to be 4.8 g/d. This value is in line with data reported by House and Bell (1993) who estimated P accretion in the conceptus to be ~ 5 g P/d during the last 4 weeks of pregnancy in Holstein dairy cows, and with Valk et al. (2002) who reported a dry dairy cattle P balance of on average 4.7 g/d at dietary P concentration of on average 2.9 g P/kg DM. The lower P balance of cows in Dry-LP than in Dry-HP is also in line with Valk et al. (2002), who reported P balance at low dietary P levels to be 0.8 g P/d (at 1.5 g P/kg DM) up to 2.9 g P/d (at 1.9 g P/kg DM). In contrast to the dry period, all cows experienced a negative P balance during the first 8 wks after calving, irrespective of dietary treatment. The negative P balance tended to be somewhat less severe at 8 wk compared with 2 wk postpartum (-9.2 and -12.7 g P/d, respectively). This change in net P balance was not caused by changes in milk P secretion. In line with Klop et al. (2014), higher milk protein content at 2 wk compared with 8 wk postpartum coincided with greater milk P content at 2 wk compared with 8 wk postpartum. This decrease in milk P content together with an increase in milk production with week of lactation resulted in a rather stable milk P secretion. Thus, the tendency of less severe negative P balance was related to a tendency of increased apparent P absorption with week of lactation. The observed negative P balances during the first two weeks are in line with expectation (Keanthao et al., 2021) because P intakes were lower than those recommended by the NRC (2001) and CVB (2012), irrespective of the P concentrations of the experimental diets. The low P-intakes are most likely explained by the low DMI during the first two weeks of lactation. For the cows fed Lac-LP, the observed negative P balances from wk4 onwards also are in line with expectation because the Lac-LP diets (i.e. 2.9 g P/kg DM) were formulated to contain a marginally deficient P concentrations (NRC, 2001; CVB, 2012). In line with negative P balance during the first 8 wks after calving in the present study, in an experiment with 3.2 g P/kg DM in the dry period (6 wks) followed by 3.6 g P/kg DM in subsequent lactation (44 wks), a negative P balance was maintained until at least wk13 postpartum (Elizondo Salazar et al., 2013). However, an uncommon negative P balance was observed already during the dry period, which may indicate a bias in P balance determination in Elizondo Salazar et al. (2013). Knowlton and Herbein (2002) reported a negative P balance in wk3 postpartum to become positive between wk5 and 7 postpartum at 3.4 g P/kg DM, and to become positive between wk3 and 5 postpartum at 5.1 g P/kg DM, indicating the significance of level of dietary P content on P balance in early lactation. Valk et al. (2002) observed approximate zero P balance in wk6 and 7 postpartum upon feeding diets with 2.6 to 2.9 g P/kg DM. In the present experiment, for the cows fed Lac-HP (i.e. 3.8 g P/kg DM), the observed negative P balances from wk4 onwards were unexpected (Keanthao et al., 2021). In theory, with the Lac LP diet upregulation of apparent P absorption from ~ 40 % of P intake (Table 2) to ~ 70 % of P intake would have been sufficient to achieve zero P balance from wk4 to 8 after calving. Martz et al. (1999) reported that cows can upregulate apparent P absorption up to 62 % of P intake. Thus, the observed negative P balance of the cows fed Lac-HP from wk4 to 8 is not easy to explain and can be interpreted in that the current levels of the negative P balance do not effectively trigger

regulatory actions to stimulate P absorption. This notion appears to be in line with the data shown in Figure 2 (Panel B) indicating that plasma 25(OH)D₃ levels were not markedly affected by the day after calving. Similarly, Puggaard et al. (2014) did not find an effect of dietary P concentration (2.3, 2.8 and 3.4 g P/kg DM) on plasma concentration of 25(OH)D₃. Irrespective of the dietary P concentration, plasma CTX values increased after calving (Figure 2, panel C), thereby, suggesting net resorption of P from bone. The greater increase in plasma CTX concentration with wk of lactation for Lac-LP compared with Lac-HP is in line with the greater bone P resorption observed with Lac-LP compared with Lac-HP. These observations may be interpreted in that P mobilization from bone is more important than upregulation of P absorption to maintain intracellular P concentrations in body fluids in periparturient cows (Grünberg et al., 2019). Obviously, this interpretation cannot be substantiated on the basis of the current data and further research is warranted to verify this suggestion. Nevertheless, the observed negative P balances during the first 8 wks after calving apparently did not compromise health and production of the cows which is corroborated by Keanthao et al. (2021), Knowlton and Herbein (2002) and Valk and Šebek (1999). It is, therefore, speculated that dairy cows are resilient to a negative P balance during early lactation, at least up to a nadir value of -14.3 g/day.

Parathyroid Hormone and Carboxy-Terminal Collagen Crosslinks

Within the current range of plasma Pi concentrations (i.e. treatment means from 0.75 mM to 1.75 mM), plasma PTH only tended to decrease with increased plasma Pi concentrations (Figure 3, panel A) and plasma Pi concentration explained little variation in plasma PTH concentration ($R^2_{\text{adj}} = 5.1\%$). This result can be interpreted in that plasma Pi is hardly involved in PTH secretion. Sherwood et al. (1968) also showed in dairy cows that plasma PTH did not respond to intravenous administration of sodium phosphate (plasma Pi concentrations ranged between ~ 1.3 and 4.0 mM) when hypocalcemia was prevented by Ca infusion to maintain plasma total Ca concentrations ~ 2.5 mM. Pfeiffer et al. (2005) summarized data from goats, where reduced P intake caused hypophosphatemia and decreased P retention without affecting plasma PTH concentrations. Thus, in view of the evidence provided here, the plasma Pi concentration does not affect the plasma PTH concentration within the normal range of plasma Pi levels, i.e. 1.3 to 2.6 mM (Goff, 1999; Grünberg, 2014). Clearly, the currently provided evidence does not exclude the possibility that plasma Pi values < 1.3 mM stimulate PTH secretion, but the data reported by Cohrs et al. (2018) indicate that, with even lower plasma Pi concentrations, plasma Pi concentrations of ~ 0.5 mM did not trigger a PTH response.

In contrast to plasma Pi concentrations, the causal relationship between plasma Ca concentrations and plasma PTH concentrations (Goff, 1999) is beyond dispute, and it is, therefore, most likely that the current observed response of plasma PTH in the course of time (Figure 2, panel A) is related to the variation in plasma Ca concentrations. Indeed, plasma Ca concentrations were found to be negatively correlated with plasma PTH values (Figure 3, panel C). It is well known that PTH orchestrates the regulatory responses to hypocalcemia, which includes promotion of bone resorption, stimulating kidneys to retain Ca for absorption, and promoting absorption of Ca from the digestive tract (Goff, 2000). As to bone resorption, serum levels of CTX have been demonstrated to be useful tools to follow bone resorption in dairy

cows (Liesegang et al., 1998), and serum levels of CTX are proportional to osteoclastic activity (Puggaard et al., 2014). However, plasma Pi concentrations, but not plasma Ca concentration were found to be related with plasma CTX values (Figure 3, panels B and D). Previously it has been shown that hypophosphatemia may induce mineral mobilization from bone independently from plasma Ca concentrations (Puggaard et al., 2014; Cohrs et al., 2018), although the underlying mechanism is not yet fully identified (Ekelund et al., 2006; Cohrs et al., 2018). Nevertheless, plasma CTX values were found to be greater when low instead of high dietary P was fed after calving (Figure 2, panel C), thereby, indicating that the low P diets caused greater mineral mobilization from bone. This result is in line with Ekelund et al. (2006) and Puggaard et al. (2014) who also reported an increase in bone resorption when low P diets (i.e. 3.2 and 2.8 g P/kg DM, respectively) were fed during early lactation.

Plasma 25(OH)D₃

Post-partum, plasma 25(OH)D₃ concentrations tended to decrease in time, irrespective of the dietary P concentration (Figure 2, panel B). The lack of response of plasma 25(OH)D₃ to P intake is corroborated by Puggaard et al. (2014) and Cohrs et al. (2018). The tendency of decrease in plasma 25(OH)D₃ concentrations in the course of time may be related to the conversion (at least in part) of 25(OH)D₃ into 1,25(OH)₂D₃. The conversion of 25(OH)D₃ into 1,25(OH)₂D₃ is mediated by a hypocalcemic induced secretion of PTH (Bergwitz and Jüppner, 2010; Anderson et al., 2017) enhancing the production of 1 α OH-ase by kidney tissue, but there are indications that the condition of hypophosphatemia also increases the activity of 1 α OH-ase and thus promotes the synthesis of 1,25(OH)₂D₃ (Perward et al., 2007). Fibroblast growth factor 23 (FGF23) has been implicated to play a role in the synthesis of 1,25(OH)₂D₃ independent from PTH (Köhler et al., 2021). Low plasma Pi values inhibit the synthesis of FGF23 and, thereby, stimulate the activity of 1 α -hydroxylase (Perward et al., 2007). Upon these theoretical considerations, we speculate that the synthesis of 1,25(OH)₂D₃ was upregulated after calving, but in view of the current observations the upregulation of 1,25(OH)₂D₃ synthesis was not sufficient to prevent a negative P balance. Perhaps the concentration of vitamin D receptors in epithelial tissues was too low (Goff et al., 1995) to effectively respond to greater plasma 1,25(OH)₂D₃ concentrations.

Apparent Total Tract OM and Fiber Digestion

Both during the dry period and the first 8 wks after calving, apparent total tract OM- and NDF digestibility was not affected by the dietary P concentration. It is generally supposed that adequate P supply to the microbial community in the rumen is crucial to safeguard OM- and NDF digestibility (Komisarczuk-Bony and Durand, 1991). The latter authors reported that a minimum P concentration of 0.5 mM in rumen fluid is required for optimum fiber digestion, at least under *in vitro* conditions. Such low rumen P concentrations are, however, difficult to achieve under most feeding conditions. Puggaard et al. (2011) reported that mean rumen P concentrations ranged between ~ 2 mM and ~ 4 mM when dairy cows were fed a diet containing 2.4 g P/kg DM, while Rodehutschord et al. (1994) reported rumen P concentrations ~ 5 mM when a diet containing 1.4 g P/kg DM was fed to goats. Next to P intake as such, rumen fluid

P concentrations are also affected by P recycled to the rumen via saliva (Tomas, 1973). Indeed, Care (1994) estimated that approximately 50% of the total P that enters the rumen originates from saliva. Valk et al. (2002) reported salivary P concentrations of 5.2 and 5.7 mM when the diet of lactating cows contained 2.26 or 2.34 g P/kg DM, respectively. Thus, even at a rather low dietary P content, cows were able to maintain a salivary P concentration ~ 5 mM. We, therefore, speculate that in the current study, the supply of P to the microbial flora in the rumen was sufficient to safeguard apparent total tract OM and NDF digestion, even in case the LP diets were fed.

CONCLUSIONS

Feeding a dietary P concentration 30% below the current CVB (2012) recommendation during the first 8 weeks postpartum does not compromise apparent total tract OM and NDF digestibility. However, this low dietary P level causes a greater negative P balance which was associated with elevated plasma CTX concentrations without increasing plasma concentrations of PTH and 25(OH)D₃, thereby, demonstrating a more prominent role of bone resorption than of increased P absorption to meet P demands in the first 8 weeks postpartum. In view of the sustained negative P balance in the first 8 wks postpartum, it remains premature to recommend dietary P concentrations below current CVB recommendations. Further studies are warranted to investigate whether cows are able to upregulate P absorption after 56 DIM when the diet contains less P than currently recommended.

ACKNOWLEDGEMENTS

The authors acknowledge the staff of Dairy Campus (Leeuwarden, the Netherlands) for their contribution to this experiment. This research was commissioned and funded by the Ministry of Agriculture, Nature and Food Quality (the Hague, the Netherlands) within the framework of Policy Support Research theme 'Feed4Foodure' (BO-31.03-005-001; TKI-AF12039), and by the Vereniging Diervoederonderzoek Nederland (Rijswijk, the Netherlands).

REFERENCES

- Akert, F. S., K. Dorn, H. Frey, P. Hofstetter, J. Berard, M. Kreuzer, and B. Reidy. 2020. Farm-gate nutrient balances of grassland-based milk production systems with full- or part-time grazing and fresh herbage indoor feeding at variable concentrate levels. *Nutr. Cycl. Agroecosyst.* 117: 383-400.
- Anderson, S. T., L. J. Kidd, M. A. Benvenutti, M. T. Fletcher, and R. M. Dixon. 2017. New candidate markers of phosphorus status in beef breeder cows. *Anim. Prod. Sci.* 57: 2291-2303.
- Bergwitz, C., and H. Jüppner. 2010. Regulation of phosphate homeostasis by PTH, vitamin D and FGF32. *Annu. Rev. Med.* 61: 91-104.
- Care, A. D. 1994. The absorption of phosphate from digestive tract of ruminant animals. *Br. Vet. J.* 150: 197-205.

- Cohrs, I., M. R. Wilkens, and W. Grünberg. 2018. Short communication: Effect of dietary phosphorus deprivation in late gestation and early lactation on the calcium homeostasis of periparturient dairy cows. *J. Dairy Sci.* 101: 9591-9598.
- CVB. 2012. CVB Farm Animal Feeding Advice 2012. CVB volume 50, the Hague, the Netherlands.
- Ekelund, A., R. Spörndly, and K. Holtenius. 2006. Influence of low phosphorus intake during early lactation on apparent digestibility of phosphorus and bone metabolism in dairy cows. *Livest. Sci.* 99: 227-236.
- Elizondo Salazar, J. A., J. D. Ferguson, D. B. Beegle, D. W. Remsburg, and Z. Wu. 2012. Body phosphorus mobilization and deposition during lactation in dairy cows. *J. Anim. Physiol. Anim. Nutr.* 97: 502-514.
- Goff, J. P., T. A. Reinhardt, and R. L. Horst. 1995. Milk fever and dietary cation-anion balance effects on concentration of vitamin D receptor in tissue of periparturient dairy cows. *J. Dairy Sci.* 78: 2388-2394.
- Goff, J. P. 1999. Treatment of calcium, phosphorus, and magnesium balance disorders. *Vet. Clin. North Am. Food Anim. Pract.* 15: 619-639.
- Goff, J. P. 2000. Pathophysiology of calcium and phosphorus disorders. *Vet. Clin. North Am. Food Anim. Pract.* 16: 319-337.
- Grünberg, W. 2014. Treatment of phosphorus balance disorders. *Vet. Clin. North Am. Food Anim. Pract.* 30: 383-408.
- Grünberg, W., P. Scherpenisse, I. Cohrs, L. Golbeck, P. Dobbelaar, L. M. Van den Brink, and I. D. Wijnberg. 2019. Phosphorus content of muscle tissue and muscle function in dairy cows fed a phosphorus-deficient diet during the transition period. *J. Dairy Sci.* 102: 4072-4093.
- Harrison, B. P., M. Dorigo, C. K. Reynolds, A. Sinclair, J. Dijkstra, and P. P. Ray. 2021. Determinants of phosphorus balance and use efficiency in diverse dairy farming systems. *Agric. Syst.* 194: 103273.
- Hollis, B. W., and R. L. Horst. 2007. The assessment of circulating 25(OH)D and 1,25(OH)₂D: where we are and where we are going. *J. Steroid Biochem. Mol. Biol.* 103: 473-476.
- House, W. A., and A. W. Bell. 1993. Mineral accretion in the fetus and adnexa during late gestation in Holstein cows. *J. Dairy Sci.* 76: 2999-3010.
- ISO. 1999. Animal feeding stuffs. Determination of moisture and other volatile matter content. ISO 6496. ISO, Geneva, Switzerland.
- ISO. 2002. Animal feeding stuffs. Determination of crude ash. ISO 5984. ISO, Geneva, Switzerland.
- Keanthao, P., R. M. A. Goselink, J. Dijkstra, A. Bannink, and J. T. Schonewille. 2021. Effects of dietary phosphorus concentration during the transition period on plasma calcium concentrations, feed intake, and milk production in dairy cows. *J. Dairy Sci.* 104: 11646-11659.
- Kebreab, E., N. E. Odongo, B. W. McBride, M. D. Hanigan, and J. France. 2008. Phosphorus utilization and environmental and economic implications of reducing phosphorus pollution from Ontario dairy cows. *J. Dairy Sci.* 91: 241-246.

- Klop, G., J. L. Ellis, A. Bannink, E. Kebreab, J. France, and J. Dijkstra. 2013. Meta-analysis of factors that affect the utilization efficiency of phosphorus in lactating dairy cows. *J. Dairy Sci.* 96: 3936-3949.
- Klop, G., J. L. Ellis, M. C. Blok, G. G. Brandsma, A. Bannink, and J. Dijkstra. 2014. Variation in phosphorus content of milk from dairy cattle as affected by differences in milk composition. *J. Agric. Sci.* 152: 860-869.
- Knowlton, K. F., and J. H. Herbein. 2002. Phosphorus partitioning during early lactation in dairy cows fed diets varying in phosphorus content. *J. Dairy Sci.* 85: 1227-1236.
- Köhler, O. M., W. Grünberg, N. Schnepel, A. S. Muscher-Banse, A. Rajaeerad, J. Hummel, G. Breves, and M. R. Wilkens. 2021. Dietary phosphorus restriction affects bone metabolism, vitamin D metabolism and rumen fermentation traits in sheep. *J. Anim. Physiol. Anim. Nutr.* 105: 35-50.
- Komisarczuk-Bony, S., and M. Durand. 1991. Effects of minerals on microbial metabolism. Pages 179-198 in *Rumen Microbial Metabolism and Ruminant Digestion*. Ed. J. P. Jouany. Institut National de la Recherche Agronomique (INRA).
- Liesegang, A., R. Eicher, M. -L. Sassi, J. Risteli, M. Kraenzlin, J. -L. Riond, and M. Wanner. 2000. Biochemical markers of bone formation, and resorption around parturition and during lactation in dairy cows with high and low standard milk yield. *J. Dairy Sci.* 83: 1773-1781.
- Liesegang, A., J. Risteli, and M. Wanner. 2006. The effects of first gestation and lactation on bone metabolism in dairy goats and milk sheep. *Bone* 38: 794-802.
- Liesegang, A., M. L. Sassi, J. Ristelli, R. Eicher, M. Wanner, and J. L. Riond. 1998. Comparison of bone resorption markers during hypocalcemia in dairy cows. *J. Dairy Sci.* 81: 2614-2622.
- Martz, F. A., A. T. Belo, M. F. Weiss, and R. L. Belyea. 1999. True absorption of calcium and phosphorus from corn silage fed to nonlactating, pregnant dairy cows. *J. Dairy Sci.* 82: 618-622.
- NRC. 2001. *Nutrient Requirements of Dairy Cattle*, 7th revised edition. National Academy of Science, Washington, DC.
- Nichols, K., A. Bannink, S. Pacheco, H. J. van Valenberg, J. Dijkstra, and H. van Laar. 2018. Feed and nitrogen efficiency are affected differently but milk lactose production is stimulated equally when isoenergetic protein and fat is supplemented in lactating dairy cow diets. *J. Dairy Sci.* 101: 7857-7870.
- Perward, F., M. Y. H. Zhang, H. S. Tenenhouse, and A. A. Portale. 2007. Fibroblast growth factor 23 impairs phosphorus and vitamin D metabolism *in vivo* and suppresses 25-hydroxyvitamin D-1 α -hydroxylase expression *in vitro*. *Am. J. Physiol. Renal. Physiol.* 293: 1577-1583.
- Peterson, A. B., M. W. Orth, J. P. Goff, and D. K. Beede. 2005. Periparturient responses of multiparous Holstein cows fed different dietary phosphorus concentrations prepartum. *J. Dairy Sci.* 88: 3582-3594.
- Pfeffer, E., D. K. Beede, and H. Valk. 2005. Phosphorus metabolism in ruminants and requirements of cattle. Pages 195 to 232 in *Nitrogen and Phosphorus Nutrition in Cattle*, E. Pfeffer and A. N. Hristov, ed. CABI Publishing.

- Puggaard, L., N. B. Kristensen, and J. Sehested. 2011. Effect of decreasing dietary phosphorus supply on net recycling of inorganic phosphate in lactating dairy cows. *J. Dairy Sci.* 94: 1420-1429.
- Puggaard, L., P. Lund, A. Liesegang, and J. Sehested. 2014. Long term effect of reduced dietary phosphorus on feed intake and milk yield in dry and lactating dairy cows. *Livest. Sci.* 159: 18-28.
- Rodehutsord, M., A. Pauen, P. Windhausen, R. Brintrup, and E. Pfeffer. 1994. Effects of drastic changes in P intake on P concentrations in blood and rumen fluid of lactating ruminants. *J. Vet. Med. A.* 41: 611-619.
- Shekarriz-Foumani, R., and F. Khodaie. 2016. The correlation of plasma 25-Hydroxyvitamin D deficiency with risk of breast neoplasms: A systematic review. *Iran. J. Cancer Prev.* 9: 4469.
- Sherwood, L. M., G. P. Mayer, C. F. Ramberg Jr., D. S. Kronfeld, G. D. Aurbach, and J. T. Potts Jr. 1968. Regulation of parathyroid hormone secretion: proportional control by calcium, lack of effect of phosphate. *Endocrinology* 83: 1043-1051.
- Tamminga, S., W. M. Van Straalen, A. P. J. Subnel, R. G. M. Meijer, A. Steg, C. J. G. Wever, and M. C. Blok. 1994. The Dutch protein evaluation system: The DVE/OEB-system. *Livest. Prod. Sci.* 40: 139-155.
- Tomas, F. M. 1973. Parotid saliva secretion in sheep: its measurement and influence on phosphorus in rumen fluid. *Quart. J. Exp. Physiol.* 58: 131-138.
- Valk, H., and L. B. J. Šebek. 1999. Influence of long-term feeding of limited amounts of phosphorus on dry matter intake, milk production, and body weight of dairy cows. *J. Dairy Sci.* 82: 2157-2163.
- Valk, H., L. B. J. Sebek, and A. C. Beynen. 2002. Influence of phosphorus intake on excretion and blood plasma and saliva concentrations of phosphorus in dairy cows. *J. Dairy Sci.* 85: 2642-2649.
- Van Es, A. J. H. 1978. Feed evaluation for ruminants. I. The systems in use from May 1977-onwards in the Netherlands. *Livest. Prod. Sci.* 5: 331-345.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74: 3583-3597.
- VSN International. 2018. Genstat for Windows, 19th Edition. VSN International, Hemel Hempstead, UK.
- Wächter, S., I. Cohrs, L. Golbeck, T. Scheu, K. Eder, and W. Grünberg. 2022. Effects of restricted dietary phosphorus supply during the dry period on productivity and metabolism in dairy cows. *J. Dairy Sci.* 105: 4370-4392.
- Wu, Z., and L. D. Satter. 2000. Milk production and reproductive performance of dairy cows fed concentrations of phosphorus for two years. *J. Dairy Sci.* 83: 1052-1063.

Chapter 4

Plasma concentrations of 1,25 vitamin D₃ are not upregulated during phosphorus deficiency in lactating dairy cows

P. Keanthao*, J. Dijkstra[‡], and J. T. Schonewille*

* Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, the Netherlands

‡ Animal Nutrition Group, Wageningen University & Research, PO Box 338, 6700 AH Wageningen, the Netherlands

To be submitted

Journal of Dairy Science

ABSTRACT

The aim of the experiment was to gain more insight on the role of $1,25(\text{OH})_2\text{D}_3$ on P absorption in dairy cows and it was hypothesized that the feeding of a P-deficient diet results in increased levels of $1,25(\text{OH})_2\text{D}_3$ and P absorption, and in addition that these effects would be more pronounced when feeding pelleted dried grass compared with coarse artificial dried grass. Eighteen multiparous (parity ≥ 2), mid-lactating cows were used in a randomized block design with 19 d experimental periods. The cows were either fed a high P (HP) diet (i.e. 2.79 g P/kg DM) containing 26% (DM basis) coarse artificially dried grass or a low P diet (LP, 2.06 g P/kg DM) also containing coarse artificially dried grass (25%) or a low P diet (2.23 g P/kg DM) containing 25% pelleted artificially dried grass. All cows were fed a restricted amount of feed (18 kg DM/d) to ensure constant P intakes within treatments. Pelleting of the artificially dried grass caused a 26.6% reduction in rumination time. Feeding LP pelleted grass diet caused greater contents (g/kg) of milk protein and lactose compared with HP coarse artificial dried grass diet. The P balance of the cows fed HP was found to be 0.1 g/d but the cows fed the LP diet were in negative P balance, irrespective of the physical form of the artificially dried grass. However, no differences were found in plasma concentrations of $25(\text{OH})\text{D}_3$ and $1,25(\text{OH})_2\text{D}_3$. Instead, there was a tendency towards greater plasma concentrations of crosslaps when the cows were fed LP and the osteocalcin to crosslaps ratio was borderline significantly greater in the cows fed HP. The latter indicates that the cows fed LP mobilized P from bone. It can be concluded that P mobilization from bone is more important than upregulation of P absorption when P deficient diets are fed.

Key words: phosphorus, saliva, ruminal activity, crosslaps

INTRODUCTION

Leaking of phosphorus (P), originating from livestock production systems, leads to eutrophication and has serious consequences for the biodiversity of all kind of water bodies (Smith and Alexander, 2000). On top of that, P resources are fossil and thus finite (Reijnders, 2014). With cattle husbandry causing half of the P pollution from all animal husbandry systems (Liu et al., 2017), it is crucial to lower its excretions. For dairy cattle, a positive linear relationship has been found between P intake and P excretion in the feces (Klop et al. 2013). Thus, reduction of P intake will lower the fecal excretion of P (Knowlton et al., 2002; Ekelund et al., 2006; Moreira et al., 2009).

Considerable variation exists in dietary P recommendations between the various international authorities (AFRC, 1991; GfE, 1993; NRC, 2001), thereby, indicating that the P requirement of dairy cows as such, is a matter of debate. The Dutch Bureau of Livestock Feeding (Valk, 2005) recommends the lowest levels of dietary P compared to the AFRC (1991), GfE (1993) and the NRC (2001). The Dutch Central Bureau for Livestock Feeding (CVB, 2005) adopted a value of 75% of true P absorption to calculate the gross P requirement of dairy cows. There are, however, indications reported in literature that the efficiency of P absorption can be as high as 90% of intake (Martz et al., 1999), thereby, suggesting that the recommend P intakes can be fine-tuned towards lower values (Keanthao et al., 2021). In

monogastric animals, calcitriol ($1,25(\text{OH})_2\text{D}_3$) plays an important role in upregulating P absorption (Lee et al., 1986; Gray et al., 1987) but in ruminants the role of $1,25(\text{OH})_2\text{D}_3$ is not settled yet. Cohrs et al. (2018) did not observe an increase in plasma $1,25(\text{OH})_2\text{D}_3$ upon the feeding of P deficient diets in dairy cows and this observation is corroborated by Breves et al. (1985), Schröder and Breves (1996), and Wächter et al. (2021) in sheep, goats, and dairy cows respectively. In contrast Host (1986), Puggaard et al. (2012), and Köhler et al. (2020) reported an increase in plasma levels of $1,25(\text{OH})_2\text{D}_3$ in response to the feeding P deficient diets in dairy cattle. It is, however, of interest to note that in the latter studies (Köhler et al., 2020), the P absorption and P balance of the animals was not determined which hinders the interpretation of a change in plasma values of $1,25(\text{OH})_2\text{D}_3$.

In ruminants, the amount of P that enters the reticulo-rumen originates not only from dietary P, but also from saliva. Typically, the amount of salivary P is quantitatively almost equally important to the amount of dietary P that enters the rumen (Bailey, 1961; Pfeffer et al., 2005). It is generally accepted that rumination time is positively related to the amount of saliva produced (Maekawa et al., 2002; Beauchemin et al., 2008). Reduction of rumination time may, therefore, lead to lower amounts of P that enter the gut, thereby, resulting in lower amounts of P available for absorption.

The current experiment was designed to gain more insight on the role of $1,25(\text{OH})_2\text{D}_3$ on P absorption. We, therefore, fed cows either at 100% or at 72% of the current CVB recommendations, i.e. P sufficient or P deficient diets, respectively. It was hypothesized that the feeding of a P-deficient diet results in increased levels of $1,25(\text{OH})_2\text{D}_3$ and P absorption. In addition, we also fed a P-deficient diet where 25% (DM basis) of the roughage was fed in the form of pelleted artificially dried grass rather than coarse artificially dried grass to reduce rumination time and we hypothesized that under these conditions the increase in plasma $1,25(\text{OH})_2\text{D}_3$ was more pronounced compared to the P-deficient diet containing coarse artificially dried grass. In the current experiment, mid-lactation cows were used in attempt to prevent a confounding effect of hypocalcemia on the plasma $1,25(\text{OH})_2\text{D}_3$ concentration (Keanthao et al., 2021).

MATERIALS AND METHODS

All experimental protocols and interventions were conducted under the Dutch Law on Animal Experimentation and approved (approval nr: ADV401002016653) by the Central Authority for Scientific Procedures on Animals (CCD, the Hague, the Netherlands).

Experimental Design and Dietary Treatment

The experiment had a randomized block design with experimental periods that lasted for 19 d. Prior to the experiment, cows were grouped in 3 blocks of 6 animals with comparable parity and milk yields. Within each block, the cows were randomly assigned to one of the three dietary treatments. For each block, the 19-d experimental period was preceded by a 14-d pre-experimental period that allowed the animals to become adapted to the control diet which consisted of corn silage, coarse artificially dried grass (Hartog B.V., Lambertschaag, the Netherlands) and a concentrate containing 4.4 g P/kg DM (**HP** concentrate, Table 1). At least

91% of the artificially dried grass had a particle length > 35 mm. During the experimental period, the proportion of dietary corn silage was kept constant for all three experimental treatments and the control cows continued to receive the diet containing coarse artificially dried grass (**CG**) and the high-P concentrate (Table 2). For the two test diets, the HP concentrate was replaced by a low-P concentrate (**LP**, Table 1) while for one of two test diets, CG was replaced by artificially dried grass in pelleted form (**PG**, diameter = 6 mm). The P concentrations of the experimental concentrates were manipulated with the use of disodium phosphate (Na_2HPO_4) and sodium bicarbonate (NaHCO_3), thereby, aiming to prevent potential confounding effects due to differences in Na intake between the low- and high P diets. The analyzed P concentrations of the experimental diets were 2.79, 2.06 and 2.23 g/kg DM for HP-CG, LP-CG and LP-PG, respectively (Table 2). The difference in dietary P concentrations between LP-CG and LP-PG was unintentional because, for logistic reasons, we were forced to use artificially dried grass that originated from two different locations. Unfortunately, the batch of artificially dried grass destined to be pelleted, contained a somewhat greater P concentration than CG (Table 2). With the exception of the P content of the two Low-P diets, the experimental diets were formulated to meet, or exceed, the nutrient requirements for maintenance and a milk yield of 25 kg fat- and protein corrected milk (CVB, 2016). TiO_2 (2.7 g/kg) was included in both concentrates as an external marker to determine apparent total tract feed digestibility.

Animals, Housing, Milking, and Feeding

Eighteen multiparous (parity ≥ 2), mid-lactating (DIM ranged from 100 to 250 days) Holstein Friesian dairy cows with a BW of 667 kg (SE = 9.5 kg) at the start of the experiment were used. Cows were housed individually in a tie-stall barn. The cubicles were covered with rubber cattle mats, with wood shavings as bedding, and were cleaned on a daily basis. Throughout the experiment, fresh drinking water was provided ad libitum. The cows were milked twice daily with a milking interval of 11 to 13 hours. Animal health was monitored by trained animal caretakers according to standard farm protocols.

Throughout the experiment, the allocated feed ingredients were fed separately to the cows and the animals received their diets in two equal portions at 0730 h and 1930 h. The cows were offered a restricted amount of feed (i.e. 18 kg DM/d) because it was anticipated that ad libitum access to feed would result in differences in DMI (Jiang et al., 2018), thereby, complicating the interpretation of the data. Feed refusals, if any, were collected daily prior to the morning meal.

Table 1. Ingredient and chemical composition of the experimental concentrates.

Item	Experimental concentrates	
	High P	Low P
Ingredient composition (g/kg)		
Beet pulp	482.3	482.3
Potato protein	210.0	210.0
Soybean meal	170.0	170.0
Molasses	50.0	50.0
Soya oil	25.0	25.0
Urea	17.0	17.0
CaCO ₃	15.0	15.0
MgO	10.0	10.0
Na ₂ SO ₄	5.0	5.0
TiO ₂	2.7	2.7
Premix ²	1.0	1.0
NaHCO ₃	-	12.0
Na ₂ HPO ₄	12.0	-
Total	1000.0	1000.0
Analyzed composition (g/kg DM)		
Dry matter (g/kg)	891.0	893.2
Crude ash	96.0	97.0
Crude protein	384.1	376.6
Neutral detergent fiber	240.1	251.0
Ca	11.27	10.96
P	4.41	1.75
Mg	7.03	7.06
Na	6.95	7.22
K	13.40	13.03
Ti	1.75	1.80
Calculated values (/kg DM) ³		
NE _L , MJ	7.42	7.42
IDP, g	217	217
RDPB, g	100	100

¹ The constant components were mixed and then divided into two parts prior to adding of sodium bicarbonate (low P concentrate) or sodium phosphate (high P concentrate).

² The premix consisted of 652.5 mg of CaCO₃, 2.0 mg of CoSO₄·7H₂O, 2.0 mg of Na₂SeO₃·5H₂O, 1.5 mg of KI, 100.0 mg of MnSO₄·H₂O, 100.0 mg of CuSO₄·5H₂O, 7.0 mg vitamin A prepare (3500 IU), 35.0 mg vitamin D prepare (3500 IU), and 100.0 mg vitamin E prepare (50 IU).

³ The net energy of lactation (NE_L) values were calculated according to the Dutch NE-system (Van Es, 1978). IDP = intestinal digestible protein; RDPB = rumen-degradable protein balance; the latter values are calculated according to the Dutch DVE/OEB-system (Tamminga et al., 1994).

Table 2. Ingredient and chemical composition of the experimental diets¹. Unless stated otherwise, values are expressed as g/kg dry matter (DM).

Item	Coarse dried grass ²		Pelleted dried grass ³
	High P	Low P	Low P
Ingredient composition			
Corn silage ⁴	470.0	473.8	475.2
Grass hay			
Coarse	256.9	251.6	-
Pelleted	-	-	249.4
Experimental concentrates			
High P	273.1	-	-
Low P	-	274.6	275.4
Analyzed composition			
Dry matter (g/kg)	486.3	484.3	481.0
Crude ash	68.9	68.5	76.3
Crude protein	171.2	173.8	178.4
Neutral detergent fiber	408.4	409.9	397.1
Ca	5.25	5.33	5.70
P	2.79	2.06	2.23
Mg	2.88	2.88	3.21
Na	2.16	2.09	2.52
K	14.3	14.4	15.2
Calculated composition values			
NE _L (MJ/kg DM) ⁵	6.52	6.53	6.50
IDP	97.3	97.6	96.5
RDPB	4.5	4.8	8.2

¹ The ingredient and chemical composition is based on the actual DM intakes of the feedstuffs.

² The analyzed composition of the coarse dried grass (924 g of DM/kg) was as follows (g/kg DM): CP, 78.4; NDF, 627.2; Ash, 91.2; Ca, 5.24; P, 2.67; Mg, 1.11; Na, 0.52; and K, 22.6.

³ The analyzed composition of the pelleted dried grass (888 g of DM/kg) was as follows (g/kg DM): CP, 95.6; NDF, 578.6; Ash, 123.0; Ca, 6.74; P, 3.35; Mg, 2.40; Na, 2.23; and K, 26.1.

⁴ The analyzed composition of the corn silage (319 g of DM/kg) was as follows (g/kg DM): CP, 102.7; NDF, 386.6; Ash, 40.5; Ca, 1.93; P, 1.91; Mg, 1.42; Na, 0.11; and K, 10.48.

⁵ The net energy of lactation (NE_L) values were calculated according to the Dutch NE-system (Van Es, 1978). IDP = intestinal digestible protein and RDPB = rumen-degradable protein balance, the latter values are calculated according to the Dutch DVE/OEB-system (Tamminga et al., 1994).

Data Collection and Sampling

Cows were weighed before the morning meal, on the first day of the pre-experimental period. During the last 5 d of each experimental period, each diet ingredient was sampled daily (~ 200 g DM) and daily samples were stored at 5 °C. Upon completion of the experimental period, the daily ingredient samples were pooled per ingredient and stored in a sealed jar at 5 °C. Feed refusals, if any, were collected quantitatively during the 5-d sampling period and subsequently pooled and stored at 5 °C. Milk yield was recorded, during four consecutive milking events, evening milkings from d15 and d18 and morning milkings from d16 and 17 of the experimental period. Directly after milking, milk was sampled and stored in tubes, containing sodium azide (0.03% m/v) for preservation, at 4 °C. Prior to chemical analysis, the two samples obtained during the morning milkings were pooled and the two samples obtained during the evening milkings were pooled as well. The samples were sent to a laboratory for analysis of their protein, fat and lactose concentrations maximally 3 h after the last milking event. Blood

samples were taken on d 19 of each experimental period. At 1100 and 1600 h, blood was sampled from the jugular vein into evacuated heparinized tubes. The blood samples were centrifuged for 15 min at $3000 \times g$, and the plasma was collected and stored at $-20\text{ }^{\circ}\text{C}$. Approximately 30 min after blood sampling, saliva samples were collected. Samples were squeezed out sponges that had been placed between the molars and the cheek (Schonewille et al., 1994). During saliva collection, synthetic surgical gloves were worn to prevent contamination of saliva with sweat. Approximately 10 ml of saliva was collected from each cow and samples were stored at $-20\text{ }^{\circ}\text{C}$. On the last day of each experimental period, between 1130 h and 1230 h, urine was collected from each individual cow by manual stimulation of the urogenital region so as to induce the micturition reflex. The individual urine collections were mixed and samples ($\sim 10\text{ ml}$) were stored at $-20\text{ }^{\circ}\text{C}$. Feces was collected on d16, 17 and 18 of the experimental period from 0800 until 1700 h. The individual 9 h feces collections were stored at $-20\text{ }^{\circ}\text{C}$. At the end of each collection period, the feces fractions of each cow were thawed, combined, and thoroughly mixed by means of a concrete mixer and sampled ($\sim 1\text{ kg DM}$). The pooled feces samples of each cow were subsequently stored at $-20\text{ }^{\circ}\text{C}$ until analysis. On d14 of each experimental period, from 0000 h onwards, cows were monitored for 24 h by means of a computer connected video recording system (Bascom[®], Utrecht, the Netherlands). Upon visual inspection of the video recordings, the time spent on eating and rumination was manually recorded with the use of a stopwatch.

Chemical Analysis

The samples of corn silage and feces were dried ($60\text{ }^{\circ}\text{C}$, 24 h) to obtain air-dry samples. Then, all feedstuff and feces samples were ground to pass a 1 mm screen in a cross beater mill (Peppink, Amsterdam, the Netherlands). The DM concentration of feed and feces samples was determined using forced-air oven drying ($105\text{ }^{\circ}\text{C}$) (ISO, 1999), and crude ash concentration after incineration at $550\text{ }^{\circ}\text{C}$ (ISO, 2002). Concentrations of NDF in samples were determined according to Van Soest et al. (1991) after pre-treatment with α -amylase and expressed without residual ash. Crude protein was calculated as $6.25 \times \text{N}$, where N was determined using the Kjeldahl method with CuSO_4 as a catalyst (ISO, 2005). The calcium, phosphorus, magnesium, sodium and potassium concentrations in feed and the phosphorus concentration of feces were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES, Eurofins Agro, Wageningen, the Netherlands). The Ti concentration of the experimental concentrates and feces was analyzed using sulfuric acid digestion in the presence of Cu at $420\text{ }^{\circ}\text{C}$. The subsequent Cu complex formed by addition of peroxide was determined spectroscopically at 408 nm as described by Nichols et al. (2018). The NE_L , intestinal digestible protein (IDP) and rumen degradable protein balance (RDPB) values of corn silage and artificially dried grass were obtained by near-infrared spectroscopy (Eurofins Agro, Wageningen, the Netherlands). The corresponding values of the experimental concentrates were calculated using tabular data (CVB, 2012). Milk samples were analyzed for fat, protein, and lactose by means of mid-infrared spectrometry (ISO, 2013; Qlip, Zutphen, the Netherlands).

All plasma samples were analyzed for Pi concentration (with ammonium molybdate) and Ca concentration (with Arsenazo III) using an automatic analyzer (ABX Pentra 400, Horiba,

Europe GmbH, Langenhagen, Germany). The concentrations of crosslaps (CL), osteocalcin (OC), parathyroid hormone (PTH), calcitriol (1,25(OH)₂D₃), and calciferol (25(OH)D₃) were determined by means of commercially available ELISA kits; CL, Serum crosslaps ELISA (Immundiagnostic Systems (ids) GmbH, Frankfurt am Main, Germany); OC, MicroVue Osteocalcin EIA Kit (Quidel Corp., San Diego, CA); PTH, Bovine Intact PTH ELISA Kit (Immunotopics Inc., San Clement, CA); 1,25(OH)₂D₃ and 25(OH)D₃, direct day ELISA (Immundiagnostik AG, Bensheim, Germany).

The salivary concentrations of P, Na, and K were measured using inductively coupled plasma optical emission spectrometry (ICP-OES) after mineralization using concentrated nitric acid. Briefly, a mixture of 200 µl of sample and 200 µl of 70% nitric acid were added to an 15 ml graduated metal-free polypropylene tube and incubated in a water bath of 70 °C for 1 hour. Then, yttrium was added as internal standard (5 mg/l in final dilution) and the volume was made up to 10 ml with distilled water. The solution, thereby, obtained was used to measure the concentrations of P, Na, and K.

Urine samples were analyzed for Ca and phosphate concentration measured colorimetrically by standard spectrometric techniques (Kawade, 1964; Ray Sarkar and Chauhan, 1967; Kruse-Jarres et al., 1979). Concentration of creatinine was determined by a standard diagnostic method in the Laboratory of Large Animals Clinics (Faculty of Veterinary Medicine, University of Leipzig, Germany).

Calculations and Statistical Analysis

Prior to statistical analysis, the daily values on DMI and milk yield were averaged per individual cow for the 5-d collection period. Fat- and protein-corrected milk (FPCM) was calculated as follows (CVB, 2012):

$$\text{FPCM (kg/d)} = \text{MY (kg/d)} \times [0.337 + 0.116 \times \text{MF (\%)} + 0.06 \times \text{MP (\%)}],$$

where MY = milk yield, MF = milk fat concentration, and MP = milk protein concentration. Feces excretion (kg DM/d) was calculated as Ti intake (g/d) / Ti concentration of feces (g/kg of DM). The apparent digestibility (% of intake) of P, OM and NDF was calculated as (Nutrient intake, g/d – Nutrient excretion with feces, g/d) / Nutrient intake, g/d × 100. For dry cows, the P balance was calculated by subtracting the daily P excretion with feces from the daily P intake. The P balance of lactating cows was calculated taking the secretion of P with milk also into account. A fixed value of 1.0 g P/kg of milk (CVB, 2005) was used to calculate absolute P excretion with milk. The amount of P excreted with urine in healthy ruminants is negligible (Grünberg, 2015) and was, therefore, ignored to calculate the P balance of the cows. With the exception of the data related to plasma and saliva, all other data were statistically analyzed by ANOVA (IBM SPSS Statistics 27th edition, 2020) using the model:

$$Y_{ijk} = \mu + B_i + T_j + e_{ij};$$

where Y_{ijk} = a response variable, μ = the overall mean, B_i = block ($i = 1$ to 3), T_j = the dietary treatment ($j = 1$ to 3; HP-CG, LP-CG or LP-PG, respectively), and e_{ijk} = residual error. The data related to plasma and saliva were analyzed with the same model including repeated measures. When the influence of treatment was significant, Tukey's t test was used to identify

diets with different effects on the variable involved. Differences were considered significant at $P \leq 0.05$ and tendencies are declared at $0.05 < P \leq 0.10$.

RESULTS

Rumination, Eating Time, DMI and Milk Production

Pelleting of the artificially dried grass caused a 26.6% reduction in rumination time ($P < 0.01$, Figure 1). The time spent on eating did not differ between HP-CG and LP-CG or LP-PG ($P \geq 0.30$) but the cows fed LP-PG spent 28.5% less time on eating ($P = 0.04$) compared to the cows fed LP-CG. All but one cows ingested their allocated diets completely. The cow that did not completely ingest the experimental diet was fed LP-CG and refused on average 0.76 kg DM of the artificially dried grass but fully consumed the allocated portions of corn silage and concentrate. Despite the aforementioned feed refusal, the data of this particular cow was taken into account when analyzing the data.

Total mean DMI and milk yield (Table 3) were similar ($P \geq 0.19$) between treatments. Upon ANOVA, FPCM yield was found to be affected ($P = 0.05$) by the factor diet, but significant differences between specific diets could not be identified by Tukey's t test. The fat content of milk was similar ($P = 0.21$) between diets but both the protein- and lactose contents of milk were greater ($P \leq 0.03$) when LP-PG instead of HP-CG was fed. The fat yield tended ($P = 0.06$) to be lower when LP-CG was fed, whereas the protein yield was 12.7% lower after the feeding of LP-CG compared to LP-PG. The yield of lactose was neither affected ($P = 0.40$) by the P content of the diet nor the physical form of the artificially dried grass (i.e. CH vs PG).

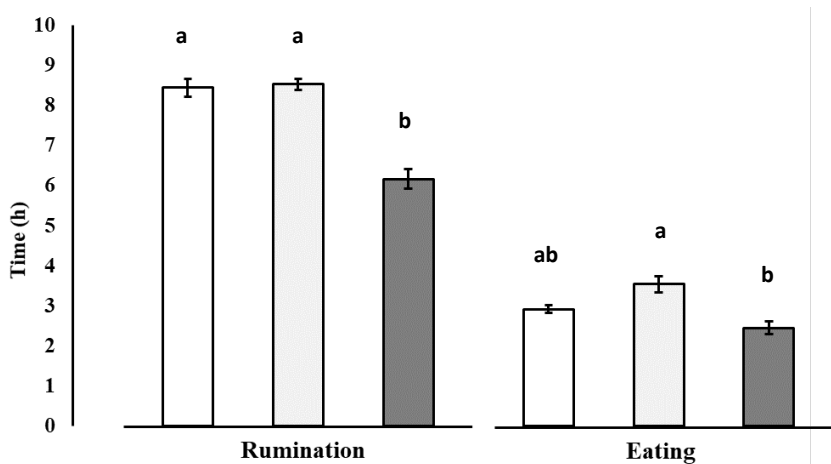


Figure 1. Effect of the physical form of artificially dried grass on rumination and eating time (h). Data were obtained after manually counting the time based on 24 h camera recordings of each individual cow (error bar = SE). Legend: white bars, coarse dried grass -high P diet (2.79 g P/kg DM); light grey bars, coarse dried grass -low P diet (2.06 g P/kg DM); dark grey bars, pelleted dried grass-low P diet (2.23 g P/kg DM). ^{a,b} within parameters, a different superscript indicates a significant difference ($P < 0.05$).

Table 3. Means of milk yield, milk composition and absolute productions of milk fat, milk protein and lactose when cows were fed experimental diets differing in dietary P concentrations¹ and dried grass fed in either a coarse or a pelleted form.

Item	Coarse dried grass		Pelleted dried grass	SEM	P-values
	High P	Low P	Low P		
DMI (kg/d)	18.0	17.9	17.8	0.07	0.19
Milk yield (kg/d)	25.3	23.9	25.2	0.97	0.57
FPCM ² yield (kg/d)	26.0	23.3	26.8	0.95	0.05
Fat (g/kg)	42.4	37.7	44.4	2.58	0.21
Protein (g/kg)	33.3 ^b	33.7 ^{ab}	36.7 ^a	0.82	0.02
Lactose (g/kg)	43.5 ^b	44.5 ^{ab}	45.6 ^a	0.48	0.03
Fat yield (g/d)	1069	897	1105	59.5	0.06
Protein yield (g/d)	841 ^{ab}	804 ^b	921 ^a	31.2	0.05
Lactose yield (g/d)	1100	1064	1148	42.7	0.40

^{a,b} Different superscripts within rows indicate significant differences at $P < 0.05$.

¹ The diets with coarse dried grass contained 2.79 g P/kg DM and 2.06 g P/kg DM for high- and low P, respectively. The diet with pelleted dried grass contained 2.23 g P/kg DM.

² FPCM = Fat- and protein-corrected milk.

SEM = Standard error of the mean.

Urinary P and Ca Concentrations

In case the LP-PG diet was fed, both the urinary creatinine concentration and the Ca concentration were lower ($P = 0.04$) compared to HP-CG, i.e. 24% and 51%, respectively (Table 4). No differences ($P = 0.20$) were found in urinary P concentrations between diets. Both the Ca to creatinine-, and the P to creatinine ratio were not influenced by dietary treatments ($P \geq 0.12$).

Table 4. Selected indices on urinary creatinine, Ca, and P concentrations when cows were fed experimental diets differing in dietary P concentrations¹ and dried grass fed in either a coarse or a pelleted form.

Item	Coarse dried grass		Pelleted dried grass	SEM	P-values
	High P	Low P	Low P		
Creatinine (mM)	7.73 ^a	7.71 ^a	5.87 ^b	0.550	0.04
Calcium (mM)	2.79 ^a	2.46 ^{ab}	1.36 ^b	0.402	0.04
Phosphate (mM)	1.64	1.46	1.35	0.124	0.20
Calcium to creatinine ratio	0.37	0.30	0.23	0.049	0.13
Phosphorus to creatinine ratio	0.21	0.19	0.24	0.017	0.12

^{a,b} Different superscripts within rows indicate significant differences at $P < 0.05$.

¹ The diets with coarse dried grass contained 2.79 g P/kg DM and 2.06 g P/kg DM for high- and low P, respectively. The diet with pelleted dried grass contained 2.23 g P/kg DM.

SEM = Standard error of the mean.

Plasma Pi and Ca and Salivary Pi, Na, and K

Neither the plasma concentrations of Pi and Ca nor the P, Na and K concentrations of saliva (Table 5) were affected by an interaction between time of sampling and dietary treatment ($P \geq 0.12$). The plasma Pi concentrations were similar between time of sampling and diets ($P \geq 0.24$). The plasma Ca concentrations were similar between diets ($P = 0.43$) but plasma Ca concentrations were found to be $\sim 3\%$ greater at 1630 h compared to 1130 h ($P = 0.03$).

The P concentration of saliva (Table 5) tended to be lower when LP-CG was fed ($P = 0.09$) but it was not affected by the time of sampling ($P = 0.16$). The salivary Na concentration was $\sim 9\%$ lower ($P = 0.03$) when LP-CG was fed instead of HP-CG, but the salivary Na concentrations of HP-CG and LP-PG were similar ($P = 0.31$). The Na concentration of saliva sampled at 1630 h was found to be $\sim 11\%$ greater compared to 1130 h ($P < 0.01$). The K concentration of saliva was neither affected by P intake nor the physical form of the artificially dried grass ($P = 0.15$) but the values were $\sim 18\%$ lower at 1630 h ($P = 0.03$) compared with 1130 h.

Table 5. Plasma Pi and Ca concentrations and concentrations of P, Na, and K in saliva when cows were fed experimental diets differing in dietary P concentrations¹ and dried grass fed in either a coarse or a pelleted form.

Item	Sampling time ²		Coarse dried grass		Pelleted dried grass	SEM	P-values		
	Morning	Afternoon	High P	Low P	Low P		Time	Diet	Time \times Diet
Plasma (mM)									
Pi	1.37	1.34	1.48	1.23	1.37	0.097	0.58	0.24	0.45
Ca	2.57 ^b	2.65 ^a	2.59	2.66	2.59	0.041	0.03	0.43	0.12
Saliva (mM)									
P	5.60	6.25	6.91	4.83	6.02	0.613	0.16	0.09	0.80
Na	133.7 ^b	148.0 ^a	147.7 ^a	134.2 ^b	140.5 ^{ab}	3.34	< 0.01	0.04	0.25
K	11.2 ^a	9.2 ^b	9.1	10.8	10.7	0.63	0.03	0.15	0.69

^{a,b} Different superscripts within rows indicate significant differences at $P < 0.05$.

¹ The diets with coarse dried grass contained 2.79 g P/kg dry matter (DM) and 2.06 g P/kg DM for high- and low P, respectively. The diet with pelleted dried grass contained 2.23 g P/kg DM.

² Plasma samples were collected at 1100 h and 1600 h while saliva was collected at 1130 h and 1630h. SEM = Standard error of the mean.

P Balance and Total Tract Digestibility of OM and NDF

The cows fed the LP-CG or LP-PG diets ingested respectively $\sim 27\%$ or $\sim 21\%$ lower amounts of P (Table 6) compared to the cows fed the HP diet ($P < 0.01$) but the amount of P excreted with feces was similar between diets ($P = 0.59$). Irrespective of the physical form of the artificially dried grass, the absolute amount of P absorbed by the cows fed the LP diets was lower compared to the cows fed the HP diet ($P < 0.01$) but P absorption expressed as a fraction of intake was similar between the experimental diets ($P = 0.31$). The amount of P secreted with milk also was not affected by dietary treatments ($P = 0.57$). In contrast, the P balance was found to be negative for both LP diets while the cows fed HP maintained a P balance around zero (P

< 0.01). The total tract digestibilities of OM and NDF were not influenced by P intake, nor the physical form of the artificially dried grass ($P \geq 0.73$).

Table 6. Phosphorus balance and total tract digestibility of OM and NDF when cows were fed experimental diets differing in dietary P concentrations¹ and dried grass fed in either a coarse or a pelleted form.

Item	Coarse dried grass		Pelleted dried grass	SEM	P-values
	High P	Low P	Low P		
P intake (g/d)	50.2 ^a	36.8 ^c	39.6 ^b	0.19	< 0.01
P feces (g/d)	24.9	22.0	22.8	2.04	0.59
Apparent P absorption					
Absolute (g/d)	25.3 ^a	14.8 ^b	16.8 ^b	2.03	< 0.01
Relative (% of intake)	50.4	40.3	42.5	4.67	0.31
Milk P (g/d)	25.3	23.9	25.2	0.97	0.57
P balance (g/d)	0.1 ^a	-9.1 ^b	-8.3 ^b	1.76	< 0.01
OM digestibility (% of intake)	70.2	70.9	71.6	1.22	0.73
NDF digestibility (% of intake)	53.5	54.9	54.5	2.21	0.89

^{a,b,c} Different superscripts within rows indicate significant differences at $P < 0.05$.

¹ The diets with coarse dried grass contained 2.79 g P/kg DM and 2.06 g P/kg DM for high- and low P, respectively. The diet with pelleted dried grass contained 2.23 g P/kg DM. SEM = Standard error of the mean.

Plasma Concentrations of PTH, Vitamin D₃ Metabolites, CL and OC

Plasma concentrations of PTH, vitamin D₃ metabolites, and CL (Table 7) were not affected by an interaction between time of sampling and dietary treatment ($P \geq 0.35$). Plasma PTH concentrations were similar between diets ($P = 0.67$) but tended to be lower at 1630 h compared to 1100 h ($P = 0.09$). Plasma concentrations of 25(OH)D₃ were similar between diets and time of sampling ($P \geq 0.33$). Plasma concentrations of 1,25(OH)₂D₃ also were similar between diets ($P = 0.95$) and tended to be greater at 1630 h compared to 1130 h ($P = 0.09$). The plasma CL concentrations tended to be affected by diet ($P = 0.08$); numerically, the lowest plasma CL concentration was found when the HP-CG diet was fed. Plasma CL concentrations were ~ 18% lower ($P < 0.01$) at 1630 h. The plasma concentrations of OC tended to be affected by time \times diet ($P = 0.07$) but not by diet and time point of sampling ($P \geq 0.15$). The OC to CL ratio (Figure 2) was not affected by an interaction between time of sampling and diet ($P = 0.19$) but at 1600 h the values were ~ 30% greater compared to 1100 h ($P < 0.01$). Upon ANOVA, the OC to CL ratio was affected by P intake ($P = 0.04$) but the difference between HP and the two LP diets just failed to reach statistical significance (i.e. $P = 0.06$ for both LP diets) when Tukey's *t* test was used.

Table 7. Plasma concentrations of parathyroid hormone (PTH), calciferol (25(OH)D₃), calcitriol (1,25(OH)₂D₃), crosslaps, and osteocalcin of cows fed experimental diets differing in dietary P concentrations¹ and dried grass fed in either a coarse or a pelleted form.

Item	Sampling time ²		Coarse dried grass		Pelleted dried grass	SEM	P-values		
	Morning	Afternoon	High P	Low P	Low P		Time	Diet	Time × Diet
PTH (pg/ml)	1973	421	1670	1249	658	800.1	0.09	0.67	0.60
25(OH)D ₃ (nM)	16.6	16.8	18.5	16.3	15.3	1.50	0.81	0.33	0.35
1,25(OH) ₂ D ₃ (pg/ml)	25.6	28.1	27.3	26.3	26.8	2.23	0.09	0.95	0.63
Crosslaps (ng/ml)	2.29*	1.88	1.25	2.25	2.74	0.432	< 0.01	0.08	0.92
Osteocalcin (ng/ml)	42.3	43.5	39.9	44.3	44.5	4.59	0.15	0.74	0.07

* Significant different ($P < 0.05$) of the concentration from the afternoon.

¹ The diets with coarse dried grass contained 2.79 g P/kg DM and 2.06 g P/kg DM for high- and low P, respectively. The diet with pelleted dried grass contained 2.23 g P/kg DM.

² Plasma samples were collected at 1100 h and 1600 h while saliva was collected at 1130 h and 1630 h.

SEM = Standard error of the mean.

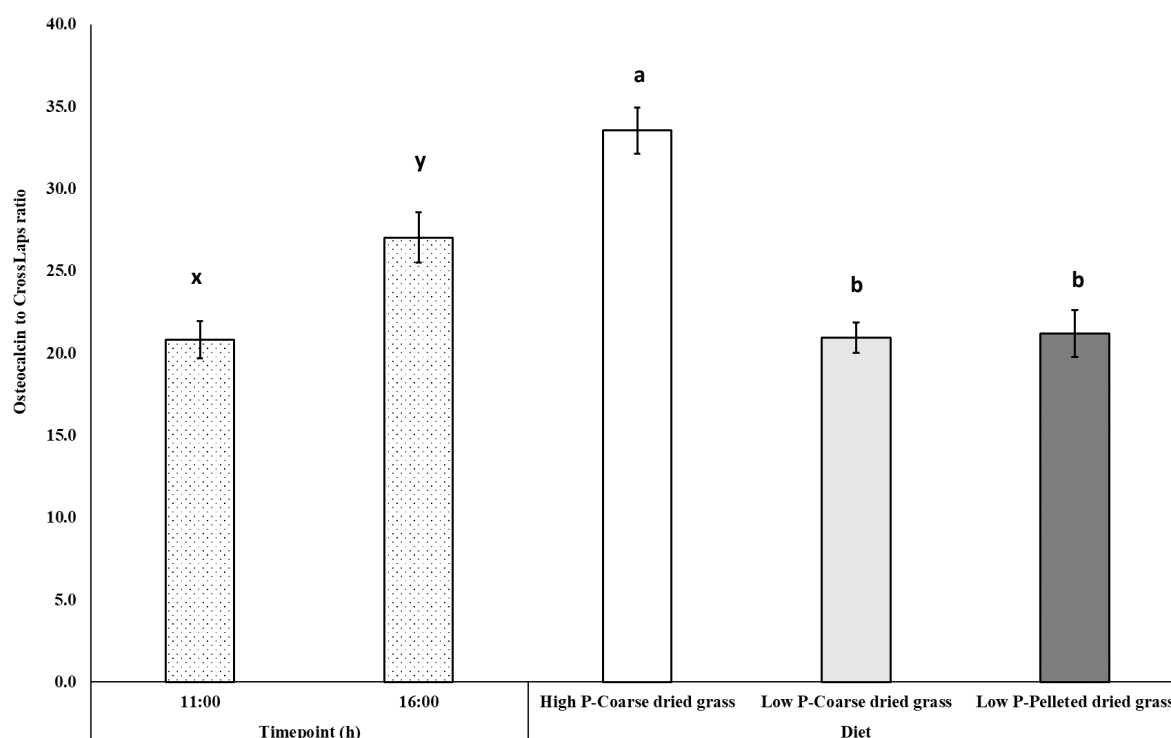


Figure 2. Osteocalcin to crosslaps ratio (error bar = SE) in response to the feeding of the experimental diets to cows, i.e. coarse dried grass-high P diet (white bars, 2.79 g P/kg DM), coarse dried grass-low P diet (light grey, 2.06 g P/kg DM); and pelleted dried grass-low P diet (dark grey, 2.23 g P/kg DM). *P*-values: Time (1100 vs 1600 h), < 0.01; Diet, 0.04; and Time x Diet, 0.19. ^{a,b} different superscript indicate a borderline significant difference between dietary treatments (*P* = 0.06). ^{x,y} different superscript indicates a significant difference between the two timepoints (*P* < 0.01).

DISCUSSION

P Balance, Plasma P, 1,25(OH)₂D₃, PTH, CL and OC

Clearly, the plasma concentration of 1,25(OH)₂D₃ did not respond to the two low P diets and we, therefore, rejected the two hypotheses. Furthermore, the P balances of the cows fed the two low P diets were clearly negative, thereby, confirming that these diets were P deficient. The current lack of response of 1,25(OH)₂D₃ upon the feeding of a P deficient diet is in line with recent data reported by Wächter et al. (2021) who also reported that the plasma 1,25(OH)₂D₃ concentration did not respond to P intake when P intake was 11 % below P requirements (CVB, 2012). Next to 1,25(OH)₂D₃, the plasma concentrations of 25(OH)D₃ did not respond to the low P intakes as well. The conversion of 25(OH)D₃ into 1,25(OH)₂D₃ is mediated by PTH (Bergwitz and Jüppner, 2010; Anderson et al., 2017) which induces the production of 1 α OH-ase in kidney tissue. In the current study, PTH did not respond to the low P intakes, i.e. the LP-CG or LP-PG diet. This observation is corroborated by Cohrs et al. (2018), who reported that plasma Pi concentrations of ~ 0.5 mM did not trigger a PTH response. Furthermore, the observed plasma Ca concentrations in the current study were similar to the upper values of the reference values of plasma Ca, i.e. ranging from 2.1 to 2.6 mM, (Goff, 1999). Moreover, plasma Ca concentrations were also similar between the diets. In view of current results on the plasma Ca

concentrations, we, therefore, speculate that a Ca induced PTH (Goff, 2000) response did not confound the current observations on $1,25(\text{OH})_2\text{D}_3$. There are, however, indications, that the condition of hypophosphatemia, independently from PTH (Köhler et al., 2021), inhibits the synthesis of fibroblast growth factor 23 (FGF23) and Perward et al. (2007) showed that FGF23 increases the activity of 1α OH-ase, thereby, promoting the synthesis of $1,25(\text{OH})_2\text{D}_3$. Perhaps, the observed plasma Pi concentrations in the current study, although the P content of LP diets was below recommended levels and P balance was negative, were not low enough to effectively increase the plasma concentrations of $1,25(\text{OH})_2\text{D}_3$. Nevertheless, the current data, and those reported by Schröder and Breves (1996) and Wächter et al. (2021) fuel the notion that $1,25(\text{OH})_2\text{D}_3$ does not play a relevant role in P absorption in dairy cows.

In contrast to $1,25(\text{OH})_2\text{D}_3$, the plasma CL concentrations tended to be increased when either low P diet was fed (Table 7) whereas the OC to CL ratios were found to be lower when LP vs HP was fed (Figure 2). The aforementioned changes in plasma CL concentrations and OC to CL ratio can be interpreted that the cows mobilized P from bone (Puggaard et al., 2011) when the low P diets were fed. This notion is in line with the negative P balances in case the LP diets were fed. It thus appears that in the current conditions, P mobilization from bone is more important than upregulation of P absorption when P deficient diets are fed. In dairy cows, mobilization of P from bone appears to be common during early lactation (Keanthao et al., 2021, Wu et al., 2000) but P stores in bone have to be replenished (Ekelund et al., 2006) to obtain a zero P balance across whole lactation. In the current study, mid lactation cows were used and it was anticipated that these cows would be susceptible to a P induced upregulation of P absorption, but that appeared to be not the case. Perhaps, the time window of the current experimental period was too short and/or the P-deficiency level not severe enough to effectively induce an upregulation of P absorption.

Salivary Pi and Total Tract Digestibility of OM and NDF

The salivary Na concentrations were found to be within the normal range (i.e. ~ 120 to ~ 155 mM) and the Na to K ratio in saliva points to a sufficient intake of Na (Schonewille and Beynen, 2005) which is in line with the dietary Na concentrations of the experimental diets, i.e. 2.16, 2.09, and 2.52 g Na/kg DM for HP-CG, LP-CG, and LP-PG, respectively.

The salivary P concentrations were not clearly affected by the experimental diets (Table 5) but numerically, the values are in line with expectation in that the lowest value was found when cows were fed the low P diet in combination with coarse dried grass. Rumination time was almost equal between HP-CG and LP-CG, thereby, implying a similar saliva production (Beauchemin et al., 2008) between the two latter diets. In case of LP-CG, lower amounts of P were absorbed compared with HP-CG, and most likely, lower amounts of P ended up in saliva. In case the feeding of low P was combined with the feeding of pelleted dried grass, rumination time was clearly lowered and thus, most likely, the amount of saliva produced. Absolute P absorption was similar between LP-CG and LP-PG, and in combination with a lower saliva production when feeding LP-PG, it will result in a greater salivary P concentration compared to LP-CG.

The salivary P concentration was found to be 6.91 mM when HP was fed. This value is considerably lower compared to the salivary P concentrations obtained from dry cows reported

by Bailey and Balch (1961), i.e. values in the range of 10-15 mM. Valk et al. (2002), however, reported salivary P concentrations obtained from lactating dairy cows and in that study, the salivary P concentrations ranged from 7.5 to 8.6 mM when P intakes were 100% matched with corresponding P requirements (Netherlands committee on mineral nutrition, 1973). The current salivary P concentration observed upon the feeding of the HP diet agree fairly well with those reported by Valk et al. (2002). The somewhat lower salivary P concentration observed after the feeding of the HP diet may be related to the fact that some P was excreted with urine (Table 4) and thus that the actual P balance of the cows fed HP-CG was ~ 1-2 g P lower (Schonewille et al., 1994) than the value presented in Table 6, i.e. 0.1 g P/d. Thus, actual P intakes did not fully match with the amount of P required when HP-CG was fed.

The apparent total tract of OM- and NDF digestibility was neither affected by P-intake nor the physical form of the dried grass. An adequate supply of P to the rumen microbes is important to safeguard OM- and NDF digestibility (Komisarczuk-Bony and Durand, 1991). Upon in-vitro assessments, Komisarczuk-Bony and Durand (1991) reported that a minimum P concentration of 0.5 mM in rumen fluid is required for optimum fiber digestion. Under most feeding conditions, however, the P concentration of rumen fluid is greater than 0.5 mM Puggaard et al. (2011) reported that mean rumen P concentrations ranged between ~ 2 mM and ~ 4 mM when dairy cows were fed a diet containing 2.4 g P/kg DM, while Rodehutsord et al. (1994) reported rumen P concentrations ~ 5 mM when a diet containing 1.4 g P/kg DM was fed to goats. Next to P intake as such, the contribution of salivary P also has a relevant impact on the P concentration of rumen fluid (Tomas, 1973; Care 1994). Valk et al. (2002) reported salivary P concentrations ranging from 5.1 to 7.3 mM when lactating dairy cows were fed 33% below their P requirement. In the current study, the cows fed LP (i.e. P intake 28% below requirement), group mean salivary P concentrations ranged between 4.83 and 6.02 mM. Thus, even in case a P deficient diet is fed, cows are able to maintain a salivary P concentration ~ 4.5 mM. It is, therefore, speculated that in the current study, the supply of P to the microbes in the rumen was sufficient to safeguard apparent total tract OM and NDF digestion, thereby, explaining the lack of effect of low P intake on the total tract digestibility of both OM and NDF.

Salivary Pi, Endogenous Fecal P and True P Absorption

The Dutch Central Bureau for Livestock Feeding (CVB, 2005) adopted the value proposed by Valk (2005) to calculate the endogenous fecal P loss, i.e., 0.81 g P/kg DM. This latter value, however, only takes into account the inevitable fecal losses originating from salivary P (Valk, 2005). Thus, within the Dutch system (CVB, 2005) the endogenous fecal P losses related to microbial residues, sloughed cells, and digestive secretions are not taken into account when calculating the endogenous fecal P loss because these factors are considered quantitatively unimportant compared to the inevitable loss of salivary P (Valk et al., 2002). When calculating the endogenous fecal P loss, the salivary P concentration is assumed to be 8 mM, but in the current study the salivary P concentrations were found to be somewhat lower, thereby, implying that the use of 0.81 g P/kg DM will overestimate the endogenous fecal loss of P. When the currently observed group mean salivary P concentrations (across the two timepoints) are used to calculate the endogenous fecal loss of P, the values (g P/kg DM) are 0.72, 0.51, and 0.61 for HP-CG, LP-CG, and LP-PG, respectively. In case the latter values are used to calculate the true

P absorption for the three experimental diets, the following values (% of intake) can be calculated; 76.1, 65.2, and 69.7 for HP-CG, LP-CG, and LP-PG, respectively. In case of the HP diet the calculated value on true P absorption (i.e. 76.1%) agrees well with the corresponding value adopted by the Dutch Central Bureau for Livestock Feeding (CVB, 2005), i.e. 75% of intake. With respect to the two low P diets, the yet calculated values seem low. Perhaps, the endogenous fecal loss of P is underestimated when the values 0.51 and 0.61 g P/kg DM are used because in case P-deficient diets are fed, the contribution of microbial residues, sloughed cells, and digestive secretions may become relevant.

Milk Production

The cows fed the diet containing pelleted dried grass had greater protein and lactose contents of milk resulting in a greater protein yield (compared with cows fed the high P coarse artificial dried grass diet). The greater yield of milk protein cannot be explained by a greater supply of intestinal digestible protein (Table 2) but it is speculated that the greater protein yield can be explained by greater glucose supply to the mammary gland. A greater glucose supply, if any, cannot be explained by the OM-, or NDF digestibility because they were similar between treatments. However, the particle size of the pelleted grass was much smaller compared to the coarse grass which most likely increased the ruminal fermentation rate of the OM (Sutton, 1985). A greater fermentation rate shifts the profile of volatile fatty acids from acetate to propionate (Krause et al., 2002), thereby, yielding more substrate for gluconeogenesis to support lactogenesis (Aschenbach et al., 2010).

CONCLUSIONS

Cows fed with grass pellets had higher milk lactose and protein contents than cows fed with coarse dried grass. However, it did not affect plasma Pi, vitamin D₃ metabolites, and CL and OC concentrations. Cows fed the high P diet had higher plasma Pi concentrations than cows fed the low P diet, and cows fed the low P diet were in negative P balance. No upregulation of 1,25(OH)₂D₃ to increase P absorption efficiency in cows fed the low P diet was observed. Instead, cows fed low P diets mobilized P from bone to satisfy their requirements.

ACKNOWLEDGEMENTS

We would like to acknowledge the staff at the research facility of the Faculty of Veterinary Medicine, Utrecht University, the Netherlands. We would also like to acknowledge the Institute of Animal Nutrition, Nutrition Diseases and Dietetics of the Faculty of Veterinary Medicine, Leipzig University, Leipzig, Germany for laboratory support.

REFERENCES

AFRC. 1991. A reappraisal of the calcium and phosphorus requirements of sheep and cattle, report 6. Nutr. Abs. Rev. B. 61: 573-608.

- Anderson, S. T., L. J. Kidd, M. A. Benvenutti, M. T. Fletcher, and R. M. Dixon. 2017. New candidate markers of phosphorus status in beef breeder cows. *Anim. Prod. Sci.* 57: 2291-2303.
- Aschenbach, J. R., N. B. Kristensen, S. S. Donkin, H. M. Hammon, and G. B. Penner. Gluconeogenesis in dairy cows: The secret of making sweet milk from sour dough. *IUBMB Life.* 62: 869-877.
- Bailey, C. B., and C. C. Balch. 1961. Saliva excretion and its relation to feeding in cattle: 2. The composition and rate of secretion of mixed saliva in the cow during rest. *Br. J. Nutr.* 15: 383-402.
- Bailey, C. B. 1961. Saliva excretion and its relation to feeding in cattle. 3. The rate of secretion of mixed saliva in the cow during eating, with an estimate of the magnitude of the total daily secretion of mixed saliva. *Br. J. Nutr.* 15: 443-451.
- Beauchemin, K. A., L. Eriksen, P. Nørgaard, and L. M. Rode. 2008. Short communication: salivary secretion during meals in lactating dairy cattle. *J. Dairy Sci.* 91: 2077-2081.
- Bergwitz, C., and H. Jüppner. 2010. Regulation of phosphate homeostasis by PTH, vitamin D and FGF32. *Annu. Rev. Med.* 61: 91-104.
- Breves, G., M. Beyerbach, H. Holler, and H. W. Lessmann. 1985. Fluid exchange in the rumen of sheep in low and adequate phosphorus administration. *Dtsch. Tierarztl. Wochenschr.* 92: 47-49.
- Care, A. D. 1994. The absorption of phosphate from digestive tract of ruminant animals. *Br. Vet. J.* 150: 197-205.
- Cohrs, I., M. R. Wilkens, and W. Grünberg. 2018. Short communication: Effect of dietary phosphorus deprivation in late gestation and early lactation on the calcium homeostasis of periparturient dairy cows. *J. Dairy Sci.* 101: 9591-9598.
- CVB. 2005. Handleiding mineralenvoorziening rundvee, schapen, geiten. the Hague, the Netherlands.
- CVB. 2012. CVB Farm Animal Feeding Advice 2012. CVB volume 50, the Hague, the Netherlands.
- CVB. 2016. CVB Table Booklet Feeding of Ruminants 2016. CVB volume 57, the Hague, the Netherlands.
- Ekelund, A., R. Spörndly, and K. Holtén. 2006. Influence of low phosphorus intake during early lactation on apparent digestibility of phosphorus and bone metabolism in dairy cows. *Livest. Sci.* 99: 227-236.
- GfE. 1993. Überarbeitete Empfehlungen zur Versorgung von Milchkühen mit Calcium und Phosphor. *Proc. Soc. Nutr. Physiol.* 1: 108-113.
- Goff, J. P. 1999. Treatment of calcium, phosphorus, and magnesium balance disorders. *Vet. Clin. North Am. Food Anim. Pract.* 15: 619-639.
- Goff, J. P., K. Kimura, and R. L. Horst. 2002. Effect of mastectomy on milk fever, energy, and vitamins A, E, and β -carotene status at parturition. *J. Dairy Sci.* 85: 1427-1436.
- Gray, R. W. 1987. Evidence that somatomedins mediate the effect of hypophosphatemia to increase serum 1,25-dihydroxyvitamin D, levels in rats. *Endocrinol.* 121: 504-512.
- Host, R. L. 1986. Regulation of calcium and phosphorus homeostasis in dairy cow. *J. Dairy Sci.* 69: 604-616.
- IBM Corp. 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.

- ISO. 1999. Animal feeding stuffs. Determination of moisture and other volatile matter content. ISO 6496. ISO, Geneva, Switzerland.
- ISO. 2002. Animal feeding stuffs. Determination of crude ash. ISO 5984. ISO, Geneva, Switzerland.
- ISO. 2005. Animal feeding stuffs. Determination of nitrogen content and calculation of crude protein content-Part 1: Kjeldahl method. ISO 5983-1. ISO, Geneva, Switzerland.
- ISO. 2013. Whole milk. Determination of milk fat, protein and lactose content-Guidance on the operation of mid-infrared instruments. ISO 9622. ISO, Geneva, Switzerland.
- Jiang, F. G., X. Y. Lin, Z. G. Yan, Z. Y. Hu, Y. Wang, and Z. H. Wang. 2018. Effects of forage source and particle size on chewing activity, ruminal pH, and saliva secretion in lactating Holstein cows. *Anim. Sci. J.* 90: 382-392.
- Kawade, M. 1964. An automatic method for determination of alkaline phosphatase in serum using p-nitrophenylphosphate as substrate. *Mie. Med. J.* 14: 41-6.
- Keanthao, P., R. M. A. Goselink, J. Dijkstra, A. Bannink, and J. T. Schonewille. 2021. Effects of dietary phosphorus concentration during the transition period on plasma calcium concentrations, feed intake, and milk production in dairy cows. *J. Dairy Sci.* 104: 11646-11659.
- Klop, G., J. L. Ellis, A. Bannink, E. Kebreab, J. France, and J. Dijkstra. 2013. Meta-analysis of factors that affect the utilization efficiency of phosphorus in lactating dairy cows. *J. Dairy Sci.* 96: 3936-3949.
- Knowlton, K. F., and J. H. Herbein. 2002. Phosphorus partitioning during early lactation in dairy cow fed diets varying in phosphorus content. *J. Dairy Sci.* 85: 1227-1236.
- Köhler, M. O., W. Grünberg, N. Schnepel, S. A. Muscher-Banse, A. Rajaeerad, J. Hummel, G. Breves, and M. R. Wilkins. 2020. Dietary phosphorus restriction affects bone metabolism, vitamin D metabolism and rumen fermentation traits in sheep. *J. Anim. Physio. Anim. Nutri.* 105: 35-50.
- Komisarczuk-Bony, S., and M. Durand. 1991. Effects of minerals on microbial metabolism. Ed. J. P. Jouany. Institut National de la Recherche Agronomique (INRA), Paris. *Rumen Microbial Metabolism and Ruminant Digestion.* 179-198.
- Krause, K. M., D. K. Combs, and K. A. Beauchemin. 2002. Effects of forage particle size and grain fermentation in midlactation cows. II. Ruminal pH and chewing activity. *J. Dairy Sci.* 85: 1947-1957.
- Kruse-Jarres, J. D. 1979. *Klinische Chemie, Vol. II: Spezielle Klinisch Chemische Analytik.* Stuttgart, Germany.
- Lee, D. B. N., M. W. Walling, and D. B. Corry. 1986. Phosphate transport across rat jejunum: influence of sodium, pH and 1,25-dihydroxyvitamin D. *Am. J. Physiol.* 251: G91-G95.
- Liu, Q., J. Wang, Z. Bai, L. Ma, and O. Oenema. 2017. Global animal production and nitrogen and phosphorus flows. *Soil Res.* 55: 451-462.
- Maekawa, M., K. A. Beauchemin, and D. A. Christensen. 2002. Effect of concentrate level and feeding management on chewing activities, saliva production, and ruminal pH of lactating dairy cows. *J. Dairy Sci.* 85: 1165-1175.
- Martz, F. A., A. T. Belo, M. F. Weiss, and R. L. Belyea. 1999. True absorption of calcium and phosphorus from corn silage fed to nonlactating, pregnant dairy cows. *J. Dairy Sci.* 82: 618-622.

- Moreira, V. R., L. K. Zeringue, C. C. Williams, C. Leonardi, and M. E. McCormick. 2009. Influence of calcium and phosphorus feeding on marker of bone metabolism in transition cows. *J. Dairy Sci.* 92: 5189-5198.
- Netherlands Committee on Mineral Nutrition. 1973. Tracing Mineral Disorders in dairy cattle. Centre for Agriculture Publishing, Wageningen, the Netherlands.
- NRC. 2001. Nutrient Requirements of Dairy Cattle, 7th revised edition. National Academy of Science, Washington, DC.
- Nichols, K., A. Bannink, S. Pacheco, H. J. van Valenberg, J. Dijkstra, and H. van Laar. 2018. Feed and nitrogen efficiency are affected differently but milk lactose production is stimulated equally when isoenergetic protein and fat is supplemented in lactating dairy cow diets. *J. Dairy Sci.* 101: 7857-7870.
- Perward, F., M. Y. H. Zhang, H. S. Tenenhouse, and A. A. Portale. 2007. Fibroblast growth factor 23 impairs phosphorus and vitamin D metabolism *in vivo* and suppresses 25-hydroxyvitamin D-1 α -hydroxylase expression *in vitro*. *Am. J. Physiol. Renal. Physiol.* 293: 1577-1583.
- Pfeffer E., D. K. Breede, and H. Valk. 2005. Phosphorus Metabolism in Ruminants and Requirements of Cattle. CAB International, Wallingford, UK. 195-231.
- Puggaard, L., N. B. Kristensen, and J. Sehested. 2011. Effect of decreasing dietary phosphorus supply on net recycling of inorganic phosphate in lactating dairy cows. *J. Dairy Sci.* 94: 1420-1429.
- Puggaard, L. 2012. The effect of dietary parameters on phosphorus metabolism and excretion in dairy cows. PhD thesis, Aarhus University, Denmark.
- Ray Sarkar, B. C., and U. P. S. Chauhan. 1967. A new method for determining micro quantities of calcium in biological materials. *Anal. Biochem.* 20: 155-166.
- Reijnders, L. 2014. Phosphorus resources, their depletion and conservation, a review. *Resour. Conserv. Recycl.* 93: 32-49.
- Rodehutsord, M., A. Pauen, P. Windhausen, R. Brintrup, and E. Pfeffer. 1994. Effects of drastic changes in P intake on P concentrations in blood and rumen fluid of lactating ruminants. *J. Vet. Med. A.* 41: 611-619.
- Schonewille, J. Th., A. Th. Van't Klooster, and A. C. Beynen. 1994. High phosphorus intake depresses apparent magnesium absorption in pregnant heifers. *J. Anim. Physiol. Anim. Nutr.* 71: 15-21.
- Schonewille, J. Th. and A. C. Beynen. 2005. Reviews on the mineral provision in ruminants (IV): Sodium metabolism and requirements in ruminants. CVB documentation Report 36. Central Bureau for Livestock Feeding, Lelystad, the Netherlands.
- Schröder, B. and G. Breves. 1996. Mechanism of phosphate uptake into brush-border membrane vesicles from goat jejunum. *J. Comp. Physiol. B.* 166: 203-240.
- Smith, R. A., and R. B. Alexander. 2000. Sources of Nutrients in the Nation's Watersheds: Managing Nutrients and Pathogens from Animal Agriculture Proceedings from the Natural Resource, Agriculture, and Engineering Service Conference for Nutrient Management Consultants, Extension Educators, and Producer Advisors. March 28-30, 2000, Camp Hill, Pennsylvania.
- Sutton, J. D. 1985. Digestion and absorption of energy substrates in the lactating cow. *J. Dairy Sci.* 68: 3376-3393.

- Tamminga, S., W. M. Van Straalen, A. P. J. Subnel, R. G. M. Meijer, A. Steg, C. J. G. Wever, and M. C. Blok. 1994. The Dutch protein evaluation system: the DVE/OEB-system. *Livest. Prod. Sci.* 40: 139-155.
- Tomas, F. M. 1973. Parotid salivary secretion in sheep- its measurement and influence on phosphorus in the rumen fluid. *Q. J. Exp. Physiol.* 58: 131-138.
- Valk, H., L. Šebek, and A. Beynen. 2002. Influence of phosphorus intake on excretion and blood plasma and saliva concentrations of phosphorus in dairy cows. *J. Dairy Sci.* 85: 2642-2649.
- Valk, H. 2005. Reviews on the mineral provision in ruminants (II): Phosphorus metabolism and requirements in ruminant. CVB documentation Report 34, Central Bureau for Livestock Feeding, Lelystad, the Netherlands.
- Van Es, A. J. H. 1978. Feed evaluation for ruminants. I. The systems in use from May 1977-onwards in the Netherlands. *Livest. Prod. Sci.* 5: 331-345.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74: 3583-3597.
- Wächter, S., Cohrs I., Golbeck L., Wilkens M. R., and W. Grünberg. 2021. Effects of restricted dietary phosphorus supply to dry cows on periparturient calcium status. *J. Dairy Sci.* 105: 748-760.
- Wilkens, M. R., C. D. Nelson, L. L. Hernandez, and J. A. A. McArt. 2020. Symposium review: Transition cow calcium homeostasis-health effects of hypocalcemia and strategies for prevention. *J. Dairy Sci.* 103: 2909-2927.
- Wu, Z., and L. D. Satter. 2000. Milk production and reproductive performance of dairy cows fed concentrations of phosphorus for two years. *J. Dairy Sci.* 83: 1052-1063.

Variation in macronutrients and minerals in Dutch bovine milk and their relationships with milk phosphorus content

P. Keanthao*, J. Dijkstra[‡], and J. T. Schonewille*

* Department of Population Health Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 7, 3584 CL Utrecht, the Netherlands

‡ Animal Nutrition Group, Wageningen University & Research, PO Box 338, 6700 AH Wageningen, the Netherlands

To be submitted

Journal of Agriculture Science

ABSTRACT

The main aim of the current study was predicting the milk P content with the use of a broader spectrum of potential predictor variables. Every week, from week 14 in 2017 up until week 13 in 2018, raw bulk tank samples of milk from 14 dairy plants located across the Netherlands were collected. Milk samples were analyzed for milk macronutrients and mineral contents. Results indicated that the milk P content is changing upon changes of macronutrients and minerals in milk which are most likely caused by the different feed regimes throughout the year. The mean P content of milk was found to be 101.2 mg/100 g which is 1.2% greater than the value commonly used in farm level analyses in the Netherlands to calculate the P excretion with manure. The contents of fat, protein, casein, Ca, Mg, and Mn were found to be highly correlated with the milk P content. Based on the current data, the preferred multiple regression equation to predict the milk P content (mg/100 g) included the predictor variables milk fat (g/100 g), Ca (mg/100 g) and K (mg/100 g); $P_{\text{milk}} = -58.6 (\pm 14.1) + 0.28 (\pm 0.11) \times \text{Ca} + 11.46 (\pm 2.56) \times \text{fat} + 0.48 (\pm 0.09) \times \text{K}$. This equation explained 80% of the variation (R^2_{adj}) in the milk P content. The contribution of the milk K content to explain variation in the milk P content was significant but cannot be physiologically explained yet.

Key words: dairy cow, phosphorus, prediction, season, milk composition.

INTRODUCTION

Excess phosphorus (P) excretion into the environment causes eutrophication of surface- and ground water and is, therefore, of major concern (Smith and Alexander, 2000). Several countries have implemented, or are currently implementing, regulations aiming at reducing the amount of P applied onto farmland with manure, in order to decrease excess P excretion into the environment (e.g., EU Water Framework Directive; EC, 2000). In dairy cattle systems, P excretion of cattle and milk P efficiency (output of P in milk as a fraction of P intake) varies widely (e.g., Harrison et al., 2021). Milk P efficiency is negatively related to dietary P content (Klop et al., 2013). Besides, milk P efficiency is related to the P content of milk, but in farm level analyses usually a fixed milk P content is assumed (e.g., 100 mg/100 g of milk; CVB, 2005).

For environmental as well as economic reasons, it is of great practical relevance to accurately calculate the amount of P secreted in milk and, therefore, not ending up in manure. The use of a fixed P content of milk to calculate the amount of P retained with milk, however, can be questioned (Lenstrup 1926; Pfeffer et al., 2005; Bannink et al., 2010; Klop et al., 2014). Indeed, the P content of milk was found to be related with milk protein content (Wu et al., 2000; Bijl et al., 2013; Klop et al., 2014) and lactose content (Klop et al., 2014). Previously, Klop et al. (2014) attempted to predict the milk P content of individual cows using only the protein, lactose and fat contents of milk as predictor variables. Some 30% of the variation in milk P content remained unexplained by their best regression models (Klop et al., 2014). Thus, other factors remain to be explored so as to fully explain the variation in milk P content. The aim of the current study was, therefore, to predict the milk P content in bovine milk with the use of a broader spectrum of potential predictor variables. In contrast to Klop et al. (2014) who

used milk production data from three individual experiments, we used data from milk that was weekly sampled from bulk tank milk of 14 dairy plants located across the Netherlands. In the process, we, therefore, also obtained information on the seasonal variation in the various measured milk constituents, analogous to a previous analysis done in 2005/2006 (Heck et al., 2009).

MATERIALS AND METHODS

Collection of Samples and Chemical Analyses

Every week, from week 14 in 2017 up until week 13 in 2018, bulk milk samples from 14 dairy plants located across the Netherlands were collected as representative samples of the average Dutch bovine milk. Then, the samples were pooled and conserved with 0.03% sodium azide. In total, 52 weekly milk samples were analyzed for their selected milk constituents at Qlip laboratory (Qlip, Zutphen, the Netherlands). Milk fat was analyzed using Röse-Gottlieb method (ISO 1211, ISO, 2010). Both, the total N content of milk and the N originating from non-protein N compounds (NPN-compounds) were analyzed with the use of the Kjeldahl method according to ISO 8968-1 (ISO, 2014). The N associated with the NPN-compounds was measured in supernatant from milk samples treated with trichloroacetic acid so as to precipitate the milk proteins (i.e. true milk protein). The N associated with true milk protein was subsequently calculated as; total milk-N – N from NPN-compounds. Finally, true milk protein was calculated as $6.38 \times \text{N}$ while NPN-compounds were calculated as $6.25 \times \text{N}$. Milk urea was analyzed using an enzymatic method according to ISO 14637 (ISO, 2004b). Milk lactose and casein content were analyzed using high-performance liquid chromatography (ISO 5548, ISO, 2004a). The content of non-casein protein in milk was calculated as the difference between the true milk protein content and the casein content of milk. The contents of Na, Mg, P, K, Ca, Mn, and Cu were determined with the use of an inductively coupled plasma mass spectrometer (ISO 17294-2, ISO, 2016)

Statistical Analyses

Prediction of the P content of milk

First, Pearson's correlation coefficients and linear regression equations were calculated to detect individual milk variables that were related to the milk P content. Then, multiple regression analyses were performed using SPSS Statistics 24.0 software (IBM SPSS Statistics 27th edition, 2020) with milk P content as dependent variable and the various milk constituents as potential predictor variables. Stepwise regression was performed by incorporating or removing into the model the milk constituents showing the highest or lowest partial correlation coefficient for its relation with the residual variance in the milk P content. Both the explained variance and Mallows' Cp (IBM SPSS Statistics 27th edition, 2020) were used as principle determinants to trace out the most suitable models to predict the milk P content.

The various equations were evaluated using the mean square prediction error (MSPE) which was calculated as: $\text{MSPE} = \sum_{i=1}^n (o_i - p_i)^2 / n$, where n is the total number of observations and

O_i and P_i are the observed and predicted values, respectively. The square root of MSPE (RMSPE) is considered an estimate of the overall prediction error and is expressed a percentage of the observed mean in question. The variance inflation factor (VIF) was used to check for multicollinearity between predictor variables in multiple regression models (IBM SPSS Statistics 27th edition, 2020) and was calculated as: $VIF_i = 1 / (1 - R^2_i)$, where R^2_i is the square of the i^{th} Pearson correlation coefficient between predictor variables in the model. The Bayesian information criterion (BIC) was used to indicate the goodness of fit, where smaller values indicate a better fit (IBM SPSS Statistics 27th edition, 2020). The residual slopes were tested for significance from zero values to verify for heteroscedasticity. Throughout, the level of statistical significance was preset at $P < 0.05$.

Monthly variation in milk constituents

For each month, the weekly values on the milk composition were used; i.e. the 4 or 5 samples that represented the month in question. All data on the monthly milk composition were subjected to ANOVA using the following model: $Y_{ij} = \mu + M_i + e_i$;

where Y_{ij} = the response variable, μ = the overall mean, M_i = month ($i = 1$ to 12), and e_i = residual error. When the influence of month was significant, Tukey's t test was used to identify the month with different effects on the variable involved. The level of statistical significance was preset at $P < 0.05$.

RESULTS

Variation in Milk Composition Throughout the Year

Despite that fact the lactose content of milk differed between months (Table 1), the variation in the lactose content of milk between months was small and the lactose contents were found to be fairly constant throughout the year (Figure 1). In contrast, the fat content of milk varied considerably between months and the lowest fat contents of milk were found during summer ($P < 0.01$). Likewise, the lowest protein contents of milk were also observed during summer but compared to the fat content of milk, the variation in protein contents between months was smaller ($P < 0.01$). The seasonal variation in the casein contents of milk, mirrored the variation in milk protein contents (Figure 1). The content of non-casein protein was found be significantly affected by month ($P < 0.01$) but, like lactose, the absolute difference between the minimum and maximum values was small. The contents of NPN-compounds were found to similar between months ($P = 0.24$) but the urea contents of milk varied between months ($P = 0.02$), the highest values were observed during winter while the lowest values were found during summer (Figure 1).

Except for the Na and Cu content of milk, all other measured minerals in milk were affected by month (Table 1; $P < 0.01$). With respect to the Ca and P contents of milk, the lowest values were found during summer (Figure 2). Likewise, the lowest Mg content of milk was found in July (Table 1) while the highest values were observed in December. For K, the time window between the lowest and highest content in milk was small, i.e. during March and April,

respectively. The Mn contents of milk varied considerably between months (Table 1; $P < 0.01$) with low values in milk during August and greatest values during March.

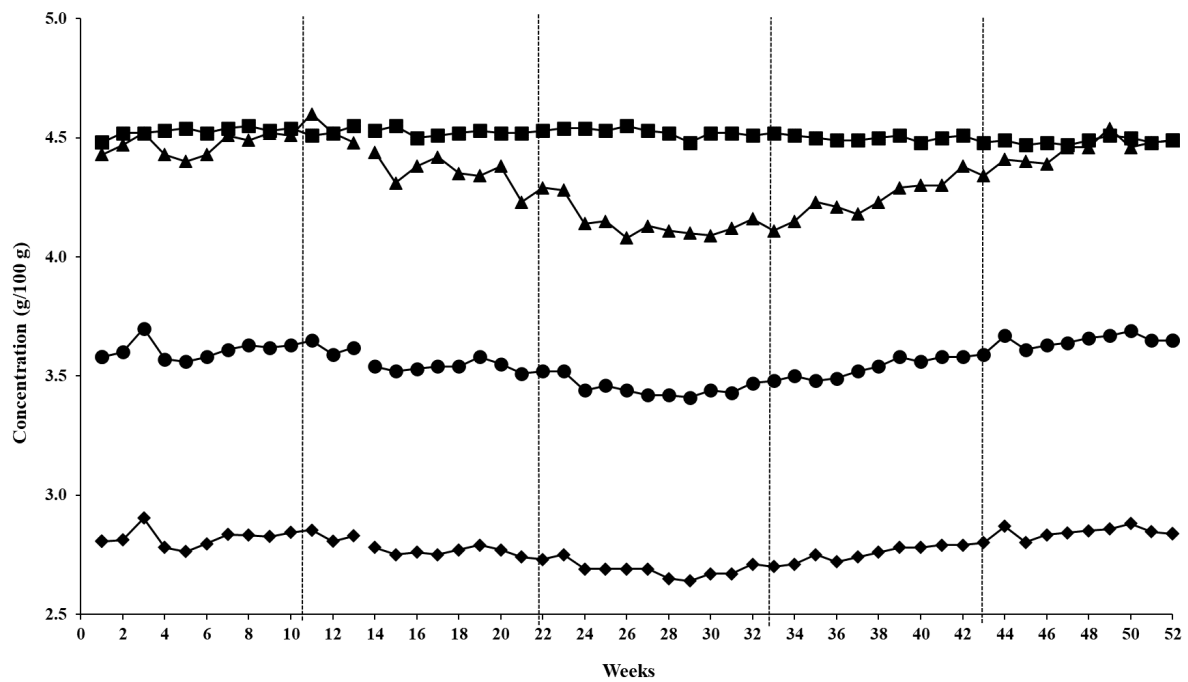


Figure 1. Weekly variation in the contents of fat- (▲), protein- (●), casein- (◆) and lactose (■) in raw bovine milk collected in the Netherlands from April 2017 up until March 2018.

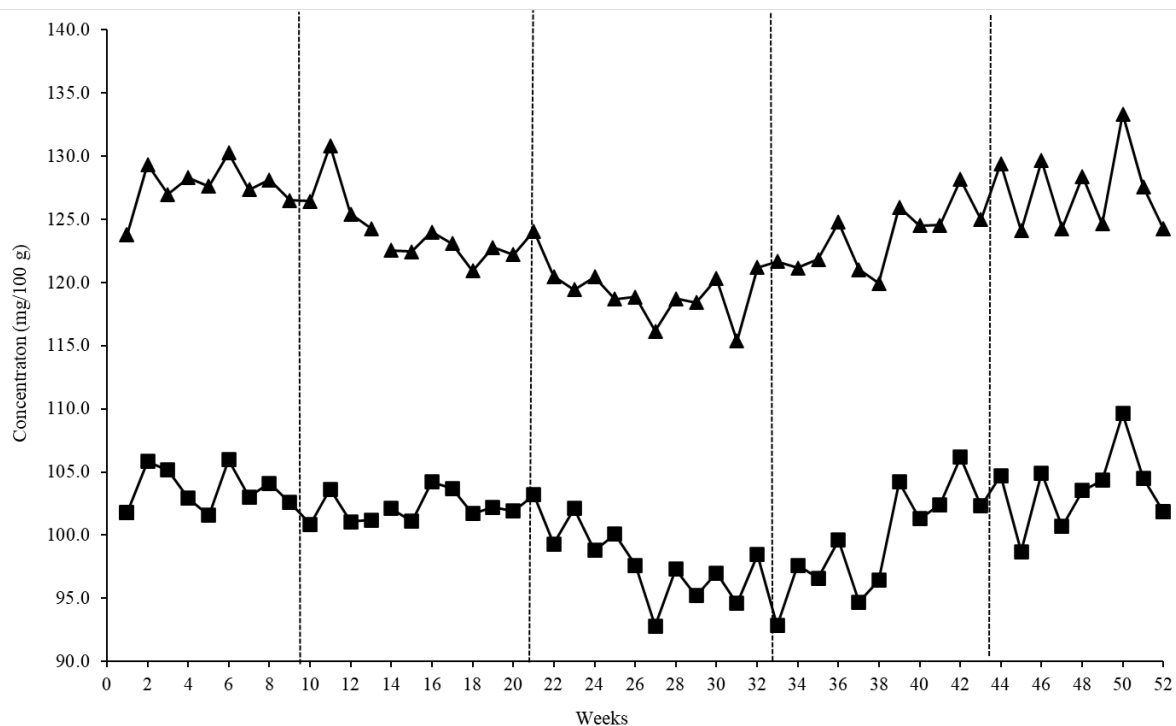


Figure 2. Weekly variation in the contents of Ca (▲) and P (■) in raw bovine milk collected in the Netherlands from April 2017 up until March 2018.

Table 1. Descriptive statistics on the pooled composition of bovine raw milk, collected in the Netherlands from April 2017 up until March 2018 from 14 dairy plants located across the country. All units are expressed per 100 g of milk ($n = 52$). The minimum and maximum values are monthly means of the 4 or 5 samples that represented the month in question.

Item	Year			Minimum		Maximum		<i>P</i> -value (month)
	Mean	SD	CV (%)	Month	Value	Month	Value	
Lactose (g)	4.51	0.02	0.49	November	4.48	>1 ¹	4.54	< 0.01
Fat (g)	4.34	0.14	3.34	July	4.11	March	4.53	< 0.01
Protein (g)	3.56	0.08	2.16	July	3.42	December	3.67	< 0.01
Casein (g)	2.77	0.06	2.26	July	2.66	December	2.86	< 0.01
NCP (g)	0.78	0.02	2.36	>1 ²	0.76	December	0.81	< 0.01
NPN (g)	0.20	0.01	2.66	May	0.19	August	0.20	0.24
Urea (mg)	22.5	1.23	5.45	March	21.6	August	24.2	0.02
Phosphorus (mg)	101.2	3.56	3.52	July	95.6	December	105.1	< 0.01
Calcium (mg)	124.0	3.87	3.12	July	118.4	February	128.6	< 0.01
Magnesium (mg)	11.3	0.32	2.82	July	10.8	December	11.6	< 0.01
Potassium (mg)	158.4	2.79	1.76	March	155.2	April	162.1	0.02
Sodium (mg)	35.5	0.80	2.27	June	34.6	December	36.1	0.45
Manganese (µg)	2.3	0.15	6.74	August	2.0	March	2.4	< 0.01
Copper (µg)	5.9	0.70	11.88	July	5.5	February	6.8	0.14

¹Equal values in February and June.

²Equal values in July and August.

NCP = Non casein protein, NPN = Non protein nitrogen compound, SD = Standard deviation, CV = Coefficient of variation, expressed as % of the mean.

Linear Relationships Between Individual Milk Constituents and the Milk P Content

Except for the lactose- and the Cu content, all other individual milk constituents were found to be related ($P < 0.05$) with the P content of milk (Table 2). The variation in either the fat-, protein- or the casein content of milk explained each ~ 55% of the variation in the milk P content ($P < 0.01$) but in case of the protein- and casein content of milk, the intercepts of the linear regressions were not different from zero ($P \geq 0.13$). The variation in milk proteins other than casein, also accounted for the variation in milk P ($P < 0.01$) but compared to fat, protein or casein to a lesser extent (Table 2). In contrast to the aforementioned milk constituents, the urea content of milk was negatively related to the milk P content but the variation in milk urea contents explained only 17% of the variation in milk P ($P < 0.01$). Likewise, the content of NPN-compounds of milk also was inversely related with the milk P content ($P < 0.01$) but the variation in the contents of NPN-compounds of milk accounted for only 9% of the variation in milk P.

Both the Ca and Mg content of milk correlated highly with the P content of milk (Table 2) and they respectively explained 67 and 62% of the variation in milk P ($P < 0.01$). The variation in the contents of Na and K as well, contributed ($P < 0.01$) to the explained variation in the milk P content but compared to both Ca and Mg, to lesser extent. In all aforementioned cases in this paragraph, the intercept of the linear regression equations did not differ from zero ($P \geq$

0.29). The variation in the Mn content of milk accounted for 49% of the variation in milk P content ($P < 0.01$).

Table 2. Pearson's correlation coefficients* and linear regression equations on the relationship between individual milk constituents and the content of P in milk¹.

Equation	Pearson's R	R ² _{adj}	P _{constant}	P _{slope}
$P_{\text{milk}} = 131.8 (\pm 102.5) - 6.78 (\pm 22.71) \times \text{lactose}$	-0.04*	-0.02	0.21	0.77
$P_{\text{milk}} = 21.1 (\pm 10.0) + 18.44 (\pm 2.30) \times \text{fat}$	0.75	0.55	0.04	< 0.01
$P_{\text{milk}} = -23.3 (\pm 15.3) + 34.98 (\pm 4.30) \times \text{protein}$	0.76	0.56	0.13	< 0.01
$P_{\text{milk}} = -16.5 (\pm 14.7) + 42.40 (\pm 5.31) \times \text{casein}$	0.75	0.55	0.27	< 0.01
$P_{\text{milk}} = 11.6 (\pm 17.2) + 114.48 (\pm 21.92) \times \text{NCP}$	0.59	0.34	0.50	< 0.01
$P_{\text{milk}} = 144.3 (\pm 17.9) - 220.25 (\pm 91.41) \times \text{NPN}$	-0.32	0.09	< 0.01	0.02
$P_{\text{milk}} = 129.9 (\pm 8.4) - 1.27 (\pm 0.37) \times \text{urea}$	-0.44	0.17	< 0.01	0.01
$P_{\text{milk}} = 7.7 (\pm 9.2) + 0.75 (\pm 0.07) \times \text{Ca}$	0.82	0.67	0.41	< 0.01
$P_{\text{milk}} = 1.0 (\pm 10.8) + 8.83 (\pm 0.95) \times \text{Mg}$	0.80	0.62	0.93	< 0.01
$P_{\text{milk}} = -13.4 (\pm 23.6) + 0.72 (\pm 0.15) \times \text{K}$	0.57	0.31	0.57	< 0.01
$P_{\text{milk}} = 20.4 (\pm 19.0) + 2.28 (\pm 0.54) \times \text{Na}$	0.52	0.25	0.29	< 0.01
$P_{\text{milk}} = 63.8 (\pm 5.3) + 16.57 (\pm 2.34) \times \text{Mn}$	0.71	0.49	< 0.01	< 0.01
$P_{\text{milk}} = 96.1 (\pm 4.3) + 0.85 (\pm 0.73) \times \text{Cu}$	0.17*	0.01	< 0.01	0.25

* Indicates a non-significant correlation ($P > 0.05$).

¹ The contents of lactose, fat, protein, casein, non-casein protein (NCP), non-protein nitrogen compounds (NPN), and urea are expressed as g/100 g of milk. The contents of Ca, Na, K, and Mg in milk are expressed as mg/100 g and the contents of Cu and Mn are expressed as µg/100 g of milk.

Multiple Regression Models to Predict the Milk P Content

Various predictor sets were used in an attempt to trace out the most suitable equation to predict the milk P content (Table 3). Upon stepwise regression of all measured milk constituents against the milk P content, only the fat-, Ca- and K contents of milk were found to significantly contribute to the explained variance in the milk P content (Table 4, equation 1). The VIF value of this model was 3.29, whereas VIF_i values ≤ 3.0 are preferred (Hair et al., 2018). Therefore, various candidate models were compiled with the aim to reduce multicollinearity between the candidate predictor variables.

All subsequent multiple regression models (equations 2-8, Table 4) had a VIF value < 3. The model containing only the protein and urea content of milk as predictor variables (equation 2, Table 4) accounted for only 59% of the variation in the milk P content. The models described with equations 3 to 7 (Table 4) were all similar to explain the variation in milk P content ($76\% < R^2_{\text{adj}} < 79\%$). The model containing, amongst others, Mn (equation 8) explained the variation in the milk P content to a somewhat lower extent ($R^2_{\text{adj}} = 73\%$) compared to equations 3 to 7.

Table 3. Overview of the candidate predictor variables used for linear stepwise, multiple regression to predict the P content (mg/100 g) of Dutch raw bulk tank milk.

Predictor set	Candidate predictor variables
1	All available candidate predictor variables
2 ¹	Protein, fat, lactose, and urea
3	Protein, lactose, urea, NPN, K, Na, and Cu
4	Casein, lactose, urea, NPN, K, Na, and Cu
5	Fat, lactose, urea, NCP, NPN, K, Na, and Cu
6	Ca, lactose, urea, NCP, NPN, K, Na, and Cu
7	Mg, lactose, urea, NCP, NPN, K, Na, and Cu
8	Mn, lactose, urea, NCP, NPN, K, Na, and Cu

¹ Within predictor sets 2 to 8, Pearson's correlation coefficient between candidate predictor variables was < 0.7. The contents of fat, protein, casein, non-casein protein (NCP), non-protein nitrogen compounds (NPN), urea, and lactose are expressed as g/100 g of milk. The contents of Ca, Mg, Na, and K in milk are expressed as mg/100 g and the contents of Cu and Mn are expressed as µg/100 g of milk.

Table 4. Overview of the regression equations to predict the milk P content, expressed as mg/100 g of milk. The multiple regression equations were obtained after stepwise regression using the candidate predictor variables¹ indicated in Table 3 (for all models $P < 0.01$).

Prediction model	Equation	R^2_{adj}	Mallow's Cp	VIF ²	RMSPE ³	BIC ⁴
1	$P_{\text{milk}} = -58.6 (\pm 14.1) + 0.28 (\pm 0.11) \times \text{Ca} + 11.46 (\pm 2.56) \times \text{fat} + 0.48 (\pm 0.09) \times \text{K}$	0.80	0.46	3.29	1.59	213
2	$P_{\text{milk}} = 0.4 (\pm 19.2) + 31.84 (\pm 4.48) \times \text{protein} - 0.55 (\pm 0.28) \times \text{urea}$	0.59	3.00	1.15	2.29	247
3	$P_{\text{milk}} = -79.8 (\pm 18.5) + 28.74 (\pm 3.31) \times \text{protein} - 0.48 (\pm 0.21) \times \text{urea} + 0.57 (\pm 0.09) \times \text{K}$	0.78	3.41	1.17	1.67	218
4	$P_{\text{milk}} = -77.0 (\pm 19.3) + 34.92 (\pm 4.30) \times \text{casein} - 0.40 (\pm 0.22) \times \text{urea} + 0.57 (\pm 0.09) \times \text{K}$	0.76	1.83	1.22	1.74	222
5	$P_{\text{milk}} = -65.8 (\pm 16.9) + 0.59 (\pm 0.08) \times \text{K} + 38.66 (\pm 16.94) \times \text{NCP}^5 + 12.16 (\pm 2.42) \times \text{fat} - 0.39 (\pm 0.22) \times \text{urea}$	0.79	2.62	2.39	1.62	217
6	$P_{\text{milk}} = -35.8 (\pm 18.0) + 0.42 (\pm 0.10) \times \text{Ca} + 0.41 (\pm 0.10) \times \text{K} - 0.63 (\pm 0.22) \times \text{urea} + 43.57 (\pm 18.56) \times \text{NCP}$	0.77	3.50	2.68	1.72	223
7	$P_{\text{milk}} = -150.6 (\pm 58.0) + 5.01 (\pm 1.17) \times \text{Mg} + 0.41 (\pm 0.10) \times \text{K} - 0.68 (\pm 0.21) \times \text{urea} + 52.38 (\pm 18.1) \times \text{NCP} + 22.97 (\pm 11.44) \times \text{lactose}$	0.78	4.37	2.50	1.68	224
8	$P_{\text{milk}} = -27.8 (\pm 20.2) + 6.79 (\pm 2.44) \times \text{Mn} + 0.52 (\pm 0.10) \times \text{K} - 0.79 (\pm 0.23) \times \text{urea} + 63.45 (\pm 18.55) \times \text{NCP}$	0.73	4.94	2.03	1.86	231

¹ The contents of fat, protein, casein, non-casein protein (NCP), urea, and lactose are expressed as g/100 g of milk. The contents of Ca, Mg, and K in milk are expressed as mg/100 g and the Mn content is expressed as µg/100 g of milk.

² VIF = Variance inflation factor.

³ RMSPE = Square root of the mean square prediction error and is expressed as a percentage of the observed mean milk P content, i.e. 101.2 mg/100 g of milk.

⁴ Bayesian information criterion.

⁵ NCP = Non-casein protein.

DISCUSSION

Variation in Milk Composition throughout the Year

Holstein-Friesian is the predominant dairy breed in the Netherlands (NRS, 2008) thus, the current data reflects the milk composition of Holstein-Friesian cows. In the Netherlands, the pattern of calving is not seasonal, thus the differences in milk composition between months is most likely related to nutrition. In the Netherlands, cows graze outside from ~ April until September and during this time span, the cow consume fresh grass either or not in combination with maize silage and, depending on the milk yield, supplemental concentrates. From October to March, grass silage instead of fresh grass is fed and compared to the grazing season, the rations contain a greater proportion of concentrates, i.e. 22 vs 31%. (Heck et al., 2009). The higher ratio of concentrate to roughage in the winter season is associated with lower levels of fiber and higher levels of starch in the diet, which stimulates the production of propionic acid in the rumen (Bannink et al., 2006) and that of the microbial protein supply (Dijkstra et al., 1998). Propionic acid is a major precursor of glucose, followed by amino acids, and the glucogenic nutrient supply result in an increased milk protein content (Wiltout and Satter, 1971; Jenkins and McGuire, 2006). In the current study, the variation in the lactose content of milk was small which is in line with Heck et al. (2009) who concluded that the lactose content of milk is virtually insensitive to changes in diet composition.

Except for the Na and Cu content of milk, the overall mean mineral contents in the current study are in line (Table 5) with the values adopted by the Central Bureau for Livestock Feeding (CVB, 2005). It is, however, of interest to note that the mean P content of milk was found to be 1.2% greater than the fixed milk P content (e.g., 100 mg/100 g of milk; CVB, 2005) used in farm level analyses to calculate P excretion with manure. It thus appears that the calculated P excretion with manure is systematically overrated.

The Na contents of milk in the current study were ~ 24% lower compared to the value reported by the CVB, whereas the Cu content of milk was ~ 47.5% greater in the current study. The CVB (2005) values on Na and Cu contents of milk are, however, means estimated on the basis of literature studies and the current observations on the Na and Cu contents of milk fall within the ranges, respectively reported by Schonewille and Beynen (2005) and Van den Top (2005). The variation of milk Ca, P and Mg generally resembles the variation in the protein content of milk and to a somewhat lesser extent the variation in milk fat content. This similarity in variation is most likely related to the process of casein and milk fat formation (see also next paragraph) in the mammary gland (Shennan and Peaker, 2000; Mather, 2011). The highest and lowest values on the milk K content were found in March and April and most likely cows are fed similar rations during these two months. Thus, it is not likely that the observed variation in the milk K content can be explained by nutrition. With respect to the Mn content of milk, a reference value is not provided by the CVB (2005) but the current mean Mn content is in line with the value reported by Castillo et al. (2013), i.e. 3.0 µg/100 g. There are indications that Mn is involved in the process of protein and fat synthesis in the mammary gland (Lönnerdal et al., 1984) but numerically the difference between the minimum- and maximum value of milk Mn is small and are not in line with the corresponding values of protein and fat content of milk. Thus, the issue on the variation in milk Mn content is not settled yet.

Table 5. Comparison between the observed mean mineral contents in bovine raw milk, collected in the Netherlands from April 2017 up until March 2018 from 14 dairy plants located across the country, and corresponding values adopted by the Central Bureau for Livestock Feeding (CVB, 2005).

Mineral	Observation	CVB
Phosphorus (mg/100 g)	101.2	100.0
Calcium (mg/100 g)	124.0	120.0
Magnesium (mg/100 g)	11.3	12.0
Potassium (mg/100 g)	158	150
Sodium (mg/100 g)	35	46
Manganese (μ g/100 g)	2.3	NP
Copper (μ g/100 g)	5.9	4.0

NP = Not provided.

Linear Relationships between Individual Milk Constituents and the Milk P Content

Inorganic P (Pi) is known to be released during the formation of lactose in the Golgi apparatus (Shennan and Peaker, 2000), thereby, indicating that variation in the P content of milk can be, at least partly, explained by variation in the lactose content of milk. In the current study, however, no relationship was found between the contents of lactose and P in milk which is in contrast with results reported by Klop et al. (2014). This discrepancy in results is probably caused by the difference in variation around the mean lactose and P content of milk between the two studies. In the study reported by Klop et al. (2014), the weighed mean lactose content of milk, was calculated to be 45.5 g/kg of milk with a coefficient of variation (CV) of 3.8%. In the current study, the mean lactose content was 45.1 g/kg milk with CV of 0.49%. Likewise, the weighed mean P content of milk and CV in the study reported by Klop et al. (2014) were 1.03 g/kg milk and 8.0%, respectively, while the mean P content of milk and CV were, respectively 1.01 g/kg milk and 3.5% in the current study.

The variations in the contents of fat or protein or casein in milk explained a significant amount of variation in the milk P content. Milk fat is known to be excreted in the form of droplets surrounded by a monolayer of phospholipids (Shennan and Peaker, 2000; Mather, 2011) which might explain the observed positive relationship between the fat and P content of milk. The process of casein synthesis in the Golgi vesicles is associated with the release of Pi (Shennan and Peaker, 2000) and esterification of calcium phosphate with the protein matrix of casein micelles also occurs (Holt, 2004; Bijl et al., 2013). The latter may explain the observed positive relationship between both casein and protein and the milk P content because the contents of milk protein and that of casein are highly correlated (Pearson's $r = 0.985$). The current data corroborate the notion that the milk P content is primarily related to the casein content of milk because the variation in the non-casein protein content explained only 34% of the variation in milk P while the variation in the contents of NPN-compounds or that of urea, explained $\leq 17\%$ of the variation in the P content of milk. The esterification of casein micelles with calcium phosphate also explains the high correlation between the Ca and P content of milk. In fact, the Ca content of milk was found to explain the greatest amount of variation in milk P compared to the other selected milk constituents. Next to calcium and phosphate, magnesium also is, at least partly, associated with the casein micelles (Holt, 2004). This finding is in line with the current data in that the calcium and magnesium contents were highly correlated

(Pearson's $r = 0.943$). The high correlation between the calcium and magnesium content of milk is corroborated by data reported by Bijl et al. (2013).

Next to lactose, Na, and K are involved in the osmotic pressure of milk, which typically resembles the osmotic pressure of blood (Bijl et al., 2013). From the perspective of the osmotic pressure of milk, an inverse relationship between Na or K and lactose is expected but in the current study, both the Na content and K content of milk were not significantly correlated with the milk lactose content ($P \geq 0.117$), most likely due to the low variation in the lactose content of milk. In contrast to the well-known role of Na and K in relation to the osmotic pressure of milk, there is yet no published information on their relationship with, if any, the formation of casein micelles or milk fat droplets. Thus, the observed positive relationship between the Na or K and the milk P content cannot be explained yet. Likewise, the current data points to a positive relationship between the Mn content of milk and milk P, but the underlying mechanistic principle, if any, is not understood.

Prediction of Milk P Content by Multiple Regression

Across the five indices used to evaluate the current multiple regression models (Table 4), 4 equations can be considered as the most promising one's to predict the P content of milk, i.e. equations 1, 3, 5, and 7. Regression of the residuals against the predicted values indicated no heteroscedasticity for any of the 4 equations in question (Figure 3). Although the range in residual milk P contents was greatest when equation 7 was used to predict the milk P content, the overall prediction errors were basically similar for the 4 regression models (Table 4). Inspection of the graphs where predicted milk P contents were plotted against the observed milk P contents (Figure 3), indicates minor underprediction of the milk P content in case of equation 7, while for equations 1, 3, and 5 milk P was slightly overpredicted. All in all, the data provided in figure 3 do not indicate a clear preference for one of 4 equations to predict the milk P content.

In equation 7, 3 out of the 5 predictor variables (i.e. K, urea, and non-casein protein) do not have a clear physiological relationship with the milk P content (previous section) and the use of lactose in equation 7 is somewhat peculiar in view of its complete lack of relationship with the milk P content (Table 2). Taken these notions into account, the use of equation 7 is not preferred to predict milk P content. Likewise, equation 5 can be considered less opportune because in this equation only the milk fat content can be causally linked to the milk P content. Following this reasoning, equation 3 is also not preferred and, also in view of its lowest prediction error (Table 4), equation 1 is preferred to predict the milk P content, thereby, taken the somewhat greater multicollinearity for granted. Furthermore, in case only the Ca and K contents are used to predict the milk P content ($VIF = 1.22$), the model already accounts for 72.0% of the variation of the milk P content ($RMSPE = 2.05\%$; Mallows's $C_p = 3.00$; $BIC = 227$) which is 13 percentage units greater compared to the use of equation 3 (Table 4). Thus, the use of Ca instead of protein and urea, is more suitable to explain the variation in the milk P content. Although it is difficult to causally link the K content of milk with that of P, the outcome of the multiple regression analysis indicates that the milk K content is an important predictor variable in the current dataset. Indeed, in 7 out of the 8 models presented in Table 4, the milk K content was found to be significantly contributing to the explained variance in the milk P

content. Perhaps, the milk K content acted as a dummy variable for a yet unidentified factor that is causally related to the milk P content.

Finally, the prediction model reported by Klop et al. (2014), i.e. $P \text{ in milk (g/kg)} = -0.64 + 0.0223 \times \text{milk protein (g/kg)} + 0.0191 \times \text{milk lactose (g/kg)}$ was used to predict the milk P content on the basis of the current dataset. The data provided in Figure 4 indicate that the model reported by Klop et al. (2014) poorly predicted the milk P contents observed in the current study. However, Klop et al. (2014) used only the contents of protein, fat, and lactose of milk as potential candidate variables to predict milk P. Thus, in view of the result shown in Figure 4, it the use of a broader spectrum of potential predictor variables to predict the milk P content was considered opportune.

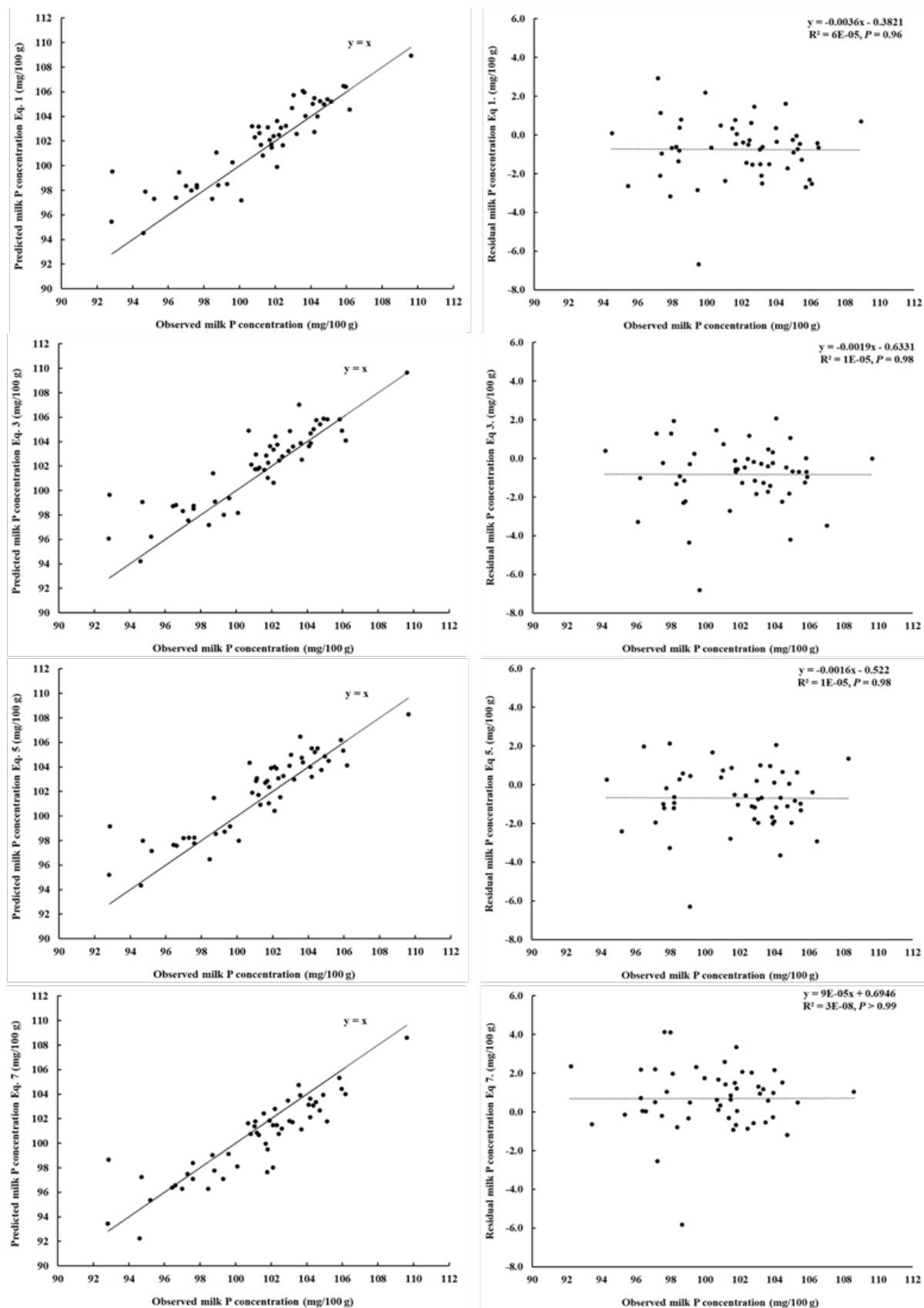


Figure 3. Predicted vs observed milk P content (left) and residuals (i.e. observed – predicted) vs predicted values for milk P content (mg/100 g) of selected multiple regression model from model 1, 3, 5, and 7 (right).

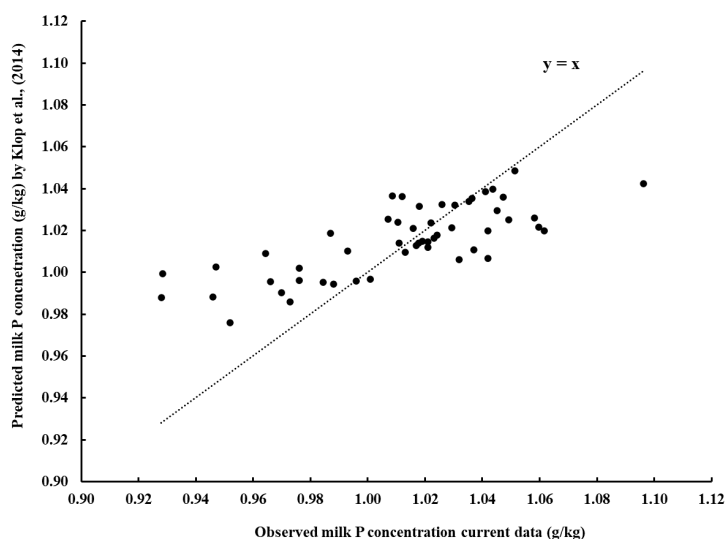


Figure 4. Predicted vs observed milk P content for milk P content (g/kg) when the multiple regression equation reported by Klop et al. (2014) is used to predict the milk P content, i.e. $P \text{ in milk (g/kg)} = -0.64 + 0.0223 \times \text{milk protein (g/kg)} + 0.0191 \times \text{milk lactose (g/kg)}$.

CONCLUSIONS

Milk P content is changing upon milk macronutrients and minerals content due to the effects from different feed regime throughout the year. The highest milk P content was found during winter and the lowest was found during summer. The contents of fat, protein, casein, Ca, Mg, in milk were highly correlated with the milk P and can also be physiologically explained. Based on the current data, the preferred multiple regression equation to predict the milk P content (mg/100 g) included the predictor variables milk fat (g/100 g), Ca (mg/100 g), and K (mg/100 g); $P_{\text{milk}} = -58.6 (\pm 14.1) + 0.28 (\pm 0.11) \times \text{Ca} + 11.46 (\pm 2.56) \times \text{fat} + 0.48 (\pm 0.09) \times \text{K}$. This equation explained 80% of the variation (R^2_{adj}) in the milk P content. The contribution of the milk K content to explain variation in the milk P content was significant but cannot be physiologically explained yet. Moreover, 20% of the variation in the milk P content could not be explained by the selected predictor variables. Thus, more research is needed to fully explain the variation in the content of P in milk.

ACKNOWLEDGEMENTS

The authors acknowledge the staff at Wageningen University & Research for contribution to milk composition and mineral content analysis.

REFERENCES

- Bannink, A., J. Kogut, J. Dijkstra, J. France, E. Kebreab, A. M. van Vuuren, and S. Tamminga. 2006. Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. *J. Theor. Biol.* 238: 36-51.

- Bannink, A., L. B. J. Šebek, and J. Dijkstra. 2010. Efficiency of phosphorus and calcium utilization in dairy cattle and implications for the environment. In *Phosphorus and Calcium Utilization and Requirements*. In: Farm Animals (Eds. Vitti D. M. S. S. and Kebreab E.), Wallingford. UK. CAB International. 151-172.
- Bijl, E., H. J. F. van Valenberg, T. Huppertz, and A. C. M. van Hooijdonk. 2013. Protein, casein, and micellar salts in milk: Current content and historical perspective. *J. Dairy Sci.* 96: 5455-5464.
- Castillo, A. R., N. R. St-Pierre, N. Silva del Rio, and W. P. Weiss. 2013. Mineral concentrations in diets, water, and milk and their value in estimating on-farm excretion of manure minerals in lactating dairy cows. *J. Dairy Sci.* 96: 3388-3398.
- CVB. 2005. Handleiding mineralenvoorziening rundvee, schapen, geiten. the Hague, the Netherlands.
- Dijkstra, J., J. France, and D. R. Davies. 1998. Different mathematical approach to estimating microbial protein supply in ruminants. *J. Dairy Sci.* 81: 3370-3384.
- European Commission. 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. *Off. J. Eur. Comm.* L327: 1-72.
- Hair, J. F., M. Sarstedt, C. M. Ringle, and S. P. Gudergan. 2018. *Advanced Issues in Partial Least Squares Structural Equation Modeling*. pp 272. SAGE Publications, Thousand Oaks, US.
- Harrison, B. P., M. Dorigo, C. K. Reynolds, A. Sinclair, J. Dijkstra, and P. P. Ray. 2021. Determinants of phosphorus balance and use efficiency in diverse dairy farming systems. *Agric. Syst.* 194: 103273.
- Heck, J. M. L., H. J. F. van Valenberg, J. Dijkstra and A. C. M. van Hooijdonk. 2009. Seasonal variation in the Dutch bovine raw milk composition. *J. Dairy Sci.* 92: 4745-4755.
- Holt, C. 2004. An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk. *Eur. Biophys. J.* 33: 421-434.
- IBM Corp. 2020. IBM SPSS Statistics for Windows, Version 27.0. Armonk, NY: IBM Corp.
- ISO. 2014. Milk and milk products-Determination of nitrogen content-Part 1: Kjeldahl principle and crude protein calculation. ISO 8968-1. ISO, Geneva, Switzerland.
- ISO. 2004a. Caseins and caseinates-Determination of lactose content-Photometric method. ISO 5548. ISO, Geneva, Switzerland.
- ISO. 2004b. Milk - Determination of urea content-Enzymatic method using difference in pH (Reference method). ISO 14637. ISO, Geneva, Switzerland.
- ISO. (2010) Milk - Determination of fat content - Gravimetric method (Reference method). ISO 1211. ISO, Geneva, Switzerland.
- ISO. 2016. Water-Application of inductively coupled plasma mass spectrometry (ICP-MS)-Part 2: Determination of selected elements including uranium isotopes. ISO 17294. ISO, Geneva, Switzerland.
- Jenkins, T. C., and M. A. McGuire. 2006. Major advance in nutrition: Impact on milk composition. *J. Dairy Sci.* 89: 1302-1310.

- Klop, G., J. L. Ellis, A. Bannink, E. Kebreab, J. France, and J. Dijkstra. 2013. Meta-analysis of factors that affect the utilization efficiency of phosphorus in lactating dairy cows. *J. Dairy Sci.* 96: 3936-3949.
- Klop, G., J. L. Ellis, M. C. Blok, G. G Brandsma, A. Bannink and J. Dijkstra. 2014. Variation in phosphorus content of milk from dairy cattle as affected by differences in milk composition. *J. Agric. Sci.* 152: 860-869.
- Lenstrup, E. 1926. The phosphorus content of human milk and cow milk. *J. Biol. Chem.* 70: 193-202.
- Lönnerdal, B., C. L. Keen, and L. S. Hurley. 1984. Manganese binding protein in human and cow's milk. *Am. J. Clin. Nutr.* 41: 550-559.
- Mather, I. H. 2011. Mammary gland, milk biosynthesis and secretion: secretion of milk constituents. In: *Encyclopedia of Dairy Sciences* (Eds. Fuquay J. W., Fox P. F., and McSweeney P. L. H). pp. 373-380. San Diego, United States: Elsevier Science Publishing Co Inc.
- NRS. 2008. Year statistics 2005. The Royal Dutch Cattle Syndicate (NRS), Arnhem, the Netherlands.
- Pfeffer, E., D. K. Beede, and H. Valk. 2005. Phosphorus metabolism in ruminants and requirements of cattle. In: *Nitrogen and Phosphorus Nutrition of Cattle and the Environment* (Eds. Pfeffer E. and Hristov A.), Wallingford, UK: CAB International. 195-231.
- Schonewille, J. Th., and A. C. Beynen. 2005. Reviews on the mineral provision in ruminants (IV): Sodium metabolism and requirements in ruminants. CVB documentation Report 36. Central Bureau for Livestock Feeding, Lelystad, the Netherlands.
- Shennan, D. B., and M. Peaker. 2000. Transport of milk constituents by the mammary gland. *Physiol. Rev.* 80: 925-951.
- Smith, R. A., and R. B. Alexander. 2000. Sources of Nutrients in the Nation's Watersheds: Managing Nutrients and Pathogens from Animal Agriculture Proceedings from the Natural Resource, Agriculture, and Engineering Service Conference for Nutrient Management Consultants, Extension Educators, and Producer Advisors. March 28-30, 2000, Camp Hill, Pennsylvania.
- Van den Top, A. M. 2005. Reviews on the mineral provision in ruminants (IX): Copper metabolism and requirements in ruminants. CVB documentation Report 41. Central Bureau for Livestock Feeding, Lelystad, the Netherlands.
- Wiltout, D. W., and L. D. Satter. 1971. Contribution of propionate to glucose synthesis in the lactating and nonlactating cow. *J. Dairy Sci.* 55: 307-317.
- Wu, Z., and L. D. Satter. 2000. Milk production and reproductive performance of dairy cows fed concentrations of phosphorus for two years. *J. Dairy Sci.* 83: 1052-106.

Chapter 6

General discussion

Model of Phosphorus Absorption in Ruminants

It has been suggested that calcitriol ($1,25(\text{OH})_2\text{D}_3$) upregulates P absorption when cows are fed P deficient diets (Host, 1986; Puggaard, 2012; Köhler et al., 2020). In contrast, Cohrs et al. (2018) did not observe an increase in plasma $1,25(\text{OH})_2\text{D}_3$ upon the feeding of P deficient diets in dairy cows and this observation is corroborated by Breves et al. (1985), Schröder and Breves (1996), and Wächter et al. (2021) in sheep, goats, and dairy cows, respectively. Therefore, there is still a debate on the regulation of P absorption by $1,25(\text{OH})_2\text{D}_3$ in ruminants.

The current model of ruminal P absorption is shown in Figure 1. This model is based on an *in vitro* study using brush border membrane vesicles of young sheep. It provides a theoretical basis to explain the outcome of feeding trials in intact ruminants in which P absorption is influenced by the level of dietary P supply. The small intestine, jejunum and duodenum are considered the major sites of P absorption. Phosphorus absorption occurs via two distinct pathways: the paracellular and transcellular. Studies in rats indicate that paracellular P absorption contributes 65 to 80% of intestinal phosphate absorption whereas the remaining part of the absorbed P is transported transcellularly (Mark et al., 2015; Vorland et al., 2020). These observations are in line with Wilkens and Muscher-Banse (2020), who reported that P is predominately absorbed via the paracellular pathway in case of high luminal P concentrations whereas transcellular, active transport is important when the luminal P concentration is low.

The electrochemical gradient across the epithelium cells provides a major driving force for paracellular P transport (Figure 1). To date, there is no information on the concentration of soluble P in the digesta of dairy cows, although Ben-Ghedalia et al. (1975) reported that the concentrations of soluble P ranged from 5.5 to 21.3 mmol/kg in sheep. The highest P concentration was observed ~ 0.05 m from the gastric pylorus (Ben-Ghedalia et al., 1975). The highest concentration of soluble P in digesta was found to be at least 10 times greater than the upper value of the reference range on plasma P_i concentrations, i.e. 1.6-2.6 mM. Thereby, implying that passive absorption of P is of major importance in the transport of P across the epithelium cells, at least in the proximal part of the ileum. This notion is in line with Pfeffer et al. (2005) who indicated that passive P absorption mainly occurs in the first 0.05 to 0.60 m of the duodenum in ruminants.

It is well known that the apical uptake of P by epithelial cells is mediated by Na/P-cotransporters, i.e. NaPi IIb and PiT1 (Hilfiker et al., 1998). Schröder and Breves (1996) reported a K_m value of 0.029 ± 0.007 mM for P absorption from an *in vitro* study of brush border membrane vesicles of goat jejunum which indicates that transcellular P absorption is active at very low P concentrations. In addition, PiT1 can also use either a Na^+ or H^+ gradient to transport P_i (Saier, 2000). The basolateral extrusion mechanism of P_i to blood is still unknown.

In Chapter 3, plasma 25 hydroxy vitamin D_3 ($25(\text{OH})\text{D}_3$) concentration was used to evaluate a P induced response on P absorption, if any, because $25(\text{OH})\text{D}_3$ is a precursor of $1,25(\text{OH})_2\text{D}_3$ (Shekarri-Foumani and Khodaie, 2016). Unfortunately, in the plasma $25(\text{OH})\text{D}_3$ concentration was not affected by the low plasma P concentration. This lack of response might be related to the stage of lactation of the cows that were used because Goff et al. (1995) reported that the concentration of vitamin D receptors in epithelial tissues are low in periparturient cows and, thus, may prevent an effective response to greater plasma $1,25(\text{OH})_2\text{D}_3$ concentrations. In Chapter 4, mid lactating cows were used but also in this study the plasma concentration of

1,25(OH)₂D₃ did not respond to the feeding of a P deficient diet. It, thus, appears that 1,25(OH)₂D₃ is not a main factor in regulating P absorption in dairy cows. This notion is, however, in line with the idea that passive, paracellular transport across the epithelial cells is a main contributor of P absorption. In line with this reasoning, it can be speculated that the concentrations of soluble P in the digesta were not low enough to cause hypophosphatemia and subsequently induce active P transport. The P concentration of digesta is not only determined by the P intake as such but also by the amounts of recycled P along the gut-parotid gland axis. In Chapter 4, cows consuming 18 kg DM may produce ~ 234 L of saliva (Valk, 2005). In case the saliva contains 8 mM soluble P, it can be calculated that the P concentration of digesta decreased by ~ 10% when the cows were fed a P deficient diet instead of a diet containing an adequate amount of P. It can, thus, be speculated that the P concentrations of the ileal digesta were not low enough to effectively reduce the amounts of P absorbed via the paracellular transport pathway. Alternatively, it might be that vitamin D receptors in epithelial tissues, or currently unknown receptors, are not sensitive enough to upregulate P absorption by dietary P concentration levels of both experiments.

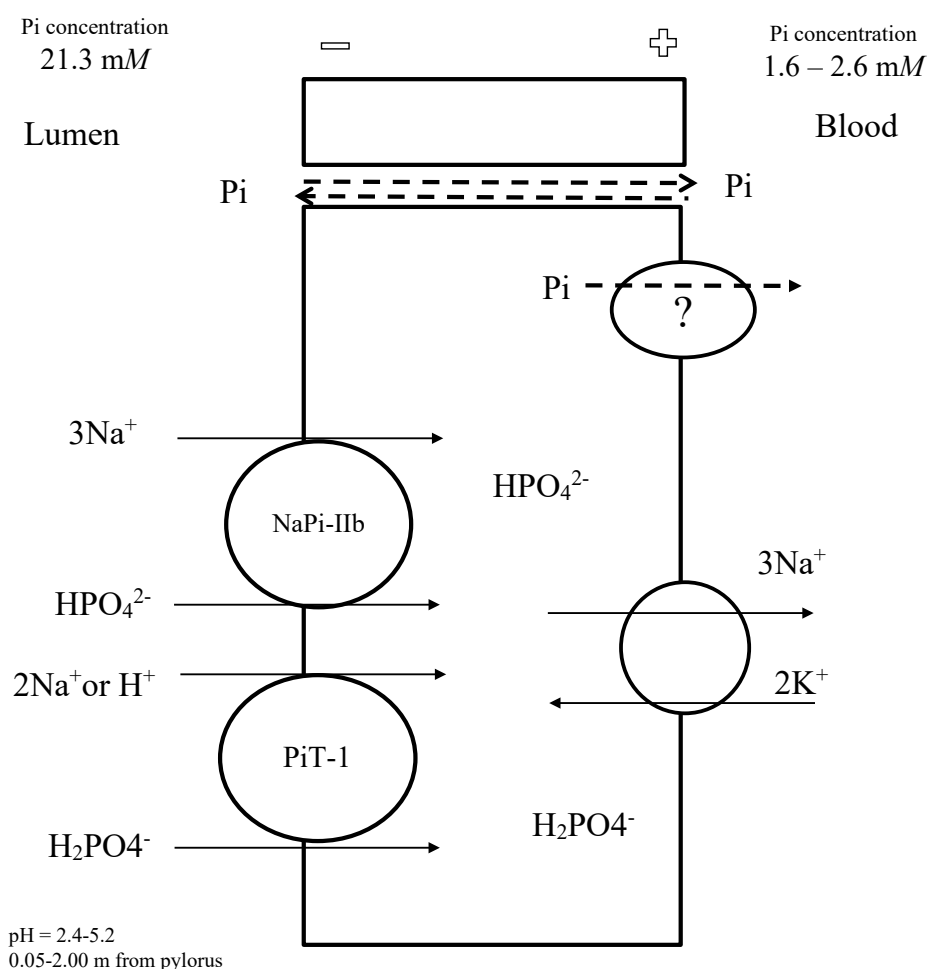


Figure 1. Current model of P absorption in ruminants (Modified from Wilkens and Muscher-Banse, 2020). Pi: inorganic P.

Bone Resorption

In animals, about 80-85% of total body P is present in bones within a collagen matrix in the form of hydroxyapatite. Net accretion of P typically occurs in growing animals, while in the case of hypophosphatemia, such as during early lactation, dairy cows resorb P from bones. The amount of P mobilized from bone can provide an amount equal to 15 - 25% of the daily P intake (Taylor et al., 2009). This indicates that bone P plays a major role in regulating the intra- and extracellular concentration of Pi. In case of severe P deficiency, long bones start to show a loss of minerals (Benzie et al., 1959). The accretion and resorption of minerals from bone are mediated by two cell types: bone formation promoting osteoblasts and bone demineralizing promoting osteoclasts (Kim et al., 2020). In general, bone resorption is regulated by both PTH and $1,25(\text{OH})_2\text{D}_3$ with hypocalcemia inducing PTH secretion and increased synthesis of $1,25(\text{OH})_2\text{D}_3$. The onset of lactation around parturition causes a decrease in plasma Ca thereby increasing PTH secretion (Goff, 1999). Subsequently, PTH triggers the synthesis of $1,25(\text{OH})_2\text{D}_3$ in the kidneys and both will stimulate bone resorption, releasing Ca and P into the blood stream. Carboxy-terminal collagen crosslinks (CTX) and osteocalcin are commonly used as biomarkers related to respectively osteoclasts and osteoblasts, in which an increase in plasma CTX concentrations is indicative of bone mineral resorption and an increase in the plasma osteocalcin concentration indicates mineralization of bone (Liesegang et al., 2000; Komori, 2020).

In Chapter 3, cows fed low dietary P showed a higher negative P balance which was associated with elevated plasma CTX concentrations, thereby, indicating P (and Ca) mobilization from the skeleton. Plasma concentrations of $25(\text{OH})\text{D}_3$, however, did not respond to the low P intake. In view of the observed negative P balances, the lack of $25(\text{OH})\text{D}_3$ response can be considered somewhat unexpected. On the other hand, the cows used in Chapter 3 were fresh cows which suffer from hypocalcemia which interfered with mineral resorption from bone. Likewise, the mid-lactating cows used in Chapter 4 tended to have higher plasma CTX concentrations when they were fed a low P diet. However, neither plasma concentrations of PTH nor plasma $1,25(\text{OH})_2\text{D}_3$ concentrations were affected by a low P intake. This finding is in line with Wu et al. (2001) who reported an increase of mineral mobilization from bone in response to a low P diet (3.1 g P/kg DM). Puggaard et al. (2014) also reported a higher bone resorption rate in cows fed with low P instead of high P during early lactation and similar to the current results, without response of the plasma $25(\text{OH})\text{D}_3$ concentration. Chapter 3 and 4 both showed that cows prefer to mobilize P from bone instead of upregulate P absorption when they are in negative P balance. Likely, the bone resorption process is more sensitive and hence shows a faster response to low plasma P concentrations than the upregulation of P absorption from the intestine tract in order to maintain normal ranges for dairy cows (Goff, 2000).

Phosphorus and Milk Composition

In milk, about 70% of P is in an inorganic form and 30% in an organic form. In milk about 70% of Ca and 50% of Pi is located in the casein micelles, preventing precipitation of calcium phosphate and providing micelles with structural stability (Holt, 2004). The ratio between milk casein and milk P is rather constant, providing a physiological explanation for the relationship

between milk protein content and milk P content. Milk serum supersaturation with calcium phosphate indicates that an increase in Pi concentration in milk is mainly related to increases in the casein content, and thus increasing Pi secretion capacity (Bijl et al., 2013). Inorganic P secretion with milk is related to synthesis of lactose and casein in the Golgi complex and endoplasmic reticulum. This physiological background of Pi, milk casein, and lactose synthesis pathway is in line with the significant correlations reported in Chapter 5 of the current thesis. Chapter 2 and 4 demonstrated that milk P concentration could not be induced by the current P-deficient diets.

Dietary P Requirement

The dietary P requirement in an adult cow is the sum of the requirement of P for maintenance, pregnancy and lactation, divided by the true absorption of P from the diet. Maintenance accounts for the endogenous fecal, salivary and urinary loss of P (NRC, 2001). The P requirement for pregnancy is the additional amount of P that is accreted into the conceptuses and the requirement for milk P excretion is equal to the milk yield multiplied by the milk P concentration (CVB, 2005). The P excretion via urine is for practical purposes not included in the estimation as the excretion with urine is considered negligible (Grünberg, 2014) which is in line with the result reported in Chapter 4.

With respect to the P requirement for pregnancy, the recommendation from CVB (2005) is in line with the results reported in Chapter 3, where the cows retained 4 to 5 g/d of P during week 4 and week 2 before calving, respectively. The current results are also in line with the P requirement for pregnancy estimated by House and Bell (1993) who measured the mass and the P concentration of the conceptus, and it was calculated to be ~ 5 g P/d in Holstein dairy cows during the last 32 weeks of pregnancy. With respect to the net P requirement for lactation, both the AFRC (1991) and NRC (2001) use a value of 0.9 g P/kg of milk whereas the Dutch Bureau for Livestock Feeding (CVB, 2005) use 1.0 g P/kg of milk. The latter value is well in line with the overall mean P content of milk reported in Chapter 5 of the current thesis and the value reported by Klop et al. (2014), i.e., 1.01 and 1.03 g P/kg of milk, respectively.

The net P requirement for maintenance in practical is determined by the endogenous P losses via urine and feces. The Dutch Bureau of Livestock Feeding (CVB, 2005) does not take the endogenous losses via urine into account because these losses are considered quantitatively unimportant. The endogenous fecal losses are completely attributed to salivary P and are estimated to be 0.81 and 1.04 g P/kg DMI for lactating and non-lactating dairy cows, respectively. NRC (2001) has been using results from Spiekens et al. (1993) suggesting that 0.8 g/kg DM is loss via feces and 0.002 g/kg BW via urine, therefore, estimate 1.0 g/kg DM for maintenance. The German recommendations (GfE, 2001) are similar to the NRC (2001) as they used the same reference research. The UK (AFRC, 1991) uses a net maintenance requirement of 1.0 or 0.7 g P per kg DMI when the metabolisability (q) of the diet is lower or higher than 0.7. Therefore, there are similar recommendations for P requirement for maintenance between various authorities.

In contrast to the estimate on the efficiency of true P absorption, there is reasonable agreement on the factors to calculate the net P requirement of dairy cows (AFRC, 1991; GfE,

2001; NRC, 2001; CVB, 2005). Thus, the range in dietary P requirements recommended by the various authorities (Figure 2) is primarily related to the values on true P absorption.

The AFRC (2001) and NRC (2001) use true P absorption of 64% for roughage and 70% for concentrate. Both in Germany (GfE, 2001) and in the Netherlands (CVB, 2005), the origin of P is not taken into account and the adopted values on true P absorption are 70 and 75%, respectively. The value adopted by the CVB (2005) is well in line with the calculated value on true P absorption reported in Chapter 4 of the current thesis. Regarding the fixed value for P absorption efficiency, this is not in line with the findings from Chapter 3 and 4 as cows showed a different P absorption at least according to the different lactation phase. For example, the fresh cows in Chapter 3 showed higher P absorption when fed low than high dietary P but contrasting results were observed in the mid-lactating cows in Chapter 4. More studies are needed to unravel the different on P absorption efficiency by different lactation stages.

The current Dutch recommendations on P intake are already the lowest compared to the other authorities (Figure 2) but the data reported in the current thesis indicate that the Dutch recommendations can be fine-tuned towards even lower values, at least during the periparturient period. The data in Chapter 2 indicate that the feeding of diets with P contents that are 30% lower than recommended by the CVB (2005) did not compromise production performance and improved the plasma Ca concentrations and thus seems to be instrumental to prevent milk fever. The P balances, however, were negative for the periparturient cows which caused mineral mobilization from bone. Although it appears that the cows were resilient to a nadir value of -14 g/d of P balance during the periparturient period, cows must replenish mineral stores in bone to maintain zero P balance across the entire lactation period. However, when mid-lactating cows were fed below the CVB (2005) dietary P recommendations, P balances were found to be negative in these mid-lactation cows as well. Interestingly, the mid-lactation cows that were fed at 100% of the CVB (2005) dietary P recommendation, were able to completely balance their P intake with total P excretion. Thus, retention of P did not occur in these cows. It, therefore, appears that the CVB (2005) dietary P recommendations are well in line with the actual P requirement of mid-lactating cows. In terms of nutrition, the history of the mid-lactation is not known, but the data presented in Chapter 3 indicate that periparturient cows are in negative P balance even when the P content of the diet is according to the CVB (2005) recommendation. Taken this notion into account, the CVB (2005) recommendations can be considered too low for cows > 14 days in milk.

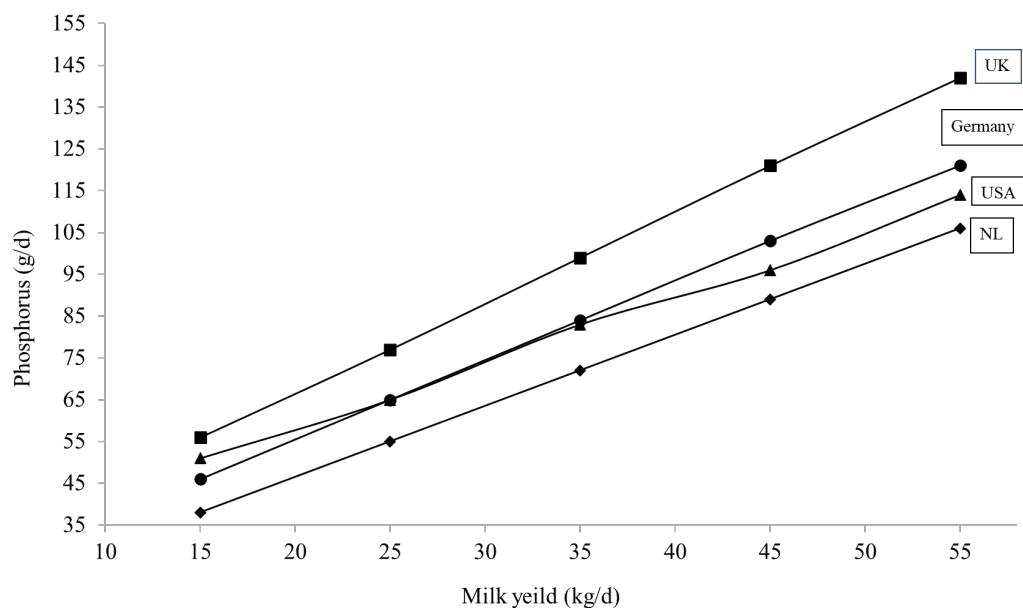


Figure 2. Four recommendation systems for P supply to non-pregnant, lactating dairy cows at different milk yield levels (modified from CVB, 2005).

Phosphorus Biomarker

Blood plasma and saliva

The concentration of inorganic P in plasma of adult dairy cows range from 1.4-2.6 mM (Goff, 1999). In practice the plasma Pi concentration is commonly used to assess the P status of an individual animal. However, with the data presented in Chapter 3 and 4 indicate that the plasma Pi concentration does accurately reflect whether cows were negative P balance. In Chapter 2, the cows showed low plasma Pi concentrations during the first 7 days after calving when they changed from a high P to a low P diet. However, plasma Pi concentrations returned to normal concentrations after 7 days in these cows while P intake was still below the required amount of P. Thus, the plasma Pi concentration poorly reflects the P status of the animal.

Saliva P concentration is highly related with plasma P concentration (Valk et al., 2002) this is in line with Chapter 4, that cows had similar saliva P concentration, although P balance result indicate cows were P-deficient.

Milk

In milk, 70% of total P is in the form of inorganic and 30% is in organic form. About 50% of Pi is in the casein micelles. The milk P concentration range 0.7-1.2 g P/kg milk (Pfeffer et al., 2005). From current finding with varies P concentration in the diet, milk P concentration is not changed as the different of P intake but with other milk constituent. In Chapter 5 described that milk P concentration is highly correlated with milk fat, protein, Ca, K, Mg, and Mn concentration. Thus, milk P is not a good maker to indicate P status of the cows.

Bone resorption

The plasma CTX concentration has been demonstrated to be a useful indicator of mineral mobilization from bone. (Liesegang et al., 1998; Cohrs et al., 2022). Pugaard et al. (2014) showed that the levels of plasma CTX are proportional to osteoclastic activity (Pugaard et al., 2014). This is in line with the current findings that plasma CTX was elevated when cows were negative P balance. Therefore, the plasma CTX concentration can be consider as a good indicator of the P status of cows. Clearly, the plasma CTX values do not provide any clue with respect to the duration of a negative P balance.

Feces and urine

The strong relation between-P intake and the concentrations of P in feces has been demonstrated in several studies (Ekelund et al., 2005; Kebreab et al., 2005; Morse et al., 1992; Wu et al., 2000). However, the P content were not yet proven to be reliably identify P-depleted in cattle. Although fecal P excretion per day was reported in Chapter 3 and 4, still fecal P show inconsistent results and cannot reflect the true P status in the cows. Therefore, the fecal P excretion is not a good indicator as it is not consistence with dietary P deficient. In healthy cows, the amount of P excreted via urine is negligible; 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)

In summary, using just a single of aforementioned parameters is not enough to identify the degree of P-deficient or true P status of the cows. Plasma CTX concentration seems to be the most reliable, reflecting the P status of animals. However, due to the limitation of plasma CTX concentration measurements, it cannot indicate the beginning of cows suffered from P-deficient, thus, together with P requirement according to the CVB (2005) recommendation and lactation phase, it could give a better overview of the P status.

Implication for Thailand

In Europe, the prevention of water pollution caused by P is the main interest to increase P efficiency in cows (EU, 2000). Next to this environmental issue, Cordell et al. (2009) forecast a phosphate rock crisis within the next 50 years. The latter will impact on the price of mineral P intended to supplement dairy diets.

In Thailand, the Department of Livestock Development (DLD) recommend 3.1 g P/kg DM in total mixed rations for dairy cows with a milk yield of 10-15 kg/d. On average, the body weight of cows is ~ 450 kg with an expected feed intake of 12 kg DM/d. Thus, the mean overall P intake is ~ 37.2 g P/d. If cows produce 15 kg/d, provided P per milk yield is 2.48 g/kg. On the basis of current CVB (2005) recommendations, cows require 33.0 g P/d, thus P provided per milk yield of 2.2 g/kg which makes a difference of 0.28 g/kg from the DLD recommendation. In 2020, DLD reported 1,291,570 tons of raw milk production which makes 361,639 kg of excess P fed to the cows. Under the assumption that the aforementioned amount of P is fed in the form of dicalcium phosphate (containing 18% of P), it can be calculated that 2,009,105 kg of dicalcium phosphate is supplied in excess of requirement. In Thailand, the price

of dicalcium phosphate is 18 Baths/kg, thus 36,163,890 Baths is lost (~ 1 million euro). Based on the current findings, it is recommended for the DLD to adjust their recommendation on dietary P, both for economic- and environmental reasons.

In Conclusion

The research described in this thesis is aimed to reappraise the P requirement of dairy cows. Based on the finding it can be concluded that;

- After calving, it is possible to feed 30% below CVB (2005) recommendation for diet P. Although P balance was negative, but no sign of reduced health and loss of production was found and it lowered fecal P excretion when dietary P was reduced. In addition, feeding cows 30% below P recommendation helps to reduce the risk of cows suffering from hypocalcemia.
- Cows prefer to mobilize bone for additional P instead of increasing P absorption by elevate plasma $1,25(\text{OH})_2\text{D}_3$ when P balance was negative.
- To assess P status of cows using plasma CTX concentration together with information on dietary P concentration from CVB (2005) recommendation seems to be a good tool.

REFERENCES

- AFRC. 1991. Agricultural and food research council technical committee on responses to nutrients. A reappraisal of calcium and phosphorus requirements of sheep and cattle. Report 6. Nutr. Abstr. Rev. B 61: 573-608.
- Ben-Ghedalia, D., H. Tagari, S. Zamwel, and A. Bondi. 1975. Solubility and exchange of calcium, magnesium and phosphorus in digesta flowing along the gut of the sheep. Br. J. Nutr. 33: 87-94.
- Benzie, D., A. W. Boyne, A. C. Dalgarno, J. Duckworth, and R. Hill. 1959. The relationship between phosphorus intake and resorption and repair of the skeleton in pregnancy and lactation. J. Agric. Sci. 52: 1-12.
- Bijl, E., H. J. F. van Valenberg, T. Huppertz, and A. C. M. van Hooijdonk. 2013. Protein, casein, and micellar salts in milk: Current content and historical perspectives. J. Dairy Sci. 96: 5455-5464.
- Breves, G., M. Beyerbach, H. Holler, and H. W. Lessmann. 1985. Fluid exchange in the rumen of sheep in low and adequate phosphorus administration. Dtsch. Tierarztl. Wochenschr. 92: 47-49.
- Cohrs, I., S. Wächter, K. Hansen, T. Scheu, M. Wilkens, and W. Grünberg. 2022. Short communication: A potential new biomarker to monitor the phosphorus balance in dry dairy cows. Anim. Feed Sci. Technol. 287: 115287.
- Cohrs, I., M. R. Wilkens, and W. Grünberg. 2018. Short communication: Effect of dietary phosphorus deprivation in late gestation and early lactation on the calcium homeostasis of periparturient dairy cows. J. Dairy Sci. 101: 9591-9598.
- Cordell, D., J. O. Drangert, and S. White. 2009. The story of phosphorus: Global food security and food for thought. Global Environ. Change 19: 292-305.

- Department of Livestock Development (DLD). TMR ration for dairy and beef cattle. Bangkok, Thailand. www.dld.go.th. (29/07/2022).
- Department of Livestock Development (DLD). 2021. Action plan for dairy cattle and milk production phase 1 (2021-2027). Bangkok, Thailand.
- European Commission. 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. Off. J. Eur. Comm. L327: 1-72.
- GfE. 2001. Recommended energy and nutrient supply for dairy cows and growing cattle. Frankfurt am Main, Germany. DLG-Verlag.
- Goff, J. P., T. A. Reinhardt, and R. L. Horst. 1995. Milk fever and dietary cation-anion balance effects on concentration of vitamin D receptor in tissue of periparturient dairy cows. *J. Dairy Sci.* 78: 2388-2394.
- Goff, J. P. 1999. Treatment of calcium, phosphorus, and magnesium balance disorders. *Vet. Clin. North Am. Food Anim. Pract.* 15: 619-639.
- Goff, J. P. 2000. Pathophysiology of calcium and phosphorus disorders. *Vet. Clin. North Am. Food Anim. Pract.* 16: 319-337.
- Grünberg, W. 2014. Treatment of phosphorus balance disorders. *Vet. Clin. North Am. Food Anim. Pract.* 30: 383-408.
- Hilifiker, H., O. Hattenhauer, M. Traebert, I. Forster, H. Murer, and J. Biber. 1998. Characterization of a murine type II sodium-phosphate cotransporter expressed in mammalian small intestine. *Proc. Natl. Acad. Sci. USA.* 95: 14564-14569.
- Holt, C. 2004. An equilibrium thermodynamic model of the sequestration of calcium phosphate by casein micelles and its application to the calculation of the partition of salts in milk. *Eur. Biophys. J.* 5: 421-434.
- Host, R. L. 1986. Regulation of calcium and phosphorus homeostasis in dairy cow. *J. Dairy Sci.* 69: 604-616.
- House, W. A., and A. W. Bell. 1993. Mineral accretion in the fetus and adnexa during late gestation in Holstein cows. *J. Dairy Sci.* 76: 2999-3010.
- Kim, J. M., C. Lin, Z. Stavre, M. B. Greenblatt, and J. H. Shim. 2020. Review: Osteoblast-Osteoclast communication and bone homeostasis. *Cell* 9: 2073.
- Köhler, O. M., W. Grünberg, N. Schnepel, A. S. Muscher-Banse, A. Rajaeerad, J. Hummel, G. Breves, and M. R. Wilkens. 2020. Dietary phosphorus restriction affects bone metabolism, vitamin D metabolism and rumen fermentation traits in sheep. *J. Anim. Physiol. Anim. Nutr.* 105: 35-50.
- Komori, T. 2020. Review: Functions of osteocalcin in bone, pancreas, testis, and muscle. *Int. J. Mol. Sci.* 20: 7531.
- Liesegang, A., M. L. Sassi, J. Ristelli, R. Eicher, M. Wanner, and J. L. Riond. 1998. Comparison of bone resorption markers during hypocalcemia in dairy cows. *J. Dairy Sci.* 81: 2614-2622.
- Mark, J., G. J. Lee, S. P. Nadaraja, E. S. Debnam, and R. J. Unwin. 2015. Experimental and regional variation in Na⁺-dependent and Na⁺-dependent phosphate transport along the rat small intestine and colon. *Physiol. Rep.* 3: e12281.
- NRC. 2001. Nutrient Requirements of Dairy Cattle, 7th revised edition. National Academy of Science, Washington, DC.

- Pfeffer, E, D. K. Beede, and H. Valk. 2005, Phosphorus metabolism in ruminants and requirements of cattle. In: Nitrogen and Phosphorus Nutrition of Cattle and Environment (Eds. Pfeffer E. and Hristov A.). CABI, Wallingford, UK, pp 195-231.
- Puggaard, L. 2012. The effect of dietary parameters on phosphorus metabolism and excretion in dairy cows. PhD Thesis. Aarhus University, Denmark.
- Puggaard, L., P. Lund, A. Liesegang, and J. Sehested. 2014. Long term effect of reduced dietary phosphorus on feed intake and milk yield in dry and lactating dairy cows. *Livest. Sci.* 159: 18-28.
- Saier, M. H. Jr. 2000. A functional-phylogenetic classification system for transmembrane solute transporter. *Microbiol. Mol. Biol. Rev.* 64: 354-411.
- Schröder, B., and G. Breves. 1996. Mechanism of phosphate uptake into brush-border membrane vesicles from goat jejunum. *J. Comp. Physiol. B.* 166: 230-240.
- Spiekens, H., R. Bintrup, M. Balmelli, and E. Pfeffer. 1993. Influence of dry matter intake on faecal phosphorus losses in dairy cows fed rations low in phosphorus. *J. Anim. Physiol. Anim. Nutr.* 69: 37-43.
- Taylor, M. S., K. F. Knowlton, M. L. McGilliard, W. S. Swecker, J. D. Ferguson, Z. Wu, and M. D. Hanigan. 2009. Dietary calcium has little effect on mineral balance and bone mineral metabolism through twenty weeks of lactation in Holstein cows. *J. Dairy Sci.* 92: 223-227.
- Valk, H., L. B. J. Šebek, and A. C. Beynen. 2002. Influence of phosphorus intake on excretion and blood plasma and saliva concentrations of phosphorus in dairy cows. *J. Dairy Sci.* 85: 2642-2649.
- Valk, H. 2005. Reviews on the mineral provision in ruminants (II): Phosphorus metabolism and requirements in ruminant. CVB documentation Report 34, Central Bureau for Livestock Feeding, Lelystad, the Netherlands.
- Vorland, C. J., A. Biruete, P. J. Lachcik, S. Srinivasan, N. X. Chen, and S. M. Moe. 2020. Kidney disease progression does not decrease intestinal phosphorus absorption in a rat model of chronic kidney disease-mineral bone disorder. *J. Bone Min. Res.* 35: 333-342.
- Wilkens, M. R., and A. S. Muscher-Banse. 2020. Review: Regulation of gastrointestinal and renal transport of calcium and phosphorus in ruminants. *Animal* 14: 29-43.
- Wu, Z., L. D. Satter, A. J. Blohowiak, R. H. Stauffacher, and J. H. Wilson. 2001. Milk production, estimated phosphorus excretion, and bone characteristics of dairy cows fed different amounts of phosphorus for two or three years. *J. Dairy Sci.* 84: 1738-1748.

Summary

Phosphorus (P) is an essential macro-mineral necessary for many body functions and needs to be supplied in sufficient quantities to optimize animal performance. With long time deficient P intake, cows will reduce feed intake, body weight and later milk production. In view of the vital role of P, in practice cows are commonly fed excess P. A high dietary P concentration leads to high P excretion via feces which can result in freshwater eutrophication. Therefore, overfeeding dietary P should stop. In the Netherlands, fecal P excretion of cattle tends to lower over time from 2017 to 2021, however, further P reduction will be needed in the future. Due to the essentialness of P, there are some limitations and criteria which need to be understood before making the decisions to lower the P concentration in the diets.

In Chapter 2 and 3, 60 pregnant multiparous Holstein Friesian dairy cows were assigned to a randomized block design with repeated measurements and dietary treatments arranged in a 2×2 factorial fashion. The experimental diets contained either 3.6 (Dry-HP) or 2.2 (Dry-LP) g P/kg DM during the dry period, and either 3.8 (Lac-HP) or 2.9 (Lac-LP) g P/kg DM during 56 days of post-calving period. Three weeks after calving, cows had negative P balance in Lac-HP group, even though dietary P concentration was at the NRC or CVB recommendation, and it was even more pronounced in the Lac-LP group. The dry matter intake, milk yield, milk constituents, body weight and plasma concentrations of non-esterified fatty acids and β -hydroxybutyric acid were not different. Plasma Ca concentrations were greater in the dry period when cows were fed Dry-LP, and were greater in the lactation period when cows previously received the Dry-LP diet or received the Lac-LP diet. The proportion of cows being hypocalcemic in the first week after calving was lowest with both Dry-LP and Lac-LP groups. Plasma PTH and 25(OH)D₃ concentration, and apparent total tract OM and NDF digestibility, were not affected by the P concentration of the experimental diets during dry and lactation period. Before calving, plasma Carboxy-terminal collagen crosslinks concentrations (CTX) decreased with Dry-HP and increased with Dry-LP. After calving, plasma CTX concentrations increased but this increase was more pronounced when Lac-LP instead of Lac-HP was fed. Overall, the results suggest that when feeding diets containing low P (2.9 g/kg DM) postpartum, cows excreted less P in the feces than at sufficient dietary P (3.8 g P/kg DM) without negative impact on feed intake, milk production and fiber digestion. Moreover, feeding postpartum diets containing low P compared with recommended P increased bone resorption, and increased plasma CTX concentrations, without increasing plasma concentrations of PTH and 25(OH)D₃, indicating a more prominent role of bone resorption than of increased P absorption to meet P demands during the first 8 weeks postpartum.

In Chapter 4, 18 multiparous, mid lactating Holstein Friesian dairy cows were used and fed high dietary P with coarse artificial dried grass (HPCG), low dietary P with coarse artificial dried grass (LPCG) and low dietary P with grass pellet (LPGP). The diets contained 2.79 (HP), 2.06 (LPCH), and 2.23 (LPGP) g P/kg DM. All cows were fed at 18.5 kg DM daily. The cows fed with grass pellet (GP) spent less time than the coarse artificial dried grass (CG) group on ruminating, but no effects were observed on saliva P, plasma Pi, PTH, 25(OH)D₃, 1,25(OH)₂D₃, CTX and osteocalcin (OC) concentration, OM and NDF digestibility, and fecal P excretion. Similarly, dietary P concentration did not affect the aforementioned parameters, except that cows fed the HP diet increased P absorption and had the highest ratio between plasma crosslaps (CL) and OC concentration. Cows fed the LP diets were in negative P balance independent of

the physical form of dried grass, however, no upregulation of P absorption was observed. Instead, those cows resorbed P from bone to fulfil their requirement.

In Chapter 5, every week, from week 14 in 2017 up until week 13 in 2018, tank milk samples from 14 dairy plants located across the Netherlands were collected. In total, 52 weekly milk samples were analyzed for macronutrients (fat, protein, lactose, casein, and urea) and minerals (Ca, P, K, Mg, Mn, Na, and Cu). The content of non-casein protein in milk was calculated as the difference between the true milk protein content and the casein content. The N associated with true milk protein was subsequently calculated as total milk-N minus N from NPN-compounds. Finally, true milk protein was calculated as $6.38 \times \text{N}$ while NPN-compounds were calculated as $6.25 \times \text{N}$. Results indicated that the milk P content is changing upon changes in macronutrients and minerals in milk which are most likely caused by the different feeding regimes throughout the year. The mean P content of milk was found to be 101.2 mg/100 g which is 1.2% greater than the value commonly used in farm level analyses in the Netherlands to calculate the P excretion with manure. The contents of fat, protein, casein, Ca, Mg, and Mn were found to be highly correlated with the milk P content. Based on the current data, the preferred multiple regression equation to predict the milk P content (mg/100 g) included the predictor variables milk fat (g/100 g), Ca (mg/100 g) and K (mg/100 g); $P_{\text{milk}} = -58.6 (\pm 14.1) + 0.28 (\pm 0.11) \times \text{Ca} + 11.46 (\pm 2.56) \times \text{fat} + 0.48 (\pm 0.09) \times \text{K}$. This equation explained 80% of the variation (R^2_{adj}) in the milk P content. The contribution of the milk K content to explain variation in the milk P content was significant but cannot be physiologically explained yet.

In Chapter 6, the result of the experimental studies reported in this thesis are discussed in light of P absorption, P requirement, P reduction in Dutch dairy cattle as well as current P used in Thai dairy cattle.

Samenvatting

Fosfor (P) is een essentieel macromineraal dat nodig is voor veel lichaamsfuncties en moet in voldoende hoeveelheden worden geleverd om de gezondheid en prestaties van de dieren te waarborgen. Om de gezondheid en de melkproductie van de koeien te waarborgen, krijgen de koeien vaak meer dan nodig gevoerd. Een hoge P-opname leidt tot grote hoeveelheden P die via de ontlasting worden uitgescheiden, waardoor eutrofiëring van oppervlaktewater ontstaat. Daarom moet het overvoeren van koeien met P worden geminimaliseerd. In Nederland zijn de aanbevolen P-concentraties in de voeding voor melkkoeien al laag in vergelijking met de waarden die worden aanbevolen in het Verenigd Koninkrijk, Duitsland en de Verenigde Staten van Amerika, maar het wordt nog steeds opportuun geacht om te onderzoeken of de huidige Nederlandse P-aanbevelingen kunnen worden verijnd of afgestemd op lagere waarden.

In Hoofdstuk 2 en 3, werden 60 drachtige Holstein Friesian melkkoeien toegewezen aan een gerandomiseerd blokontwerp met herhaalde metingen en dieetbehandelingen gerangschikt op een 2×2 factoriële manier. De experimentele diëten bevatte ofwel 160% van de CVB (2012) aanbeveling of de precieze aanbeveling tijdens de droogstand, en ofwel op CVB (2012) aanbeveling of 30% onder de aanbeveling gedurende 56 dagen na het afkalven. Tot drie weken na het afkalven hadden de koeien die hoog P kregen een negatieve P-balans, maar de P-balansen waren negatiever bij de koeien die laag P kregen na het afkalven. Ondanks de negatieve P-balansen bleek de droge stof (DS)opname en melkgift vergelijkbaar te zijn tussen de koeien die hoog of laag P kregen. Plasma Ca-concentraties waren hoger wanneer koeien het laag P-dieet kregen en het aandeel koeien dat hypocalciëmie had tijdens de eerste week na het afkalven was het laagst wanneer de koeien het laag P-dieet kregen. Plasma PTH- en $25(\text{OH})\text{D}_3$ -concentraties en de schijnbare totale organische stof- en NDF-verteerbaarheid van het kanaal werden niet beïnvloed door de P-concentratie van de experimentele diëten tijdens de droog- en lactatieperiode. Vóór het afkalven nam de plasma-CTX-concentratie af met hoog P en verhoogd met laag P. Na het afkalven namen de plasma-CTX-concentraties toe, maar deze toename was meer uitgesproken wanneer laag P in plaats van hoog P werd gevoerd. Over het algemeen laten de resultaten zien dat bij het voeren van diëten met een lage P (2,9 g/kg DS) postpartum, koeien minder P in de feces uitscheiden dan bij voldoende P in de voeding (3,8 g P/kg DS) zonder negatieve invloed op de voeropname, melkproductie, en vezelvertering. Melkkoeien lijken bestand te zijn tegen een negatieve P-balans tijdens de vroege lactatie, ten minste tot een dieptepunt van -14,3 g/dag door extra P uit gemineraliseerd bot te gebruiken.

Bij éénmagige dieren is $1,25(\text{OH})_2\text{D}_3$ belangrijk bij het (op)reguleren van de P-opname, maar bij herkauwers is de rol van $1,25(\text{OH})_2\text{D}_3$ nog onduidelijk. Hoofdstuk 4 is een experiment opgezet om meer inzicht te krijgen in de rol van $1,25(\text{OH})_2\text{D}_3$ bij melkkoeien. Achttien midden lacterende Holstein Friesian melkkoeien werden gebruikt waar de controlekoeien een dieet kregen met een hoge P-concentratie (2,79 g P/kg DS) in combinatie met grof gedroogd gras (HPCG). Er werden twee testdiëten samengesteld om lage P-concentraties in de voeding te bevatten in combinatie met ofwel grof gedroogd gras (2,06 g P/kg DS, LPCG) of gepelletiseerd gedroogd gras (2,23 g P/kg DS, LPPG). De hypothese was dat het voeren van een P-deficiënt dieet resulteert in verhoogde concentraties van $1,25(\text{OH})_2\text{D}_3$ en P-absorptie. Er werd ook verondersteld dat het voeren van een P-deficiënt dieet in combinatie met gepelletiseerd gedroogd gras de herkauwtijd verkortte en een meer uitgesproken effect zou hebben op de $1,25(\text{OH})_2\text{D}_3$ -plasmaconcentratie. In het huidige experiment werden koeien in het midden van de lactatie gebruikt om een verstorend effect van hypocalciëmie op de plasmaconcentratie van

1,25(OH)₂D₃ te voorkomen (Keanthao et al., 2021). Het pelletiseren van gedroogd gras resulteerde in een reductie van 26,6% in herkauwtijd, maar plasma 1,25(OH)₂D₃-concentraties reageerden niet op de lage P-opname. In plaats daarvan gaven biomarkers die verband houden met botmetabolisme aan dat de koeien P uit bot mobiliseerden wanneer één van beide laag P-dieet werd gevoerd. Dit laatste idee kwam overeen met het feit dat de P-balans in beide LP-groepen negatief was. Het blijft onduidelijk waarom de P-deficiënte koeien de efficiëntie van de P-opname niet verhoogden.

In Hoofdstuk 5 was het doel om het melk P-gehalte te voorspellen met behulp van een breed spectrum van potentiële voorspellende variabelen. Wekelijks, van week 14 in 2017 tot en met week 13 in 2018, werden tankmelkmonsters verzameld van 14 melkveebedrijven verspreid over heel Nederland. In totaal zijn 52-wekelijkse melkmonsters geanalyseerd op vet, eiwit, niet-eiwit stikstof, lactose, caseïne, niet-eiwit caseïne, en ureumconcentratie, mineralen (Ca, P, Mg, Mn, Na, en Cu). De resultaten gaven aan dat het P-gehalte van melk verandert door veranderingen van macronutriënten en mineralen in melk, die hoogstwaarschijnlijk worden veroorzaakt door de verschillende voerregimes gedurende het jaar. Het gemiddelde P-gehalte van melk bleek 101,2 mg/100 g te zijn, wat 1,2% hoger is dan de waarde die gewoonlijk wordt gebruikt in analyses op bedrijfsniveau om de P-excretie met mest te berekenen. De gehalten aan vet, eiwit, caseïne, Ca, Mg, en Mn bleken sterk gecorreleerd te zijn met het P-gehalte van melk. Op basis van de huidige gegevens omvatte de geprefereerde meervoudige regressievergelijking om het P-gehalte van melk (mg/100 g) te voorspellen de voorspellende variabelen melkvet (g/100 g), Ca (mg/100 g) en K (mg/100 g); $P_{\text{melk}} = -58,6 (\pm 14,1) + 0,28 (\pm 0,11) \times \text{Ca} + 11,46 (\pm 2,56) \times \text{vet} + 0,48 (\pm 0,09) \times \text{K}$. Deze vergelijking verklaarde 80% van de variatie (R^2_{adj}) in het melk P-gehalte. De bijdrage van het melk-K-gehalte aan de verklaring van variatie in het melk-P-gehalte was significant, maar kan fysiologisch nog niet worden verklaard.

In Hoofdstuk 6 worden de resultaten van de experimentele studies beschreven in dit proefschrift besproken in het licht van de P-absorptie, P-vereiste, en P-reductie in Nederlands melkvee en het huidige P-gebruik in Thais melkvee.

Acknowledgement

It has been 5 years of pursuing a PhD. A doctoral thesis is often described as a solitary endeavor. However, during my PhD studies, I received support from many people. Without going into a long list of those who supported me, I would specifically like to thank the following people and organizations.

First and foremost, I am deeply grateful for the continuous support, insight and patience of my supervisors, Dr. Jan Thomas Schonnewille and Dr. ir. Jan Dijkstra without their constant trust and, sometimes, gentle prodding, this thesis would not have been completed. I also thanks Prof. dr. ir. Wouter H. Hendriks for the opportunities and letting me do a PhD at Utrecht University as well as his kind support in everything that I did.

Second, I would like to thank the Ministry of Higher Education, Science, Research and Innovation and Rajamagala University of Science and Technology Lanna, Thailand for providing me the financial support as well as the time. I would not have been able to come to Utrecht University without their support.

I would like to thank all my friends and colleagues who have been providing me a warm positive and supportive environment during my stay in Utrecht University. I wish I could accommodate you all in Thailand someday.

Last but not least, my wife Phonthipa, who I first met at Wageningen University, has been a never-ending source of love, encouragement and motivation. I would like to thank my family for their huge spiritual support. Especially my father who has passed away when I received this scholarship but still told me to continue pursuing a PhD without worrying about him. Even though, he is not with me anymore, he will be in my heart always.

Pornsini Keanthao

Curriculum Vitae

Pornsir Keanthao was born on the 20th of September 1985 in Udon Thani province, Thailand. He obtained his Bachelor degree of Science from Van Hall Larenstein University of Applied Science, the Netherlands in 2009. During 2011-2013 he worked as an extensionist at the CPF company in Thailand. He obtained a scholarship from Rajamagala University of Technology Lanna to pursue his Master degree in Animal Science at Pingtung University of Technology, Taiwan. Since August 2014, he has been a lecturer at the Faculty of Science and Agriculture Technology, Rajamagala University of Technology Lanna, Phitsanulok campus in Thailand. In 2017, he was granted a scholarship from the Ministry of Higher Education, Science, Research and Innovation of Thailand for his PhD program at Utrecht University, the Netherlands. During his PhD, he conducted research on phosphorus metabolism of high productive dairy cows.

List of publications

1. **Keanthao P.**, R. M. A. Goselink, J. Dijkstra, A. Bannink, and J. T. Schonewille. 2021. Effects of dietary phosphorus concentration during the transition period on plasma calcium concentrations, feed intake, and milk production in dairy cows. *J. Dairy Sci.* 104: 11646-11659.

Contributions to conferences and symposia

1. **Keanthao, P.**, R. M. A. Goselink, J. T. Schonewille, J. Dijkstra, and W. H. Hendriks. 2019. Effect of phosphorus intake during the transition period on plasma phosphorus content and hypocalcemia in dairy cows. The 17th International Conference on Production Disease in Farm Animals, Bern, Switzerland (oral presentation).