# Can the dynamic of milk Ca content throughout lactation be an indicator of the effects of management system and diets on bone mobilization in dairy cows?

Pierre Gaignon

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*Ecole doctorale Ecologie, Géosciences, Agronomie et Alimentation*
Spécialité : « *Biologie et physiologie animales* »

Par

## « **Pierre GAIGNON**»

## « **Can the dynamic of milk Ca content throughout lactation be an indicator of the effects of management system and diets on bone mobilization in dairy cows?** »

**Thèse présentée et soutenue à Rennes, le 22 Octobre 2018**
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**THESE / AGROCAMPUS OUEST**

Sous le label de l'Université Européenne de Bretagne

pour obtenir le diplôme de :

**DOCTEUR DE L'INSTITUT SUPERIEUR DES SCIENCES AGRONOMIQUES, AGRO-ALIMENTAIRES, HORTICOLES ET DU PAYSAGE**

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**Ecole Doctorale : « Ecologie Géologie Agronomie Alimentation »**

présentée par :

**PIERRE GAIGNON**

**CAN THE DYNAMIC OF MILK CA CONTENT**

**THROUGHOUT LACTATION BE AN INDICATOR**

**OF THE EFFECTS OF MANAGEMENT SYSTEM AND DIETS**

**ON BONE MOBILIZATION IN DAIRY COWS?**

soutenue le 22 Octobre 2018 devant la commission d'Examen

Composition du jury :

| | |
|---|---|
| Annette Liesegang | Rapporteur |
| Stephen Anderson | Rapporteur |
| Agnès Narcy | Examinatrice |
| David Causeur | Examinateur |
| Jean-Yves Dourmad | Examinateur |
| Michel Duclos | Examinateur |
| Catherine Hurtaud | Directrice de thèse |
| Anne Boudon | Co-encadrante |
| Anca-Lucia Laza-Knoerr | Membre invitée |

*"First of all, to tell you that I am immensely fond of you all, and that eleventy-one years is too short a time to live among such excellent and admirable hobbits.*

*I don't know half of you half as well as I should like; and I like less than half of you half as well as you deserve.*

*Finally, I wish to make an ANNOUNCEMENT. I regret to announce that - though, as I said, eleventy-one years is far too short a time to spend among you - this is the END. I am going. I am leaving. NOW. GOOD-BYE!"*

Bilbo Baggins, *The Lord of The Rings: The Fellowship of the Ring*. J.R.R. Tolkien

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# Abbreviation - Glossary

**5HT :** 5-hydroxytryptamine or serotonin

**ACA :** Apparent Coefficient of Absorption

**BW :** Body Weight

**CaSR :** Calcium Sensing Receptor

**CTX :** Carboxy-Terminal cross-linking telopeptides of collagen type I

**DCAD :** Dietary Cation-Anion Difference

**DPD :** deoxypuridinoline

**DMI :** Dry Matter Intake

**DIM :** Days in Milk

**FGF 23 :** Fibroblast Growth Factor 23

**ITPR :** Inositol 1,4,5-triphosphate receptors

**MEC :** Mammary epithelial cells

**MIR :** Medium Infra-Red

**MP :** Milk Production

**NPT2b:** Sodium-dependent phosphate co-transporter 2b

**NTX :** Amino-Terminal cross-linking telopeptides of collagen type I

**OC :** Osteocalcin

**PDI :** Protein Digestible in the Intestine

**Pi :** inorganic Phosphorus

**PICP :** Carboxy-terminal of propetides of collagen types I

**PINP :** Amino-terminal of propetides of collagen types I

**PMCA :** Plasma Membrane $Ca^{2+}$-ATPase

**PTH :** Parathyroid hormon

**PTHrP :** Parathyroid hormon-related-Protein

**PYD :** Pyridinoline

**RCA :** Real Coefficient of Absorption

**SERCA2 :** Sarco Ebdoplasmic reticulum $Ca^{2+}$-ATPase

**SPCA :** Secretory Pathway $Ca^{2+}$ ATPase

**TMR :** Total Mixed Ration

**TRPV :** Transcient Receptor Potential Voltage

**VDR :** 1,25-$(OH)_2$-vitamin D Receptor

# List of Communications

## Scientific Articles

Gaignon P., Gelé M., Hurtaud C., Boudon A. 2018. Characterization of the nongenetic causes of variation in the calcium content of bovine milk on French farms. *Journal of Dairy Science*. 101:4554-4569

P.Gaignon, P.Faverdin, A.Laza-Knoerr, K. Le Grand , C.Hurtaud , A.Boudon 2018. Effects of parity and individual variability on bone accretion and resorption and milk calcium and phosphorus content during lactation in dairy cows. *animal*. Submitted.

P. Gaignon, K. Le Grand, A. Laza, C. Hurtaud, A. Boudon. 2018. Effect of calcium intake and dietary cation anion difference in early lactation on bone mobilization dynamics all over lactation in dairy cows. *Journal of dairy Science*. Submitted.

P.Gaignon, L.Delaby, A.Laza-Knoerr, K. Le Grand, C.Hurtaud, A.Boudon. 2018. Effects of breed and feeding strategies on bone accretion and resorption and milk calcium and phosphorus content during lactation in dairy cows. *animal*. In preparation.

## Oral Presentations in International Congress

Gaignon P., Gelé M., Hurtaud C., Boudon A. Characterization of the non-genetic causes of variation of bovine milk calcium concentrations on French farms. *Annual Meeting of American Dairy Science Association*, 25-18 June 2017, Pittsburgh,PA (USA). **Journal of Dairy Science**, 100, Supplement 2:423. **Appendix 1**

Gaignon P., Gelé M., Hurtaud C., Boudon A. Characterization of the non-genetic causes of variation of bovine milk calcium concentrations on French farms. *12th International Meeting on Montain Cheese*, 20-22 June 2017, Padova (Italy). p25-28. **Appendix 2**

Gaignon P., Le Grand K., Laza-Knoerr A., Hurtaud C., Boudon A. Effect of parity and age at first calving of dairy cows on dynamics of milk calcium contents and blood biomarkers of bone accretion and resorption throughout lactation.*International Symposium on the Nutrition of Herbivores (ISNH)*, 2-6 September 2018, Clermont-Ferrand (France).

Gaignon P., Le Grand K., Laza-Knoerr A., Hurtaud C., Boudon A. Effet d'une restriction des apports en calcium en début de lactation sur la production laitière, la composition du lait et les dynamiques de mobilisation et de reconstitution osseuse au cours de la lactation chez la vache laitière. *Rencontres Recherches Ruminants (3R)*, 5-6 December 2018, Paris (France). **Appendix 5**

### Posters in International Congress

Gaignon P., Le Grand K., Laza-Knoerr A., Hurtaud C., Boudon A. Effect of parity and age at first calving of dairy cows on dynamics of milk calcium contents and blood biomarkers of bone accretion and resorption throughout lactation.*International Symposium on the Nutrition of Herbivores (ISNH)*, 2-6 September 2018, Clermont-Ferrand (France). **Appendix 4**

### Internal Oral Presentations

Gaignon P., Hurtaud C., Boudon A. Étude de la variabilité individuelle des dynamiques de teneur en calcium du lait et des teneur sanguines en biomarqueurs d'accrétion et de résorption osseuse au cours de la lactation chez la vache laitière. *XVI$^e$ Journée de l'animation transversale de "Glande mammaire, lait"*. 14th November 2017, Paris (France)

Gaignon P., Faverdin P., Sidaner D., Hurtaud C., Boudon A. Etude de l'effet de la parité et de la variabilité individuelle sur les dynamiques de mobilisation et de reconstitution osseuse au cours de la lactation en relation avec la teneur en calcium du lait. *Journée d'Animations Scientifique du département PHASE*. Avril 2018, Rennes (France). **Appendix 3**

# Introduction

Chez la vache laitière, le calcium (Ca) et le phosphore (P) sont des éléments essentiels à la croissance et à la production laitière. Les conséquences des carences alimentaires de ces éléments ont déjà été démontrées au début du XX$^e$ siècle (Becker et al., 1934, Suttle, 2010). Pour cette raison, une supplémentation minérale, fournissant entre autres Ca et P, est généralement fournie aux vaches laitières en fonction de leurs besoins. Les estimations actuelles des besoins de Ca et de P sont basées sur des approches factorielles dans plusieurs systèmes d'alimentation (AFRC, 1991, NRC, 2001, INRA, 2010). Pour les vaches laitières, ces approches factorielles consistent à diviser les besoins en Ca et en P en quatre composantes : entretien (excrétions fécale et urinaire inévitables), croissance (accrétion osseuse), gestation (accrétion osseuse fœtale) et production de lait (sécrétion dans le lait). Pour estimer les besoins totaux, les quatre composantes des besoins sont estimées indépendamment, puis additionnées. Le principe de ces approches factorielles est de remplacer les pertes ou les rétentions de Ca ou de P dans les os ou les tissus des vaches laitières à l'échelle de la journée.

Cependant, toute la complexité de l'homéostasie de Ca et de P n'est pas prise en compte dans ces approches factorielles d'estimation des besoins. Plus précisément, le fait que les vaches laitières soient soumises à des cycles importants de mobilisation et de reconstitution osseuses n'est pas considéré (Ekelund et al., 2006, Puggaard et al., 2014). La prise en compte

de ces cycles pourrait permettre une plus grande précision dans l'estimation des besoins en Ca et P et une intégration de ces exigences à l'échelle du cycle de production, en tenant compte de la mobilisation et de la reconstitution des réserves corporelles. Le premier objectif de l'homéostasie de Ca et de P est de maintenir une stabilité relative des concentrations plasmatiques de ces éléments et d'éviter l'hypocalcémie ou l'hypophosphatémie lorsque les besoins ne sont pas couverts. Les os jouent un rôle important dans cette homéostasie car ils constituent la première réserve de Ca et de P dans l'organisme. La mobilisation de Ca et de P à partir des os est permet la restauration des concentrations plasmatiques de Ca et de P lorsque l'apport de ces éléments est trop faible, (Horst et al., 2005). Chez les vaches laitières, les situations de sous-apports sont courantes pour le Ca après le vêlage, à la mise en place de la lactation, en raison de la forte augmentation des exportations de Ca dans le lait. Une conséquence est une mobilisation osseuse en début de lactation, difficile à éviter (Horst et al., 2005). Cette mobilisation est compensée après trois mois de lactation par une reconstitution osseuse. Actuellement, la mobilisation osseuse en début de lactation n'est pas considérée comme un apport de Ca ou de P dans les approches factorielles de détermination des besoins en Ca et en P et la reconstitution osseuse comme un besoin.

La prise en compte des cycles de mobilisation et de reconstitution osseuses dans la définition des besoins en Ca et P permettrait également d'intégrer une partie des interactions entre le métabolisme de Ca et de P. En effet, Ca et P sont liés ensemble dans l'os sous forme d'hydroxyapatite (Moreira et al., 2009, Elizondo Salazar et al., 2013). Ainsi, un apport insuffisant en un minéral pourrait avoir des conséquences sur la quantité à fournir pour l'autre. Par exemple, on peut considérer que la mobilisation osseuse, souvent considérée comme inévitable en début de lactation, induit une libération de P par l'os et que, par conséquent, l'apport alimentaire de P pourrait être réduit à ce stade. Au contraire, il faudrait peut-être augmenter les apports de P, avec l'apport en Ca après le troisième mois de lactation, lorsque les vaches reconstituent leurs os. L'approche devrait permettre à la fois une meilleure utilisation de P avec moins de risques de rejet dans l'environnement et d'eutrophisation et une meilleure reconstitution osseuse en fin de lactation, peut-être en relation avec la prévention des problèmes de santé et la longévité des vaches.

Pour intégrer les cycles de mobilisation et de reconstitution osseuse dans les

estimations des besoins et des apports en Ca et P pour les vaches laitières, il est nécessaire de disposer de bases de données conséquentes quantifiant ces cycles par rapport à la lactation. Actuellement, il n'existe pas de méthode rapide, économique et efficace pour suivre l'évolution de la mobilisation osseuse et qui pourrait être utilisée à grande échelle. Les méthodes actuellement disponibles ne peuvent pas être utilisées sur un grand nombre d'animaux en raison de coûts élevés (biomarqueurs sanguins de l'os, Liesegang et al., 2007), d'un besoin important en main d'œuvre (bilan minéral total, Taylor et al., 2009), d' enjeux environnementaux (radio-isotopes, Ramberg et al., 1970) ou de questions éthiques liées à l'expérimentation animale (biopsies osseuses répétées, Beighle, 1999). Les résultats publiés au cours des 15 dernières années suggèrent que la teneur en Ca du lait pourrait refléter un apport insuffisant en Ca et une mobilisation osseuse accrue chez les mammifères (VanHouten et al., 2004). Des résultats récents suggèrent également qu'une partie de la variabilité de la teneur en Ca du lait pourrait être liée à la stratégie alimentaire des vaches (Hurtaud et al., 2014, Boudon et al., 2016). La variation de la composition minérale du lait, et plus particulièrement de la teneur en Ca et peut-être en P du lait, pourrait permettre une prédiction de la mobilisation osseuse des vaches. Avec l'utilisation croissante de la technologie du moyen infrarouge (**MIR**) pour analyser la composition du lait, notamment la teneur en Ca (Soyeurt et al., 2009), le Ca pourrait être un biomarqueur simple et peu coûteux de la mobilisation osseuse chez les vaches laitières.

L'objectif de cette thèse était de comprendre comment les variations de la teneur en Ca du lait pourraient être associées aux variations de l'accrétion et de la résorption osseuses durant la lactation chez les vaches laitières, en relation avec plusieurs facteurs de variation de ces dynamiques tels que l'individu, la parité, la race ou la stratégie d'alimentation. La première partie de cette thèse a consisté à établir un état des lieux sur l'homéostasie et les besoins en Ca et P, les cycles de mobilisation et de reconstitution des os et la variabilité des teneurs en Ca et P du lait chez les vaches laitières. Dans une seconde partie, une analyse des principaux facteurs de variation de la teneur en Ca du lait de vache a été réalisée grâce à une base de données des résultats de la composition laitière issue d'une enquête réalisée avant le début de ma thèse dans environ un millier d'exploitations françaises. Dans les troisième, quatrième et cinquième parties, les résultats de trois expériences consistant à caractériser simultanément les cycles de mobilisation et de reconstitution osseuses et la

variabilité laitière des teneurs en Ca et P du lait chez la vache laitière pendant toute la lactation sont présentés. Un essai a évalué l'effet de la parité et de la variabilité individuelle. Le deuxième essai s'est intéressé à l'effet de la race et de la stratégie d'alimentation alors que dans le dernier essai, nous avons suivi la lactation complète des vaches ayant un apport en Ca insuffisant en début de lactation.

# Chapitre I : Homéostasie de Ca et de P, cycles de mobilisation et reconstitution osseuses et variabilité des teneurs en Ca et P du lait chez la vache laitière

La calcémie, c'est à dire la concentration du plasma en Ca, est très finement régulée avec de très faibles variations. Cette régulation très fine est permise par l'action de trois hormones, la parathormone (PTH) et la vitamine D en cas d'hypocalcémie et la calcitonine en cas d'hypercalcémie. Ces trois hormones modifient l'activité des principaux organes impliqués dans la régulation de la calcémie, notamment l'intestin (pour l'absorption du Ca alimentaire), le rein (pour la ré-absorption du Ca excrété dans l'urine) et l'os (pour la mobilisation/reconstitution des réserves). La phosphatémie est, quant à elle, moins finement régulée que la calcémie, avec des variations plus importantes à l'échelle de la journée. Une hormone participant à sa régulation, le FGF 23 a été découverte récemment. Les régulations de la calcémie et de la phosphatémie sont liées, car Ca et P sont liés dans l'os sous forme d'hydroxyapatite. La régulation de la calcémie et de la phosphatémie est particulière chez les animaux en lactation, en lien avec les grandes quantités de Ca et P qui sont sécrétées dans le lait.

Des apports inappropriés en Ca et/ou P peuvent avoir des conséquences sur la santé et les performances des vaches laitières mais aussi en termes de résultats économiques et environnementaux pour les élevages. Des estimations des besoins en Ca et P ont été proposées par plusieurs systèmes (AFRC, INRA et NRC), menant à des estimations relativement proches à l'échelle de la lactation. L'objectif des systèmes actuels est de pallier aux pertes journalières, liées aux Ca urinaire, fécal, endogène ou secrété dans le lait. La plus grande différence entre les systèmes concerne les parts de Ca et de P

alimentaires qui sont absorbés par la vache. De fortes disparités existent entre les trois systèmes étudiés sur la part absorbable, notamment pour le Ca, menant donc à des pratiques de supplémentation minérale disparates selon le système considéré.

Le squelette de la vache laitière est seul organe de stockage de Ca dans l'organisme, et le principal de P. Sa structure séparée en une phase minérale et une phase protéique est à l'origine de sa structure et sa solidité. La possibilité de mobiliser et de reconstituer ces réserves vient du fait que l'os est sans cesse en renouvellement, par des phénomènes d'accrétion et de résorption, sous l'effet de cellules, les ostéoblastes et les ostéoclastes. Pour suivre ces phénomènes d'accrétion et de résorption osseuses, plusieurs méthodes ont été employées, comme les radio-isotopes, des bilans entrée-sortie, des biopsies ou des biomarqueurs sanguins. Ces méthodes ont notamment permis d'étudier les variations existantes de mobilisation osseuse au cours de la lactation chez la vache laitière. Pour la sécrétion de Ca et de P dans le lait, la glande mammaire doit faire face à d'importants flux de ces éléments. Pour éviter de fortes variations des concentrations intracellulaires au sein de la cellule épithéliale mammaire et une altération de l'activité de ces dernières, plusieurs voies de sécrétions ont été mises en place. Ca et P peuvent être sécrétées sous plusieurs formes, solubles ou liés à des composant du lait comme les caséines, dans le lait, qui résultent d'équilibres entre leur différentes formes. Cependant, la teneur en minéraux peut varier selon les animaux et les conditions environnementales.

## Stratégie de thèse

L'organisme des vaches laitières en lactation fait face à d'importants flux de Ca et de P en raison de la sécrétion de lait. Pour cette raison, la plupart des vaches laitières en lactation reçoivent une supplémentation minérale en Ca et P après évaluation des apports et des besoins quotidiens en Ca et en P grâce à des modèles élaborés dans des systèmes d'alimentation publiés. Les recommandations actuelles de ces systèmes d'alimentation sont de remplacer les pertes quotidiennes des vaches, à savoir les excrétions fécales et urinaires et la sécrétion de lait par un apport alimentaire adéquat. Cependant, il est probable que les vaches en lactation acquièrent la capacité de mobiliser Ca et P à partir de leurs os au début de leur lactation et qu'elles restaurent leurs pools de Ca et de P à la fin

de leur lactation. On pourrait alors considérer qu'il serait préférable de raisonner la supplémentation minérale à l'échelle de la lactation en tenant compte des cycles de mobilisation et de reconstitution osseuses pendant la lactation et la gestation. Pour y parvenir, une première étape consisterait à disposer de méthodes permettant de quantifier ces cycles pendant une longue période sur un nombre représentatif de vaches.

Comme il a été démontré chez la souris que la glande mammaire peut en même temps diminuer la quantité de Ca secrétée dans le lait et augmenter la résorption osseuse en cas d'hypocalcémie, l'hypothèse de cette thèse est que la teneur en Ca du lait pourrait être un biomarqueur de la mobilisation osseuse. Ainsi, le but de ce travail était d'étudier si la teneur en Ca du lait pouvait refléter la mobilisation osseuse chez les vaches laitières en lactation. Comme on sait que la génétique est un déterminant majeur de la teneur en Ca du lait, il est hautement prévisible que la teneur en Ca du lait ne pourra être utilisée que comme biomarqueur dynamique. **Ainsi, la question principale de cette thèse était de déterminer si la dynamique de la teneur en Ca du lait au cours de la lactation peut permettre de prédire celles des biomarqueurs de la résorption osseuse, ou peut-être un ratio entre marqueurs de résorption et d'accrétion osseuses.** Trois sous-questions ont été traitées dans cette thèse.

**La première sous-question était de déterminer si la teneur en Ca du lait était variable chez les vaches laitières, en excluant l'effet génétique, et à quantifier l'importance relative des facteurs de variation de la teneur en Ca du lait.** La première étape était donc de caractériser les facteurs de variations non génétiques de la teneur en Ca du lait chez les vaches laitières. Cette caractérisation a été réalisée en utilisant les résultats d'une grande enquête réalisée dans les 3 principales régions françaises de production laitière, impliquant environ 1 000 exploitations. Les données obtenues ont permis d'étudier les facteurs de variations non génétiques de la teneur en Ca du lait à grande échelle et d'identifier ainsi les facteurs conduisant à de faibles variations de la teneur en Ca du lait. L'existence d'une variabilité de la teneur en Ca du lait expliquée par un facteur de variation autre que celui lié à la race et à la génétique était une première condition permettant d'identifier la teneur en Ca du lait comme un biomarqueur de la résorption osseuse. L'analyse de l'enquête a été réalisée en collaboration avec IDELE, ce qui nous a permis de calculer la teneur en Ca du lait à partir des spectres MIR.

**La deuxième sous-question était de déterminer si une relation pouvait être identifiée entre la dynamique des biomarqueurs plasmatiques de l'accrétion et de la résorption osseuses pendant la lactation et celle de la teneur en Ca du lait.** La deuxième étape de ma thèse a donc consisté en un travail expérimental consistant à suivre les deux dynamiques pendant la lactation dans diverses conditions chez les vaches laitières. Les principaux facteurs de variation considérés étaient la parité, le stade de lactation et l'individu. Une première expérience a eu lieu dans la ferme expérimentale de Méjusseaume (INRA, Bretagne). Elle a consisté à mesurer les deux dynamiques dans un troupeau de 33 vaches laitières Holstein (50% de primipares), toutes nourries avec la même ration. Une seconde expérience a été menée au domaine expérimental du Pin-au-Haras (INRA, Normandie). Elle a consisté à mesurer les deux dynamiques dans un troupeau de 13 vaches laitières Holstein et 17 vaches laitières Normande nourries avec avec des régimes à densité énergétique élevée ou faible en adéquation avec deux stratégies d'alimentation,. La dynamique de l'accrétion et de la résorption osseuses a été mesurée grâce aux biomarqueurs plasmatiques. Ces expériences ont été initialement conçues pour deux projets qui n'étaient pas directement liés à ma thèse.

**La dernière sous-question était de déterminer si une mobilisation osseuse accrue en début de lactation par un apport en Ca plus faible et / ou un faible bilan alimentaire cation-anion (BACA) entraînait une diminution de la teneur en Ca du lait.** La dernière étape de ma thèse a consisté en un essai qui a eu pour objectif d'induire une plus forte mobilisation osseuse grâce à une faible teneur en Ca alimentaire ou à une faible teneur en Ca alimentaire et un BACA favorisant la mobilisation osseuse et à comparer les dynamiques d'accrétion et résorption osseuses et celles des teneurs en Ca du lait pendant la lactation avec celles de 5 vaches témoins. Un deuxième objectif de cette expérience était de déterminer comment les vaches reconstituent leurs os après une mobilisation osseuse accrue au début de la lactation. Cette expérience a été conçue spécifiquement pour ma thèse et a été réalisée à la ferme expérimentale de Méjusseaume. La mobilisation osseuse a été mesurée à l'aide de deux méthodes : les biomarqueurs osseux plasmatiques et le bilan entrée-sortie en minéraux des vaches au cours de la lactation.

# Chapitre II : Facteurs de variations non-génétiques de la teneur en Ca du lait

Le lait est une source importante de Ca dans les régimes alimentaires occidentaux. Le Ca du lait est important pour la fabrication du fromage et pourrait être un biomarqueur utile de la régulation du Ca chez la vache laitière. L'objectif de cette étude était d'identifier et de quantifier les facteurs de variation non génétiques affectant la teneur en Ca du lait de vache. Lors du programme PhénoFinLait, une enquête a été menée dans 3 grands zones géographiques de production de lait en France. Cette enquête consistait à collecter des échantillons de lait, ainsi que des informations sur la gestion des troupeaux et l'alimentation des vaches laitières, à partir de 924 exploitations commerciales. Plus de 200 000 échantillons de lait individuels ont été prélevés et leurs spectres MIR ont été mesurés. Chaque ferme a été enquêtée à plusieurs reprises au cours de l'année et 3 à 6 échantillons de lait ont été prélevés sur chaque vache. Une équation permettant de prédire la teneur en Ca du lait à partir des spectres MIR a été développée sur la base des teneurs en Ca de 292 échantillons de lait, et les teneurs en Ca dans les 200 000 échantillons ont ensuite été prédites. La teneur en Ca du lait était la plus faible chez les vaches Holstein, intermédiaire chez les vaches Montbéliardes et la plus élevée chez les vaches Normandes. Pour toutes les 3 races, le Ca du lait a diminué pendant le premier mois de lactation et augmenté après le 4ème mois de lactation, l'écart entre valeurs minimales et maximales étant le plus grand chez les Holstein, intermédiaire chez les Montbéliardes et le plus petit chez les vaches Normande. La teneur en Ca du lait diminue également avec la parité dans les trois races. En utilisant l'analyse factorielle multiple, 6 grandes stratégies d'alimentation existant dans les exploitations laitières françaises ont été caractérisées sur la base des données de l'enquête. La stratégie d'alimentation par mois et par vache a eu une incidence sur la teneur en Ca du lait, qui a baissé au printemps pendant la période de pâturage et était plus faible lorsque les vaches étaient nourries avec de l'herbe fraîche et conservée. En conclusion, en plus de la génétique des vaches, des facteurs environnementaux affectent la la teneur en Ca du lait. Dans plusieurs des conditions testées, des augmentations de la production de lait et de la quantité de Ca sécrétée quotidiennement dans le lait étaient associées à une diminution de la teneur en Ca du lait

comme si la glande mammaire limitait l'exportation de Ca lorsque la production laitière augmentait rapidement. Ce résultat suggérerait que la teneur en Ca du lait pourrait être un biomarqueur de la régulation du Ca chez les vaches laitières.

# Chapitre III : Effets de la parité et de la variabilité individuelle sur l'accrétion et la résorption osseuses et la teneur en Ca et P du lait chez la vache laitière en lactation

Les recommandations actuelles concernant la supplémentation en Ca et en P chez la vache laitière ne tiennent pas compte de la dynamique de mobilisation/reconstitution osseuse qui se produit pendant la lactation. Cette étude visait à déterminer si la dynamique de la teneur en Ca du lait pendant la lactation pouvait permettre de prédire la dynamique de la mobilisation/reconstitution osseuse. Elle consistait à mesurer les teneurs mensuelles en Ca et en P du lait et les concentrations plasmatiques des biomarqueurs de l'accrétion (OC) et de la résorption osseuses (CTX) chez 33 vaches laitières Holstein en première (n = 17), deuxième (n = 10) et troisième ou plus (n = 6) lactations à partir de 15 jours avant le vêlage jusqu'à la fin du $9^{ème}$ mois de lactation. Les vaches ont reçu la même ration complète pendant toute l'expérience. Les vaches primipares ont présenté des concentrations plasmatiques en OC et en CTX plus élevées que les vaches multipares (P <0,01). L'augmentation du CTX au cours des premiers mois de lactation a été plus importante également chez les primipares (P <0,05). Les vaches primipares ont également montré une plus forte diminution de la teneur en Ca du lait du soir (P <0,03), concomitante à l'augmentation du CTX, suggérant qu'une réduction de la sécrétion de lait pourrait permettre à l'animal de réguler sa calcémie au cours du premier mois de lactation. Cependant, la dynamique individuelle de la teneur en Ca du lait n'a pas permis d'estimer la forme de la dynamique individuelle de l'accrétion et de la résorption osseuses. Le ratio Ca/P du lait semblait être un indicateur prometteur du rapport OC/CTX plasmatique chez les individus. La cohérence de cet indicateur reste à évaluer dans des

situations difficiles pour l'homéostasie du Ca chez les vaches laitières.

# Chapitre IV : Effets de la race et de la stratégie d'alimentation sur l'accrétion et la résorption osseuses et la teneur en Ca et en P du lait chez la vache laitière en lactation

Les recommandations actuelles pour la supplémentation en Ca et en P chez la vache laitière ne tiennent pas compte de la dynamique de la mobilisation/reconstitution osseuses qui se produit pendant la lactation. Cette étude a consisté à déterminer si la dynamique de la teneur en Ca du lait pendant la lactation pouvait permettre de prédire la dynamique de la mobilisation/reconstitution osseuses chez les vaches nourries avec deux stratégies d'alimentation différentes. Elle a consisté à mesurer les teneurs mensuelles en Ca et P du lait et les concentrations plasmatiques des biomarqueurs de l'accrétion osseuse (OC) et de la résorption (CTX) sur 30 vaches laitières Holstein et Normande nourries avec avec des régimes à densité énergétique élevée ou faible en adéquation avec deux stratégies d'alimentation pendant toute leur lactation. Les vaches multipares ont présenté des concentrations plasmatiques en OC et CTX plus élevées chez les vaches Normande que chez les vaches Holstein (P <0,01), mais aussi des concentrations plasmatiques plus élevées dans la stratégie d'alimentation "*Haut*" (P <0,01). Ce résultat était lié à une augmentation importante de la production de lait (P <0,01) chez les vaches recevant une alimentation avec une densité énergétique plus élevée. Les vaches primipares ont montré un effet de la stratégie d'alimentation mais sur l'accrétion osseuse (P = 0,05), mais aucune différence due aux races ou sur la résorption osseuse. Cependant, les différences liées à la stratégie d'alimentation et la race sur l'accrétion et la résorption osseuses n'ont pas pu être liées aux variations de la teneur en Ca du lait. La possibilité d'utiliser le ratio Ca/P du lait pour estimer le ratio plasmatique OC/CTX, comme cela a été suggéré dans la littérature, a été également insatisfaisante, notamment pour les vaches recevant une alimentation avec une faible densité énergétique.

# Chapitre V : Effets de l'apport en Ca et du bilan alimentaire cation-anion en début de lactation sur les dynamiques de mobilisation osseuse au cours de la lactation chez la vache laitière

Cette étude a visé à évaluer les conséquences d'une mobilisation osseuse accrue en début de lactation sur la dynamique de la teneur en Ca du lait pendant la lactation et la reconstitution osseuse en fin de lactation. Quinze vaches Holstein multipares ont été réparties entre 3 traitements 5 semaines avant leur date de vêlage prévue. Ces 3 traitements ont consisté en 3 supplémentation minérales distinctes entre 5 jours après le vêlage et 10 semaines de lactation. Pendant cette période, le traitement témoin (NCa) a consisté à couvrir 100% des besoins en Ca, avec un bilan alimentaire cation-anion (BACA/DCAD) de 200 mEq/kg MS. Les traitements LCa (Bas Ca) et LCaLD (Bas Ca, Bas BACA) ont consisté à couvrir 70% des besoins en Ca, avec un BACA de 200 et 0 mEq/kg de MS respectivement pour LCa et LCaLD. Après 10 semaines de lactation, toutes les vaches ont reçu la même ration qui a été formulée pour couvrir à 100% des besoins en Ca, avec un BACA de 200 mEq/kg MS. LCa et LCaLD ont eu tendance à diminuer la rétention corporelle de Ca à 3 semaines de lactation par rapport à NCa (-0,95 vs 8,10 g/j, P <0,09), mais n'ont pas affecté pas la dynamique des biomarqueurs sanguins de l'accrétion osseuse (OC) et de la résorption (CTX) pendant les 32 semaines de lactation ou la rétention corporelle de Ca à 17 semaines de lactation. Les vaches ont compensé presque entièrement la diminution de l'apport en Ca dans les traitements LCa et LCaLD par rapport au traitement NCa en augmentant leur absorption digestive apparente de Ca à 3 semaines de lactation (39,6 vs 30,1 %, P = 0,03), alors que l'absorption digestive apparente n'était pas affectée par les traitements à 17 semaines de lactation. La teneur en Ca du lait du matin était plus élevée avec les traitements LCa et LCaLD qu'avec NCa, mais comme cette différence n'est apparue qu'après 10 semaines de lactation, elle peut être attribuée aux différences génétiques entre les vaches. La production de lait a été plus faible (P = 0,09) pendant la lactation avec LCa et LCaLD par rapport à NCa, avec une différence moyenne de 2 kg/j, bien que la production laitière n'ait pas été différente au cours de la

lactation précédente entre les groupes de vaches. Cette étude a montré que la mesure de la dynamique de la teneur en Ca du lait pendant la lactation ne peut pas être utilisée pour estimer indirectement la dynamique de l'accrétion et de la résorption osseuses des vaches. Elle a montré qu'à 3 semaines de lactation, une augmentation de l'absorption digestive apparente du Ca était le principal moyen pour les vaches de s'adapter à un faible apport en Ca. Ce résultat suggère qu'un apport limité de Ca en début de lactation peut avoir un effet délétère sur la production de lait.

# Chapitre VI : Discussion générale

En complément du chapitre II, les facteurs de variations non-génétiques de la teneur en P du lait ont été étudiés. Pour cela, une équation de prédiction de la teneur en P du lait a été réalisée et appliquée aux spectres MIR de la base de données PhénoFinLait. Le même modèle d'analyse que pour le Ca a été utilisé pour étudier les variations de P et Ca/P du lait. Il en a résulté que la teneur en P du lait est plus affectée par la parité que par le stade de lactation, contrairement au Ca. Comme pour le Ca, les effets de la saison et de l'alimentation étaient moins importants. L'hypothèse sur le lien entre Ca/P et OC/CTX est que le sens dépend du facteur à l'origine de la mobilisation osseuse. Si le Ca est limitant, la relation pourra être positive. Si c'est le P, la relation deviendrait négative, mais cela reste à vérifier.

Une analyse conjointe des trois essais réalisés au cours de cette thèse a été réalisée, pour avoir un aperçu plus global des variations des phénomènes d'accrétions et de résorptions osseuses au cours de la lactation. Pour cela, 13 groupes de vaches transversaux aux essais ont été constitués et chaque groupe consistait en un arrangement d'un traitement (alimentation dans chaque essai), d'une parité (primi ou multipare) et d'une race (Normande ou Holstein). L'accrétion osseuse, suivie par la teneur plasmatique en OC a montré peu de variations, quelles que soit les conditions expérimentales. Les dynamiques étaient similaires, avec juste des différences de concentrations moyennes. Cependant, de fortes variations dans les dynamiques de CTX (marqueur de la résorption) ont pu être observées entre les essais, notamment avec de fortes variabilités sur les groupes de vaches issus de l'essai du Pin au Haras. De même, les dynamiques de Ca du

lait étaient plus variables pour les groupes issus de l'essai du Pin au Haras. Les variations du rapport Ca sur taux protéique (TP) ont aussi été étudiées car VanHouten et al. (2004) ont démontré que l'effet sur le Ca du lait était obtenu sur le Ca rapporté par gramme de protéine. Cependant, les variations de Ca et du TP n'étaient pas concomitantes au cours de la lactation, et le Ca ne peut être déterminé par le TP même si ces deux éléments du lait sont liés. Les régressions de Ca en fonction du TP ont montré que la relation était individu-dépendante. Le lien entre Ca et TP demeure à cause de la place du Ca dans la structure des micelles des caséines. Cependant, l'incorporation du Ca dans ces structures résulte d'un équilibre global du lait. La part de Ca associé aux caséines reste donc variable. L'hypothèse du lien entre Ca/P et OC/CTX a été testé de façon plus globale, mais a montré de mauvais résultats en terme de qualité de régression, que ce soit sur l'ensemble des données ou en intra-essai. Il serait possible d'utiliser le P inorganique du lait plutôt que le P total du lait, car il refléterait mieux les variations de la phosphatémie à l'échelle de l'organisme aux vues des connaissances actuelles. Ces dernières restent trop incomplètes actuellement pour permettre une utilisation à bon escient d'un ratio Ca sur P inorganique dans le lait.

Dans la dernière partie de cette discussion générale, il s'est agi de mieux définir la place de la glande mammaire dans le système de régulation de la calcémie. Si la vitesse de mise en place des différentes réponses à une hypocalcémie chez un animal non lactant a été bien décrite, la place de la glande mammaire et la rapidité de son action par la PTHrP est moins connue. Sa vitesse de mise en place peut être cependant considérée comme plus lente que celle de la PTH qui se fait dans l'ordre de la minute. Cependant, les réponses mises en place pour une hypocalcémie ou un déficit d'apport dans le temps ne semblent pas être les mêmes. Dans un tel cas, l'augmentation de l'absorption digestive semble être privilégiée à la mobilisation osseuse. L'hypothèse de suivre le Ca comme indicateur de la mobilisation osseuse ne semble cependant pas adaptée à l'échelle de la lactation, mais à des échelles de pas de temps plus courts, comme cela a pu être observé dans les chapitres II, IV et V. A l'aide de la technologie MIR, il serait possible de suivre les variations journalières et donc de détecter d'éventuels problèmes dans les apports de Ca.

# Conclusion

Cette thèse a montré que les variations de la teneur en Ca du lait, à l'échelle d'une mesure par mois, pendant la lactation ne peuvent pas constituer un bon indicateur de l'amplitude de la mobilisation osseuse pendant la lactation. Il est possible que la pression de mesure, avec un intervalle d'échantillonnage d'un mois dans la plupart des cas, soit trop faible pour permettre de détecter les variations de la teneur en Ca du lait liées à la régulation de la calcémie chez la vache laitière. Une pression d'échantillonnage plus élevée, avec de plus grands défis de régulation de la calcémie, pourrait être nécessaire pour étudier comment les animaux régulent la sécrétion de Ca dans le lait pour maintenir la calcémie. Cependant, nos résultats suggèrent qu'avec une fréquence d'échantillonnage mensuelle et dans certaines conditions, la dynamique du rapport Ca / P du lait pourrait donner une idée grossière de l'évolution de l'équilibre entre l'accrétion osseuse et la résorption tout au long de la lactation. Cette thèse a permis d'identifier des facteurs pouvant affecter le remodelage osseux ou l'amplitude de la mobilisation osseuse pendant la lactation chez la vache laitière. Le régime alimentaire des vaches et notamment la densité énergétique de l'alimentation ont fortement influencé l'amplitude de la mobilisation osseuse pendant la lactation, en relation avec l'augmentation de la production de lait induite par ces régimes alimentaires. Au contraire, aucun effet de la teneur en Ca alimentaire n'a été observé. Les races laitières présentaient également des différences d'amplitude de la mobilisation osseuse pendant la lactation, avec une mobilisation osseuse plus élevée chez les Normandes que chez les Holstein. Le remodelage osseux également plus élevé chez les primipares que chez les multipares et les vaches primipares pouvaient également avoir une plus grande amplitude de mobilisation osseuse pendant la lactation que les vaches multipares. Ce dernier résultat reste néanmoins à confirmer et semble dépendre fortement des conditions environnementales.

Cette thèse a également montré que la mobilisation osseuse n'est pas la seule réponse à une situation d'hypocalcémie au cours de la lactation. Il a ainsi été clairement montré, que sur des vaches laitières à 3 semaines de lactation, une augmentation de l'absorption digestive de Ca peut suffire à maintenir la calcémie sans mobilisation osseuse du moins si la source de Ca est facilement absorbable par l'animal. Cela signifierait que la

mobilisation osseuse ne constitue pas une réponse préférentielle à un déficit de Ca durable, du moins si l'absorption digestive peut être améliorée. Les résultats obtenus au cours de cette thèse ont également soulevé la question d'un effet possible d'un apport alimentaire faible en Ca en début de lactation sur la production laitière dans l'ensemble de la lactation. Ce résultat surprenant doit être confirmé à plus grande échelle. S'il était vérifié, ce résultat serait une démonstration de la nécessité de complémenter les vaches laitières pour couvrir leurs besoins en Ca au début de leur lactation. Les conséquences d'un faible apport de Ca au début de la lactation sur la santé et les performances reproductives des vaches devraient également être étudiées. Enfin, il serait également intéressant de déterminer si la variation quotidienne de la teneur en Ca du lait peut refléter un défaut de régulation de la calcémie en début de lactation, et donc constituer un outil de détection d'hypocalcémie subclinique. Cette possibilité permettrait mieux adapter les conduites de préparation au vêlage et d'anticiper les conséquences des hypocalcémies post-partum sur la santé et les performances des vaches.

In dairy cows, Ca and P are essential elements for both growth and milk production and consequences of the dietary deficiencies in these elements have been already demonstrated at the beginning of the XXth century (Becker et al., 1934, Suttle, 2010, McDowell, 2017). For that reason, a mineral supplementation, providing, inter alia, Ca and P is generally supplied to dairy cows according to their requirements. Current estimations of requirements of Ca and P are based on factorial approaches that have been included in several feeding systems (AFRC, 1991, NRC, 2001, INRA, 2010). For dairy cows, these factorial approaches consist in splitting Ca and P requirements into four components: maintenance (inescapable fecal and urinary excretions), growth (bone fixation), gestation (fetal bone fixation) and milk production (secretion into milk). To estimate total requirements, the four components of requirements are estimated independently and then summed. The idea of these factorial approaches is to replace mineral losses or fixations of dairy cow day-to-day.

However, the whole complexity of Ca and P homeostasis is not taken into account in the factorial approaches of determination of requirements. More specifically, the fact that dairy cows are submitted to important cycles of bone mobilization and reconstitution (Ekelund et al., 2006, Puggaard et al., 2014) is not considered. The consideration of those cycles could allow a higher precision of the Ca and P requirements and an integration of those requirements at the scale of the whole cycle of production, with a consideration of the body reserve mobilization and reconstitution. The first aim of Ca and P homeostasis is to maintain a relative stability of plasma concentrations of those elements and to avoid

hypocalcemia or hypophosphatemia when requirements are not covered. The bones play a prominent role in this homeostasis because they constitute the first reserve of Ca and P in the organism. The mobilization of Ca and P from bones is a leverage to allow restauration of plasma Ca and P concentrations when supply of those elements are too low (Horst et al., 2005). In dairy cows, situations of undersupply are common for Ca after calving, at the set up of lactation, due to the sharp increase in exportation of Ca in milk. A consequence is a bone mobilization in early lactation, which is difficult to avoid (Horst et al., 2005). This mobilization is compensated after three months of lactation by a bone reconstitution. Currently, the bone mobilization at the beginning of lactation is not considered as a supply in the factorial approaches of determination of requirements of Ca and P and the bone reconstitution is not considered as a requirement.

The consideration of the cycles of bone mobilization and reconstitution in the definition of Ca and P requirements would also allow integrating a part of the interactions between Ca and P metabolism. Indeed, Ca and P are bound together in bone in the form of hydroxyapatite (Moreira et al., 2009, Elizondo Salazar et al., 2013). Thus, insufficient supply of one mineral could have consequences of the amount to be supplied for the other. For instance, it can be considered that bone mobilization, often considered as unavoidable at the beginning of lactation, induces a release of P and thus that P supply could be reduced at this stage. On the contrary, P supply could have to be reallocated, with Ca supply after the third month of lactation, when cows reconstitute their bones. The approach should allow both a better use of P with less rejection and eutrophication risks and a better bone reconstitution in late lactation, maybe in relation with prevention of health problems and impaired longevity of cows.

To integrate bone mobilization and reconstitution cycles in estimations of Ca and P requirements and supply in dairy cows, it will be necessary to dispose of consequent sets of data quantifying these cycles over lactation. Currently, there is no fast, cheap and efficient method to follow evolution of bone mobilization, which could be used in large scale. Current available methods cannot be used on large number of animals due to either high cost (blood bone biomarkers, Liesegang et al., 2007), time-consuming procedure (total mineral balance, Taylor et al., 2009), environmental issues (radio-isotopes, Ramberg et al., 1970) or ethical issues related to animal experimentation (repeated bone biopsies, Beighle, 1999). Results

published in the last 15 years suggest that milk Ca content could reflect insufficient Ca supply and bone mobilization in mammals (VanHouten et al., 2004). Recent results also suggest a variability of milk Ca content that could be linked to the feeding strategy of the cows (Hurtaud et al., 2014, Boudon et al., 2016). These results highlight the possibility that the variation of milk mineral composition, and more specifically that of Ca and maybe of P contents of milk, could allow a prediction of the bone mobilization of cows. With the increasing use of med infra-red (**MIR**) technology to analyze milk composition, notably Ca content (Soyeurt et al., 2009), milk Ca could be a cheap and simple biomarker of bone mobilization along lactation in dairy cows.

The objective of this PhD thesis was to understand how variations of milk Ca content and bone accretion and resorption during the lactation, could be linked in dairy cows, in relation with several factors of variation of those dynamics such as, the individual, the parity, the breed or the feeding strategy. The first part of this thesis consist in establishing a state of the art about Ca and P homeostasis and requirements, cycles of mobilization and reconstitution of bones and variability of milk Ca and P contents in dairy cows. In a second part, an analysis of the main factors of variation of milk Ca content of cow was performed thanks to a database of results of milk composition obtained from a survey performed before the initiation of my PhD thesis in about a thousand of French commercial farms. In the third, fourth and fifth parts, the results of three experiments consisting in characterizing simultaneously bone mobilization and reconstitution cycles and variability milk of Ca and P contents in dairy cows during the whole lactation are presented. The third part consisted in evaluating the effect of the parity and the individual variability. The fourth part consisted in evaluating the effect of the breed and the feeding strategy whereas the last part consisting in following whole lactation of cows with inadequate Ca supply in early lactation.

About Ca and P homeostatis, cycles of mobilization and reconstitution of bones and variability of milk Ca and P contents in dairy cows
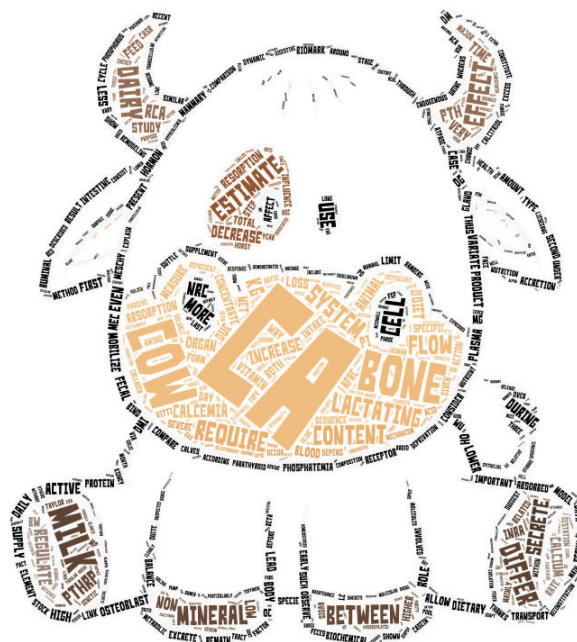
# A)   Regulation of Calcemia and Phosphatemia

Ca and P are quantitatively the most important mineral elements of the human, or bovine body, representing together more than 70% of the mineral content of the body (McDowell, 2017). The regulations of their content in the plasma are partly joined, notably because they are stocked together in bone as hydroxyapatite, with a constant ratio between the two minerals (Bullough, 2010). In this section, the main roles of Ca and P in the organism will be briefly described. Then, the principles of the systemic regulation of the body contents of these elements will be given for non-lactating animals before a description of the specificities of lactating animals is given.

## 1   Repartition of Ca and P in body pools and major roles

### a   Calcium repartition and roles in mammals

Ca is the fifth most present chemical element in the body, after C, O, H and N (Blanco and Blanco, 2017a). Between 95 and 99% of body Ca is present in bones and teeth (Flynn and Cashman, 1997, Mundy and Guise, 1999). The remaining part is split between non-blood extra-cellular fluids and within cells. In an adult dairy cow, total body Ca is estimated around 10 kg (Martín-Tereso and Martens, 2014).

$Ca^{2+}$ is a common messenger in cells. Indeed, $Ca^{2+}$ extra-cellular concentration is ten thousand times more important than intracellular concentration (Brown et al., 1995), and it can be increased by ten in just few milliseconds (Blanco and Blanco, 2017a). The concentration of $Ca^{2+}$ is around 100 nM in cytosol and 1.2 mM in extra-cellular compartments (Hennings et al., 1980). Ca is involved in muscle contraction, nervous transmissions, (Brown, 1991, Reinhardt et al., 1988) and so, cardiac contraction (Lakatta et al., 2010).

Calcemia is defined as the plasma concentration of total Ca. Variations of calcemia are very low, under 3% for most mammals (Blanco and Blanco, 2017b). In dairy cows, calcemia is considered to be in a normal range between 80 to 120 mg/L (McDowell, 2003, Goff, 2008, Reinhardt et al., 2011, Martinez et al., 2012), but the lower limit has been recently questioned. Some authors proposed 85 mg/L as a threshold for hypocalcemia with clinical consequences (Neves et al., 2017, Rodríguez et al., 2017). Around the onset of

lactation, the main difficulty in the choice of the threshold for hypocalcemia is to identify the moment when the organism is affected by hypocalcemia with clinical manifestations. Rodriguez et al. (2017) found several threshold of hypcalcemia around calving according to animals, and more specifically parity. In blood, 40 to 45% of Ca is linked to plasma proteins, 5% is bonded to other organic elements like citrate or inorganic element, and around 50 % is circulating as soluble, or ionized $Ca^{2+}$ (NRC, 2001).

### b   Phosphorus repartition and role in mammals

Unlike Ca, only 80% of body P is present in bone whereas almost 20% is present in cells and about 0.3% is present in extra-cellular fluids (Blanco and Blanco, 2017b). P is a constituent of several structural components of cells such as DNA, RNA, ATP and other molecules involved in energy metabolism (ADP, UTP, CTP, GTP, glusose-6-P), and phospholipids allowing the double lipid layer structure in membranes (Soares Jr, 1995).

Phosphatemia is the concentration of total plasmatic inorganic P (**Pi**). For a normal adult dairy cow, normal range of phosphatemia is between 40 and 60 mg/L (NRC, 2001). This range of variation is high compared to that of Ca. Only 30% of blood P is in the form of Pi, with 2 forms $HPO_4^{2-}$ and $H_2PO_4^-$, which proportions depend on blood pH. The remaining 70% of plasma P is present in proteins. Total blood P, including Pi, P in plasma and in red blood cells, range from 350 to 450 mg/L (McDowell, 2003).

## 2   Systemic regulation of calcemia in non-lactating animals

### a   Flows susceptible to affect calcemia in non-lactating animals

Even for a non-lactating cow, calcemia could be easily affected, in absence of regulation, by important Ca flows that can be quantified at the scale of the animal or its organs. This is illustrated on figure I.1, representing Ca flows from the blood pools of a non-lactating cows that were quantified thanks to radio-isotopes of Ca ($^{45}$Ca, Ramberg et al., 1970). It clearly appears from these data that the flows that affect more the non-skeletal Ca pools are the flow of Ca absorption from the feed by the digestive tract (21 g/d), that relies on the amount of Ca ingested by the animal (67.7 g/d) and on the part of Ca absorbed from the diet (0.31 g/g), the flows of bone accretion and resorption (9.78 and -4.3 g/d respectively) and more marginally the flow of endogenous loss in the feces (6.3 g/d). Urinary losses are very low. The flow of total Ca fecal losses (53 g/d) consists in the sum of the Ca flow that has not been absorbed

the digestive tract (46.7 g/d) and of that of Ca endogenous loss. Endogenous Ca loss in the feces consists mainly in desquamation debris of cells from the digestive tract mucosa and digestive secretions and is mostly determined by the importance of intestine flow and total dry matter intake (**DMI**, Meschy, 2010, Vitti and Kebreab, 2010). In this example, the fact that the cow was a multiparous cow considered during a dry period may explained the high flow of bone accretion compared to bone resorption, leading to a net Ca flow of 15 g/d from blood to bone and thus a net flow of bone reconstitution.
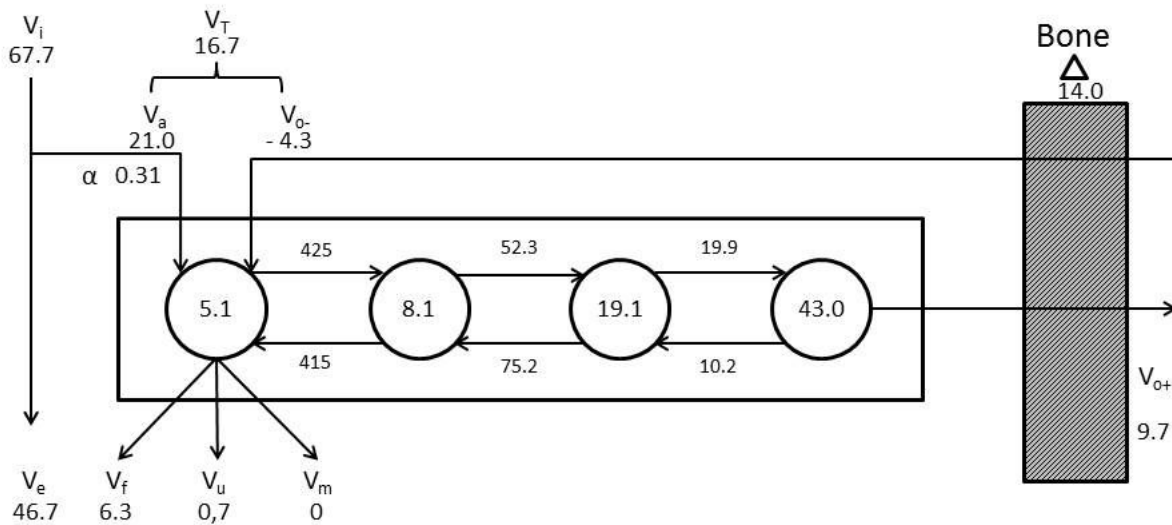


Figure I.1: Intern Ca flows in a non-lactating dairy cows (Ramberg et al., 1970). The central box represents repartition of non-skeletal Ca in the model. Repartition of non-skeletal Ca between compartiments cannot be alocated to one specific anatomic or physiological entity (blood for example). Compartiments (within rounds) are in grams and rate of Ca transport (arrows) in g/d. $V_i$: Ca intake; $V_e$: non-absorbed Ca in fecal excretion; $\alpha$: part of Ca absorbed from diet; $V_a$: Absorbed Ca from diet; $V_{o-}$: Bone resorption; $V_T$: Total Ca inflow; $V_{o+}$: Bone accretion; $V_f$: Fecal endogeneous losses; $V_u$: Urinary losses, $V_m$: Secretion in milk; $\triangle$: difference between accretion and resroption (g/d)

Bone is the only organ that can stock Ca in the organism. Two cell types are involved in bone Ca accretion and resorption: osteoblasts and osteoclasts. Osteoblasts are responsible of bone accretion and fixation of Ca in hydroxyapatite and osteoclasts are responsible of bone resorption, releasing Ca into blood (Durand and Beaudeux, 2011). These two types of cells are always conjunctively active, and the net flow between blood, leading to bone mobilization or reconstitution is dependent on the relative difference of activities of both cells. In an organism that is not either mobilizing or reconstituting bone, equivalents flow of bone accretion and resorption are maintained thanks to activity of both osteoblasts and osteoclasts, which constitues bone remodeling (Ross et al., 2011). A specific part of

this bibliography will later focus on the dynamics of the equilibrium of bone accretion and resorption during the lactation-gestation cycle in dairy cows (Section C of this chapter). Excretion of Ca in urine represents a small flow at the level of the organism.

### b   Systemic hormonal regulation of calcemia

Major effectors of calcemia regulation are resumed in figure I.2. Calcemia is very finely regulated at the level of the organism thanks to a complex system of three hormones, the parathormon secreted by the pathyroid glands (**PTH**), the 1,25-(OH)$_2$-vitamin D at the level of the kidney and the calcitotin secreted by the thyroid. These hormones allow the maintenance of a constant calcemia, by modulating activities of kidney, bone and intestine that modify Ca flows from and into blood.

The parathyroid glands are able to detect very low and rapid variation of calcemia, which contributes to the accuracy of the calcemia regulation (Brown, 1991). Hypocalcemia is mainly detected by a Ca-Sensitive Receptor (**CaSR**) in chief cells of parathyroid glands that secrete PTH. This constitutes the first step of the response of the organism to a decreased calcemia. PTH is a 84 length amino acids peptide (Mundy and Guise, 1999), obtained after several cleavages of an initial 115 length amino acids peptide (Durand and Beaudeux, 2011). This hormone has a very short half-life, below 4 minutes, because of fast degradation by the Kuppfer liver cells (Mundy and Guise, 1999). Normal or high calcemia in plasma activates the CaSR on chief cell, leading to an inhibition of PTH transcription (Brown et al., 1993). The rate of secretion of PTH by the parathyroid glands is very sensitive to variations of calcemia (Brown, 1991), as illustrated in figure I.3. Brown (1991) even suggested that the rate of decrease of calcemia may also influence the rate of PTH secretion, with higher immediate release of PTH with faster decrease of calcemia. PTH is secreted in blood by exocytosis. Chief cells of parathyroid glands have a stock allowing PTH secretion during 1 to 1.5 hours, but 12 hours are necessary to replace the entire stock. After 40 min of PTH secretion, the rate of PTH secretion decreases to avoid a lack of PTH in the chief cells, even with persistent hypocalcemia (Brown et al., 1995). If calcemia remains low for a long time, chief cells can multiply, but this requires several days. PTH action on bone and kidney mainly is mediated by several receptors, but PTHR1 (PTH receptor of type 1) is the main and is coupled with a G protein (Durand and Beaudeux, 2011). PTH promotes the activation of 25-OH-vitamin D into 1,25-(OH)$_2$ vitamin D at the level of the kidney (Taylor
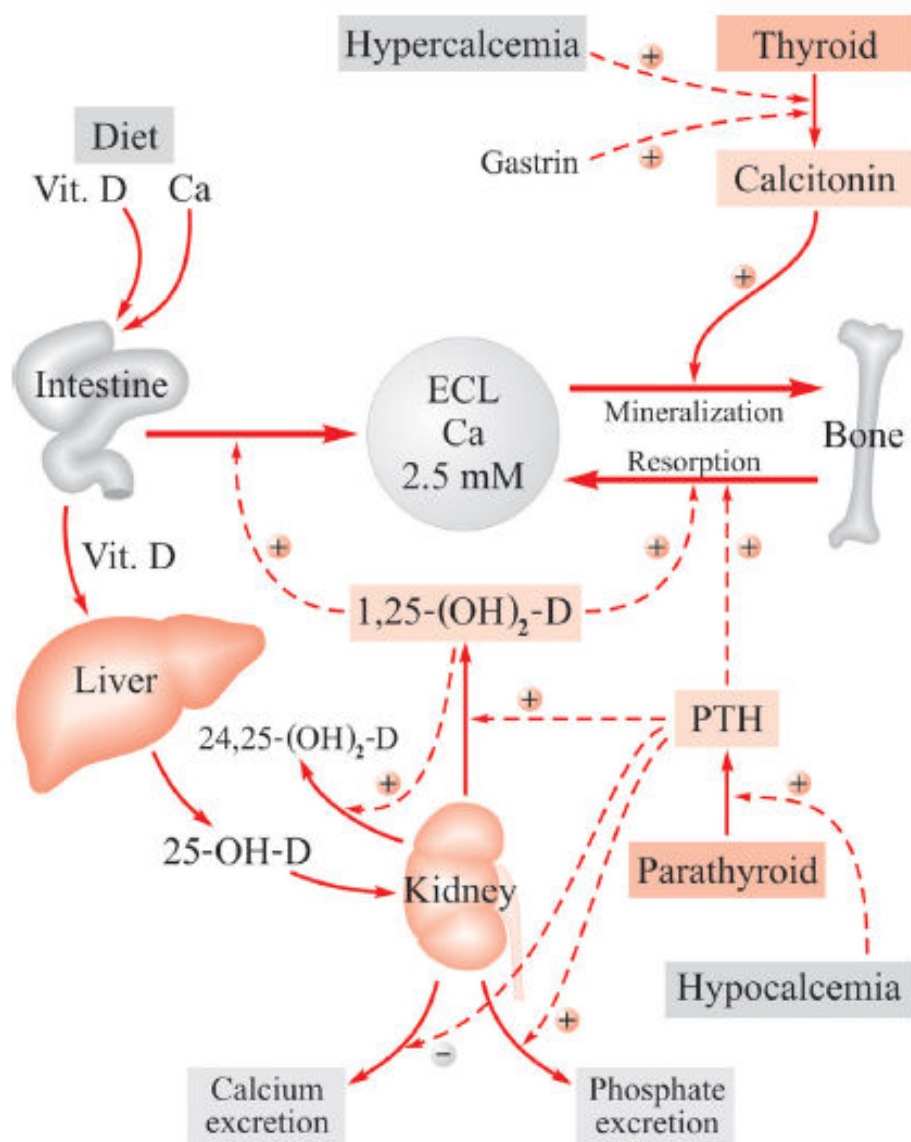
Figure I.2: Major effectors of calcemia regulation in mammals (Blanco and Blanco, 2017a). PTH: Parathyroid hormon; ECL: Extra-cellular liquid

et al., 2008), bone resorption and renal tubulat re-absorption of Ca. It also increases activity of osteoclasts, and thus bone resorption (Mundy and Guise, 1999).

The involvement of vitamin D is a second step in the regulation of calcemia in case of hypocalcemia. It allows a more durable action than PTH alone. The main circulating form of vitamin D is 25-OH-vitamin D which is inactive for the regulation of calcemia. The blood concentration of 25-OH-vitamin D defines the vitamin D status of the organism. The blood concentration of 25-OH-vitamin D in the organism depends either on ingestion of vitamin $D_2$ (ergocalciferol) of plant origin, or on ingestion of vitamin $D_3$ (cholecalciferol) mainly of animal origin, or on endogenous synthesis of vitamin $D_3$ from ultraviolet radiation of sterols
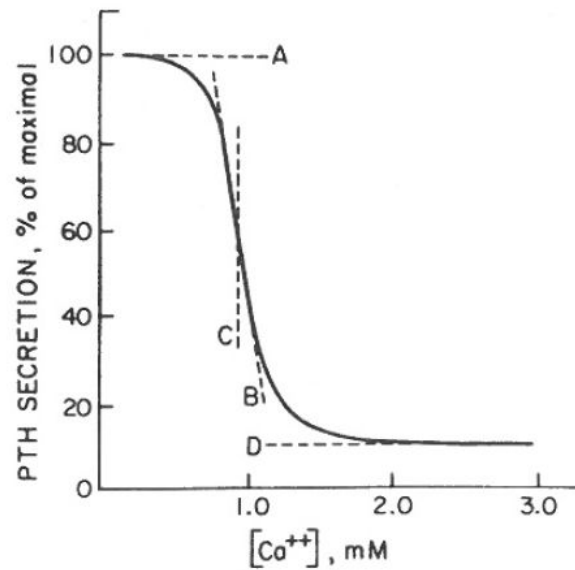
Figure I.3: PTH secretion rate (Brown, 1991) . Four-parameter model of inverse sigmoidal relationship between extracellular $Ca^{2+}$ and PTH release: $V_s = \frac{A-D}{1+\frac{Ca}{C})B}$, with A : maximal rate of secretion; B : slope of curve at its midpoint; C : Ca concentration producing half maximal change in PTH release; D : minimal secretory rate

(7-dehydrocholestol) present in the skin (Hymøller and Jensen, 2010). Two hydroxylations of vitamin D are necessary to activate vitamin D. These transformations are resumed in figure I.4. The first one is performed in the liver by 25-hydroxylase-cytochrome P45 and transforms vitamin D into 25-OH-vitamin D, evocated earlier in this text and also called calcidiol. The product of this first hydroxylation inhibits its own production. 25-OH-vitamin D is transferred from liver to kidneys by vitamin D-binding protein (Reinhardt et al., 1988). Only around 5% of 25-OH-vitamin D circulates without being bonded because it is a hydrophobic vitamin (Mundy and Guise, 1999). The second hydroxylation occurs in the kidney and produced 1,25-(OH)$_2$-vitamin D, the calcitriol, under the action of 25-OH-vitamin D-1$\alpha$-hydroxylase (Henry, 2011). This second hydroxylation is enhanced by PTH but is inhibited by its own product. Other hydroxylated forms of 25-OH-vitamin D are produced in kidney, notably the 24,25-(OH)$_2$-vitamin D that is the second major product of hydroxylation in kydneys . It may have a specific role on systemic regulation of calcemia or it may constitute a way to eliminate excess of 25-OH-vitamin D (Bouillon et al., 1995). The position of second hydroxylation of 25-OH-vitamin D, i.e. 1 or 24, seems to be mainly determined by the potential necessity of the organism to increase calcemia. In case of hypocalcemia, renal hydroxylation on position 1 is more important, whereas renal hydroxylation on position 24

```
                        7-Dehydrocholesterol

        Ultraviolet light              Skin
                                │
                                │
                                ▼
                             Vitamin D₃  ◄── intestine ── Diet
        25-OHD₃ ──── − ──►
                                         Liver
        Phenobarbital ── + ──►
                                │
                                │
                                ▼
                             25-OHD₃
                                         Kidney

    Hypovitaminosis D ── + ──►┌──────────┐
      Low plasma Ca ── + ──►  │          │ ◄── + ── Hypervitaminosis D
     Low plasma Pᵢ ── + ──►   │          │ ◄── − ── Parathyroid hormone
  Parathyroid hormone ── + ──►│          │ ◄── + ── 1,25-(OH)₂D
         Estrogens ── + ──►   │          │ ◄── + ── High plasma Ca
          Prolactin ── + ──►  │          │
      1,25-(OH)₂D ── − ──►     │          │
      High plasma Ca ── − ──► └────┬─────┘
                                 │     │
                                 ▼     ▼
                          1,25-(OH)₂D₃   24,25-(OH)₂D₃
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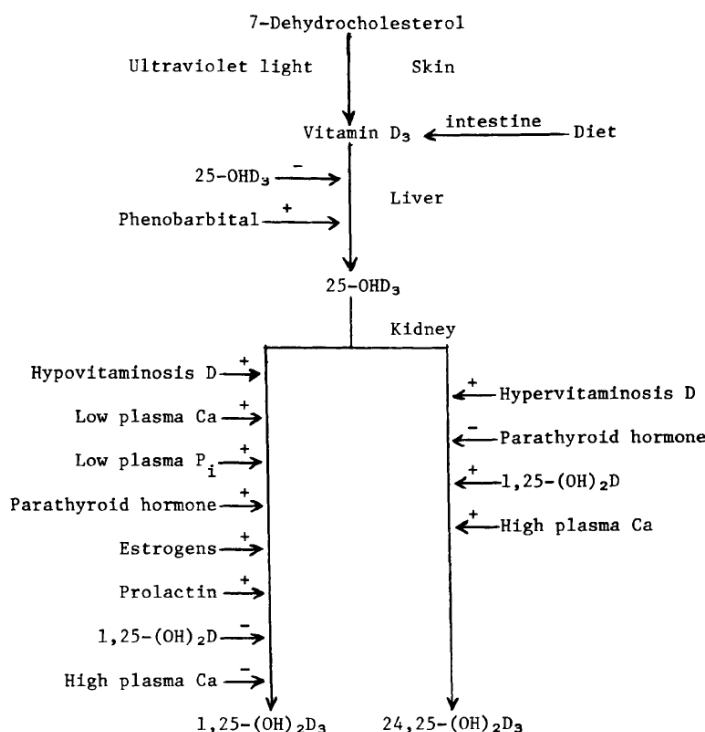
Figure I.4: Resume of factors affecting hydroxylation of vitamin D (Horst, 1986)

will be predominant when calcemia is high (Henry, 2011).

$1,25\text{-}(OH)_2$-vitamin D receptors (**VDR**) are present on enterocytes, osteoblasts and osteoclasts. $1,25\text{-}(OH)_2$-vitamin D stimulates TRPV6, calbindinin 9K and ATP-Ca pump activities in enterocytes. $1,25\text{-}(OH)_2$-vitamin D receptors may also be phosphorylated after binding to $1,25\text{-}(OH)_2$-vitamin D, which allows them to bind to 9-cis-retinoic acid receptor (RXR) and to form a VDR/RXR heterodimer. This induces a conformational change, allowing the VDR/RXR heterodimer to enter the nucleus (Blanco and Blanco, 2017a). The VDR/RXR heterodimer presents a strong affinity for promoter areas of several genes (Durand and Beaudeux, 2011). By this mechanism, $1,25\text{-}(OH)_2$-vitamin D stimulates active Ca and P absorption by action of the VDR/RXR heterodimer on nucleus, but the effectiveness of this mechanism decreases with aging (Goff, 2000, McDowell, 2003). The fixation of the VDR/RXR heterodimer on promoter areas results in more synthesis of calbindin, which is involved in Ca transport through intestine cells (McDowell, 2003, Vitti and Kebreab, 2010). In bones, $1,25\text{-}(OH)_2$-vitamin D promotes cell differentiations of osteoclasts, leading to a more important bone resorption and bone Ca and P release in blood (Bouillon et al., 1995, Horst et al., 1997). $1,25\text{-}(OH)_2$-vitamin D also increases Ca reabsorption in the kidney, while decreasing P reabsorption to avoid hyperphosphatemia

(Vitti and Kebreab, 2010).

In case of hypercalcemia, calcitonin, a 32 amino acids peptide, is secreted by the parafollicular cells of the parathyroid glands (Muff et al., 1988, Mundy and Guise, 1999). Its secretion rate is determined by the ionized Ca concentration in plasma (Vitti and Kebreab, 2010). Calcitonin actions are opposed to that of 1,25-(OH)$_2$-vitamin D. Receptors of calcitonin are present in kidney, where their stimulation inhibits renal tubular reabsorption of Ca. Calcitonin also favors the second hydroxylation of 25-(OH)-vitamin D on position 24 and inhibits osteoclast activity, which decreases bone resorption. The action of calcitonin is quite fast, as its effect can be observed within minutes after secretion (Mundy and Guise, 1999). Reponses of the organism to hypercalcemia were less studied than responses to hypocalcemia because hypercalcemia is less frequent. However, it is likely that calcitonin action is not limited to cases of hypercalcemia.

### c    Effect of 1,25-(OH)$_2$-vitamin D on intestinal absorption of Ca

As described previously, a systemic regulation of calcemia is possible thanks to the action of 3 hormones on intestinal absorption of Ca, bone accretion and resorption and renal tubular reabsorption of Ca. The flow of urinary excretion of Ca is very low compared to that of excretion of Ca in feces or associated to bone accretion and resorption and thus, the specific mechanisms of action of PTH on this flow will not be detailed in this thesis. The effect of PTH will be described in section C of this bibliography. This part will focus on the mechanisms of intestinal absorption of Ca and the implication of 1,25-(OH)$_2$-vitamin D on them.

Two mechanisms of Ca absorption have been described in the intestine and consist in either an active transcellular transport of Ca or a passive paracellular absorption of Ca (Bronner, 1987, Bronner, 2003, Vitti and Kebreab, 2010, Puggaard et al., 2011). Both mechanisms of Ca absorption co-exist simultaneously but the predominant mechanism depends on intestinal Ca concentration. Passive paracellular absorption of Ca is negative when intestinal Ca concentration is lower than 1 mmol/l, whereas active transcellular transport of Ca is predominant but active transport becomes overloaded when intestinal Ca concentration is over 4 mmol/l. Both ways are resumed in figure I.5.

The active transcellular transport allows an increased absorption rate when Ca concentration in intestine is low (Vitti and Kebreab, 2010). Ca is transported through
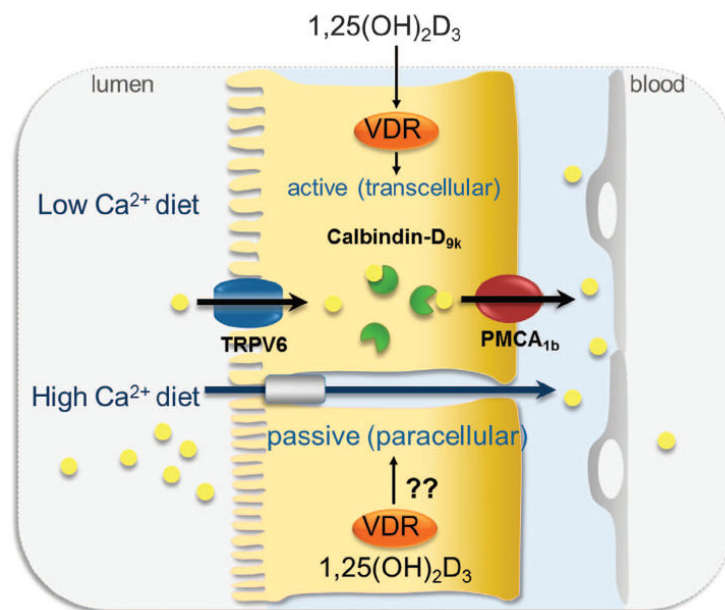
Figure I.5: Intestinal Ca absorption according to the model of Christakos et al. (2014) . This model comprises a transcellular active mechanism allowing transport of Ca when dietary Ca intake is normal or low and a paracellular passive mechanism triggered when dietary Ca intake is high. VDR: 1,25-$(OH)_2$-vitamin D Receptor; PMCA: Plasma Membrane $Ca^{2+}$-ATPase; TRPV: Transcient Receptor Potential Voltage

intestinal mucosal cell from apical to basal surface with the intervention of calbindin that is a Ca-binding protein allowing the transport of Ca across the cell (Feher et al., 1992). This mechanism of absorption involved three steps. At a first step, Ca penetrates the enterocytes by a Ca transporter, the transcient receptor potential voltage 6 (**TRPV6**), under the action of calmodulin that is a multifunctional intermediate Ca-binding messenger protein (Bronner, 2003, Christakos et al., 2014). The TRPV6 are situated on the brush border of intestinal epithelium. The presence of TRPV6 greatly increases permeability of cell membranes to Ca. Permeability to Ca through Ca transport is also regulated by intracellular Ca concentration that determines the fixation of calmodulin to TRPV6, with a Mickaelis-Menten relationship. At a second step, Ca is transported to basal surface of the enterocyte bound to calbindin-$D_{9k}$, a Ca binding protein (**CaBP**, Bronner, 1987, Feher et al., 1992). The main function of calbindin-$D_{9k}$ is to limit the increase in intracellular Ca concentration. About 90% of transported Ca through the cell is bound to calbindin, to avoid toxic concentration for the cell. At a third step, Ca is expulsed from enterocytes at basal surface, by active transport, against the gradient of Ca concentration, Ca being more concentrated in extracellular compartment than in cell. The active transcellular transport is performed by Mg-ATPase, exchanging Ca from cytoplasm

and extracellular Na.

As evocated before, the active transcellular transport of Ca is particularly efficient for low Ca concentration in intestine lumen and can be upregulated by the action of 1,25-$(OH)_2$-vitamin D in case of Ca deficiency at the level of the organism. However, when the Ca concentration in intestine lumen becomes high, the active transcellular transport of Ca become overloaded, and then the passive paracellular absorption becomes predominant (Bronner, 1987). This passive paracellular absorption is enhanced by Ca concentrations difference between blood and intestinal lumen, with Ca transfer through intercellular tight junctions. It requires, to be effective, a luminal Ca concentration of at least 1 mmol/L (Meschy, 2010). As Ca passive paracellular absorption is directly dependent on difference between two concentrations, there is no theoretical superior limit for Ca transfer by paracellular transport (Bronner, 1987). It is suspected that the passive transport of Ca could be also modulated by the action of 1,25-$(OH)_2$-vitamin D (Christakos et al., 2014). As passive Ca absorption is negative for low Ca concentration in intestinal lumen, 1,25-$(OH)_2$-vitamin D would decrease the opening of tight junctions to limit Ca losses.

Ca can be absorbed at multiple sites along the digestive tract and a specific question for ruminants is the relative contributions of rumen and intestine in Ca absorption (Bronner, 2003, Meschy, 2010). The intestine has been considered for a while as the only site of Ca absorption in dairy cows, but it has been established in the early 1980s that Ca can also be absorbed through rumen wall (Höller et al., 1988). However, intestinal absorption of Ca has been well described whereas the mechanism of ruminal Ca absorption remains uncertain (Khorasani et al., 1997).

## 3 Systemic regulation of phosphatemia in non-lactating animals

### a Flows susceptible to affect phosphatemia in non-lactating animals

As in the case of Ca flows, daily P flows at the scale of the organism are important enough to potentiate high variations of phosphatemia in absence of regulation, even for a non-lactating cow (Hill et al., 2008). Even though the amounts of P supplied to cows are generally lower than that of Ca, at least if current feeding recommendations are respected, most P flows in the organism are higher than those of Ca, except maybe those related to

bone accretion and resorption. This is mainly explained by a higher absorption rate of P compared with Ca and the presence of a flow of P recycling in the rumen. The importance of the flow of P recycling in the rumen is illustrated in figure I.6 for a dairy cow (Hill et al., 2008). As flows of P were estimated for a lactating cow in this figure, they cannot be directly compared to the flows of Ca illustrated in figure I.1. It clearly appears that the flow of P exiting the rumen (128 g/d) is higher than that of P ingested (75 g/d) and that the difference between both flows arise from salivation and P recycling. About 47 g/d of Pare excreted in feces, resulting in a net absorption of 28 g/d but a real absorption of more about 82 g/d of P. In the case of P and unlike that of Ca, P saliva losses in feces are an important source of endogenous loss of Ca that have to be added to P losses due to desquamation debris of cells from the digestive tract mucosa and digestive secretions.

Figure I.6 also illustrated important flows of P between bone and blood with comparable flow of accretion and resorption. In this example, the cow is almost in a situation of strict bone remodeling with a negligible net bone reconstitution (483 g/d of P for bone resorption vs 487 g/d offor bone accretion). Flows of Ca and P from bones are largely correlated due to fact that the mineral matrix of bone is mainly constituted of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ with a mass ratio Ca/P of 2.15. However, bone mobilization occurs after a longer time of deficiency for P than for Ca (Suttle, 2010). Bone allows P storage, as Ca, but, in case of hyperphosphatemia, saliva can also constitute a storage pool. For P, blood can be considered as a storage pool, due to its capacity to endure higher variations of phosphatemia than for calcemia (Suttle, 2010).

A part of P is also excreted in urine but this represents a very small flow, around 1 g/d, even if variations are more important than for Ca. Urine P losses are a way of excretion of excess of P. However, in the specific case of ruminants, fecal excretion of saliva P can also have an excretory function in case of hyperphosphatemia (Puggaard et al., 2011), even though saliva P flows hardly decrease under a certain limit in case of hypophosphatemia (Puggaard et al., 2011). This last point may constitute a protective mechanism to maintain rumen function given that saliva brings at least 50% of P needed by micro-organisms in normal feeding conditions (Vitti and Kebreab, 2010) and that, activity of micro-organisms in rumen depends on P supply (Meschy, 2010). The first effect of a low dietary P supply for ruminants is a deterioration of ruminal micro-organisms activity and a decrease of dry

matter intake (Meschy, 2010, Suttle, 2010).
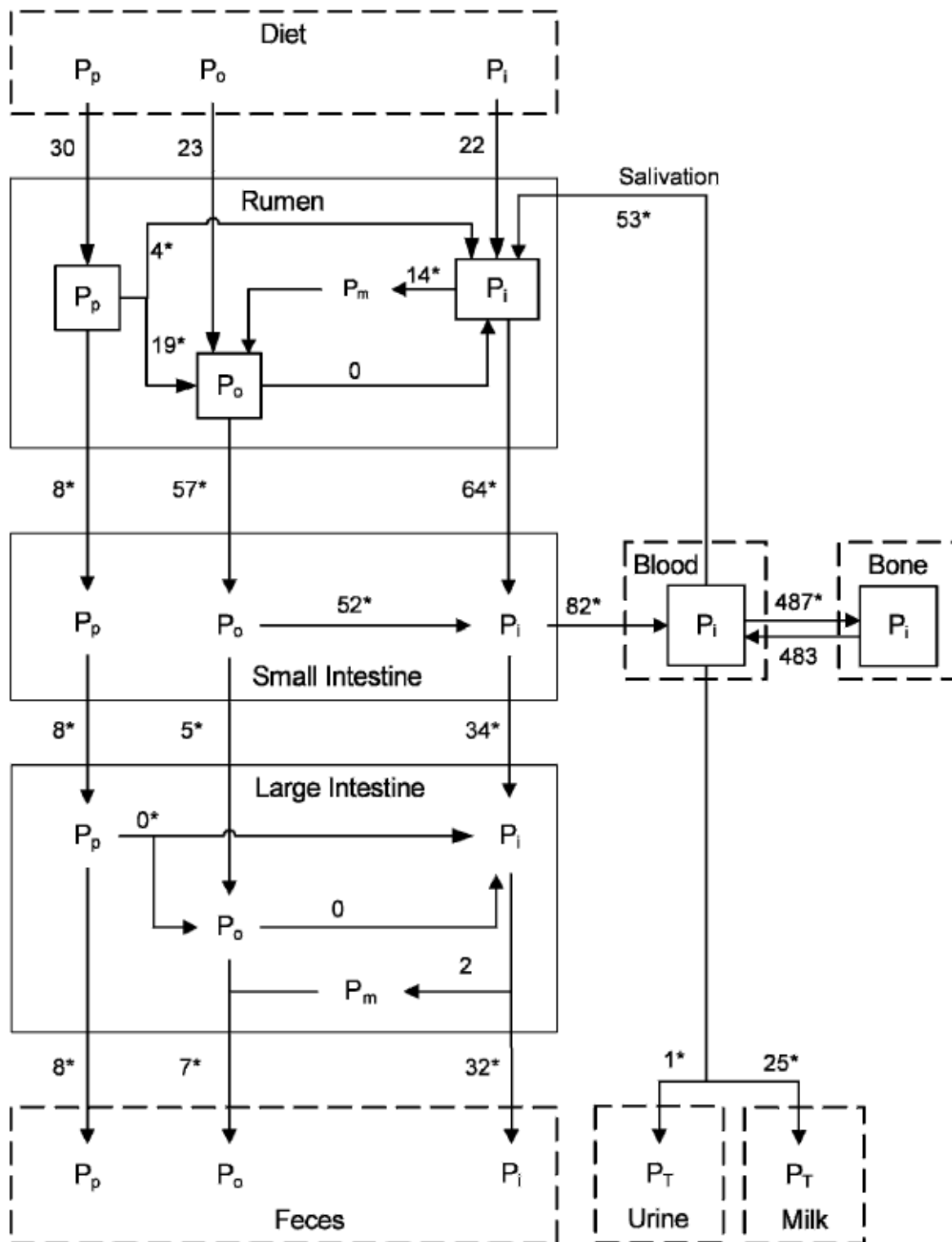


Figure I.6: P flows in a lactating cow obtained thanks a dynamic mechanistic and compartimental model (Hill et al., 2008). Boxes with solid lines represent pools, boxes with dashed lines represent compartments, and solid arrows represent fluxes. Numbers associated with arrows are the predicted flows (g/d). $P_T$ = total P; $P_P$ = phytic P; $P_O$ = Organic P excluding phytate; $P_i$ = inorganic P; $P_m$ = microbial P

### b   Systemic hormonal regulation of phosphatemia

Variations in phosphatemia are far more important than for calcemia. In humans, variations of phosphatemia can be as high as to 25 mg/L within a day whereas it barely exceeds 5 mg/L for calcemia (Jubiz et al., 1972). Even though feed intake can explain an important part of the variations of phosphatemia, many variations remain unexplained and the specific mechanisms of regulation of phosphatemia remain far less described than those of regulation of calcemia. However, as P and Ca are linked in the bone structure, it is clearly established that hormones that affect bone accretion and resorption to regulate calcemia also affect phosphatemia and that regulation of both calcemia and phosphatemia are linked (Horst, 1986). More specifically, it has been established that phosphatemia can influence the hydroxylation of 25-OH-vitamin D in $1,25\text{-}(OH)_2$-vitamin D (active form) in the kidney (Cunningham and Klein, 2007). Phosphatemia may also directly influence PTH secretion. Phosphorus restriction prevents parathyroid gland growth. High P diet directly stimulate PTH secretion *in vitro* (Slatopolsky et al., 1995). However the higher variation of phosphatemia compared with that calcemia let think that the regulation of phosphatemia has less priority than regulation of calcemia.
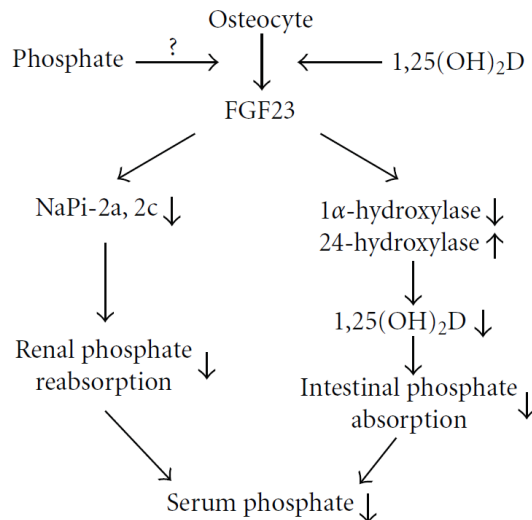
Figure I.7: Actions of FGF 23 on phosphatemia regulation (Saito and Fukumoto, 2009)

Hyperphosphatemia is not really a problem for ruminants as they have multiple ways to excrete excess P, i.e. saliva recycling increase and intestinal absorption decrease that were evocated earlier in this text (Puggaard et al., 2011). An hormone specific to phosphatemia

regulation has been recently discovered, the fibroblast growth factor 23 (FGF23, Hardcastle and Dittmer, 2015). This hormone is secreted by osteocytes and osteoblasts as a 227 amino acids peptide (Durand and Beaudeux, 2011). Its synthesis is upregulated under the action of $1,25\text{-}(OH)_2$-vitamin D (Saito and Fukumoto, 2009) but recent discoveries suggest that its synthesis can be directly regulated by high phosphatemia (Figure I.7, Hardcastle and Dittmer, 2015).  Even if FGF23 is classified as a fibroblast, its endocrine action is more similar to an hormonal action.  The active form of FGF23 reduces the formation of $1,25\text{-}(OH)_2$-vitamin D, in favor of the $24,25\text{-}(OH)_2$-vitamin D, by regulating expression of genes of enzymes catalyzing the reactions (Saito and Fukumoto, 2009).  FGF23 also limits P reabsorption in proximal tubes and P absorption in intestine, by suppressing expression of NPT2B in intestine (Hardcastle and Dittmer, 2015).

### c   Effect of $1,25\text{-}(OH)_2$-vitamin D and FGF 23 on intestinal absorption of P

Absorption of P in the digestive tract has been less described than that of Ca.  It seems that ruminal absorption of P is not quantitatively important and that most P absorption occurs at the beginning of the small intestine (duodenum and jejunum, Meschy, 2010). As for Ca, two mechanisms of absorption of P have been described, a passive paracellular absorption and an active transcellular absorption (Figure I.8).  The passive paracellular absorption is dependent on differences between intestinal lumen and blood concentrations and occurs through tight junctions (Reinhardt et al., 1988).  It has been recently stated that in case of hyperphosphatemia, P is still absorbed from the diet but with a lower efficiency (Christakos et al., 2014).  This decrease in passive absorption rate is under the control of FGF 23, a hormone secreted by the osteocytes and osteoblasts in situation of hyperphosphatemia (Christakos et al., 2014), that may close tight junction (Saito and Fukumoto, 2009).  The active transcellular absorption of P is less known and P has been thought to be only passively absorbed until recently (Meschy, 2010, Christakos et al., 2014).   This active absorption occurs principally on the brush border of intestinal epithelium. It occurs with Na absorption thanks to the NPT2B transporter (Na-dependent phosphate co-trasnporter 2b, Christakos et al., 2014).   It has been suggested that $1,25\text{-}(OH)_2$-vitamin D can interfere on P absorption (Horst, 1986, Vitti and Kebreab, 2010, Christakos et al., 2014).
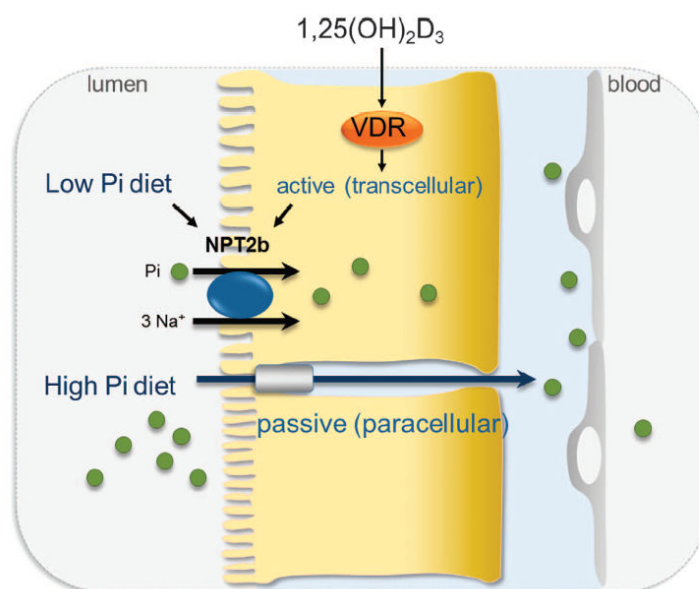
Figure I.8: Intestinal P absorption. Model of intestinal P transport (Christakos et al., 2014). VDR : 1,25-(OH)$_2$-vitamin D receptor. NPT2b: Na-dependent phosphate co-transporter 2b

## 4 Specificities of calcemia and phosphatemia regulations during lactation

### a The high exportation of Ca and P in milk is a challenge for calcemia and phosphatemia regulation

At the beginning of lactation, dairy cows face an homeostasis challenge in the relation with the high increase in Ca and P secretions with milk production (Figure I.9, Horst, 1986, Horseman and Hernandez, 2014). At the peak of lactation, a dairy cow can secrete up to 80 g/d of Ca in milk, leading a total blood Ca replacement between 20 and 30 times a day (Horst et al., 2005). This increase in Ca and P requirements for milk production is fast during the first days of lactation and the cows need to adapt quickly. When they do not adapt quickly enough, a decrease in calcemia can induce milk fever, which prevalence is comprised between 3 and 7% (Reinhardt et al., 2011). This metabolic disease is quite specific to dairy cows, maybe because the Ca demand is relatively low in that specie before parturition and increases very sharply after calving (DeGaris and Lean, 2008). Even though the beginning of lactation is an acute phase of challenge for Ca homeostasis, the quantity of Ca exported in milk remains important throughout the lactation. It has been estimated that a Holstein cow producing 9,000 kg of milk over a lactation, secreted 11 kg of Ca in milk

for the lactation, which represents more than its total body Ca content (Horst, 1986, Martín-Tereso and Martens, 2014).

Even though dairy cow faces a specific challenge that can drive to Ca homeostasis failure at the beginning of lactation, it remains impossible for all mammals to cover the increase in Ca requirements at the very beginning of lactation by only increasing intake. Thus, a first answer at the onset of lactation is the mobilization of Ca stock, i.e. bone, before a progressive increase in intake and in intestinal capacity of Ca absorption (Horst et al., 2005, Kovacs, 2016). In ruminants, this was first demonstrated in ewes thanks to the use of radio-istopes of Ca and P (Braithwaite, 1983a, Braithwaite, 1983b). Since the early 2000s, the existence of the increase in bone mobilization has been illustrated also in several publications with dairy cows thanks to the development of plasma biomarkers (Liesegang et al., 2000, Taylor et al., 2008, Sato et al., 2011).
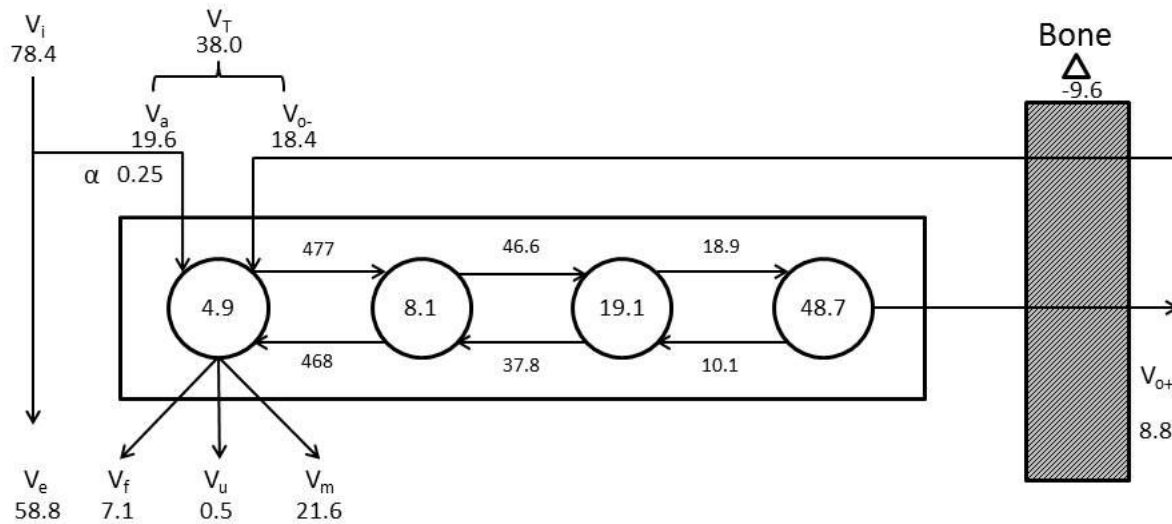


Figure I.9: Intern Ca flows in a lactating dairy cows (Ramberg et al., 1970). The central box represents repartition of non-skeletal Ca in the model. Repartition of non-skeletal Ca between compartiments cannot be alocated to one specific anatomic or physiological entity (blood for example). Compartiments (within rounds) are in grams and rate of Ca transport (arrows) in g/d. $V_i$: Ca intake; $V_e$: non-absorbed Ca in fecal excretion; $\alpha$: part of Ca absorbed from diet; $V_a$: Absorbed Ca from diet; $V_{o-}$: Bone resorption; $V_T$: Total Ca inflow; $V_{o+}$: Bone accretion; $V_f$: Fecal endogeneous losses; $V_u$: Urinary losses, $V_m$: Secretion in milk; $\triangle$: difference between accretion and resroption (g/d)

Even though, P secretion in milk sharply increased at the onset of lactation, clinical signs specific to failure of P homeostasis are anecdotic. However, physiological responses to hypophosphatemia are less known (Klop et al., 2013). It is believed that low phosphatemia per se is barely a specific factor of bone mobilization (Elizondo Salazar et al., 2013), but it

is known that excessive dietary P could aggravate lack of plasma Ca by inhibiting the hydroxylation of 25-OH-vitamin D into 1,25-(OH)$_2$-vitamin D, leading to subclinical hypocalcemia or milk fever (Meschy, 2010). As for Ca, an increase in P absorption could also be an answer to this increase in P requirements (Christakos et al., 2014).

### b  Place of the mammary gland in the regulation of calcemia and phosphatemia

Even though an important amount of Ca is secreted in milk during lactation, several studies found that hormones involved in the homeostasis of non-lactating organisms do not affect milk production or milk Ca content (Horst et al., 1997, Kovacs, 2016), suggesting a missing link in the Ca homeostasis of lactating cows. More specifically, it has been shown that ablation of parathyroid glands in rats did not affect calcemia and phosphatemia directly, suggesting that mammary gland has a function in regulation of calcemia and phosphatemia during lactation (Garner et al., 1990). In the early 90s, the discovery of a specific hormone, the PTH-related protein (**PTHrP**), allows characterizing a specific role of the mammary gland in calcemia regulation. PTHrP was first detected in hypercalcemic cancer (Thiede and Rodan, 1988, Law et al., 1991, Yamamoto et al., 1992, Uemura et al., 1997), because its serum concentration is vely low in healthy non-lactating animal (Sato et al., 2014). In the first days of lactation, blood PTHrP concentration has been shown to increase by a factor 4 in dairy cows (Kocabagli et al., 1995). Even if it is present in blood, PTHrP is five to ten thousand times more concentrated in milk (Thiede, 1994, Uemura et al., 1997, Wojcik et al., 1998). First demonstrated role of PTHrP was to favor the development of the mammary gland during embryonic growth (Dunbar et al., 1999) and during adolescence in human (Hiremath and Wysolmerski, 2014).

PTHrP was named after its sequence homology with PTH (Mundy and Guise, 1999). In bovine, the amino acids sequence 1-34 is almost identical between PTH and PTHrP (Thiede, 1994). The figure I.10 shows the very good homology of sequence between PTHrP and PTH for several species (chicken, human and rat). This figure, as well as the figure I.11, illustrates that PTHrP sequences have also been highly conserved between species (Thiede, 1994, Wojcik et al., 1998), which can be explained by a same ancestor gene (VanHouten et al., 2004). This sequence homology between species is particularly high at the beginning of the amino acids chains. For example, the sequence homology on 1-112 is 90% between

```
              10          20          30          40
cPRP  A V S E H Q L L H D K G K S I Q D L R R R I F L Q N L I E G V N T A E I R A T S
rPRP  * * * * * * * * * * * * * * * * * * * * F * * H H * * A E I H * * * * * * * *
hPRP  * * * * * * * * * * * * * * * * * * * * F * * H H * * A E I H * * * * * * * *
cPTH  S * * * M * * M * N L * E H R H T V E * Q D W * Q M K L Q D * H S * L E D * R T
rPTH  * * * * I * * M * N L * * H L A S V E * M E W * R K K L Q D * H N F V S L G V Q
hPTH  S * * * I * * M * N L * * H L N S M E * V E W * R K K L Q D * H N F V A L G A P
Con   - V S E - Q L - H - - G K - - - - - - R - - - L - - - - - - - H - - - - - - - -

              50          60          70          80
cPRP  E V S P N P K P A T N T K N Y P V R F G S E D E G R Y L T Q E T N K S Q T Y K E
rPRP  * * * * * S * * * P * * * * H * * * * * * D * * * * * * * * * * * V E * * * *
hPRP  * * * * * S * * S P * * * * H * * * * * * D * * * * * * * * * * * V E * * * *
cPTH  Q R P R * K E D I V L G E I R N R * L L P * H L R A A V Q K K S I D L D K A Y M
rPTH  M A A R E G S S Q R P * * K E E N V L V D G N S K S L G E G D K A D V D V L V K
hPTH  L A P R D A G S Q R P R * K E D N V L V E S H * K S L G E A D K A D V N V L T K

              90          100         110         120
cPRP  Q P L K V S G K K K K A K P G K R K E Q E K K K R R A R S A W L N S G M Y G S N
rPRP  * * * * T P * * * * * G * * * * * R * * * * * * * * T * * * * P G T T G S * L L
hPRP  * * * * T P * * * * * G * * * * * * * * * * * * * * T * * * * * D * * V T * * *
cPTH  N V * F K T K P
rPTH  A K S Q
hPTH  A K S Q

              130         140
cPRP  V T E S P V L D N S V T T H N H I L R
rPRP  E D P Q * H T S P T S * S L E P S S * T H
hPRP  L E G D H L S * T * T * S L E L D S * R H
```

Figure I.10: Comparison of PTH and PTHrP amino acids sequences (Thiede, 1994). Comparison of the amino acid sequences of parathyroid hormone-related protein (PTHrP) and parathyroid hormone (PTH) from chicken (c), rat (r), and human (h). The top line (cPRP) represents the amino acid sequence of cPTHrP that was deduced from a cDNA isolated from a 10-d chicken embryo library. This sequence is compared with the corresponding sequences of the rPRP and hPRP PTHrP and the cPTH, rPTH and hPTH. Residues that show identity with the cPTWP are designated by an asterisk. A consensus (Con) sequence for those residues between 1 and 40 that are shared by cPTHrP, rPTHrP and hPTHrP and cPTH, rPTH and hPTH is presented on the bottom line.

human and bovine PTHrP but falls to 67% on 112-141 (Figure I.11, Wojcik et al., 1998).

The sequence homology between PTHrP and PTH makes that PTHrP has similar effect than PTH, i.e. an increase in bone resorption, a decrease in renal tubular reabsorption of Ca and an increase in 1,25-(OH)$_2$-vitamin D formation and Ca absorption in the intestine (Cornish et al., 1997). In particular, it has been shown, in mice, that a depletion of PTHrP gene leads to a conservation of bone mass during lactation (VanHouten et al., 2003). It has also been shown that PTHrP may modify blood flow (Roca-Cusachs et al., 1991), and particularly increase blood flow in mammary gland (Davicco et al., 1993). Due to its large scale of action, PTHrP is classified as cytokine (VanHouten et al., 2004). An important effect of PTHrP is also to modulate Ca and P secretion in milk, which was first shown in goats by Barlet et al. (1992). These authors observed than injecting supraphysiological dose of PTHrP increased Ca and P contents in milk in the following hours, possibly due to high Ca and P release from bone. However, a more integrated view of the part of mammary

```
bovine    AVSEHQLLHDKGKSIQDLRRRFFLHHLIAEIHTAEIRATSEVSPNSKPAP    ( 50)

human     ...............................................S.    ( 50)

rat       .................................................    ( 50)

mouse     .................................................    ( 50)

canine    .................................................    ( 50)

chicken   ....................I..QN..EGVN..............P...T    ( 50)



bovine    NTKNHPVRFGSDDEGKYLTQETNKVETYKEQPLKTPGKKKKSKPGKRKEQ   (100)

human     ...............R............................G........   (100)

rat       ...............R............................G.....R..   (100)

mouse     ...............R............................G.....R..   (100)

canine    ...............R............................G........   (100)

chicken   ....Y......E...R........SQ........VS.....A........   (100)



bovine    EKKKRRTRSAWLTSYVAGTGLEEDYLSDISATS--LELNSRRH          (141)

human     ...........D.G.T.SN..G.H...T.T..--...D....          (141)

rat       ...........--PGTT.S..N..PQPHT.P..TS..PS..T.          (141)

mouse     ...........--PST.AS..L..P.PHT.R..--..PSL.T.          (139)

canine    ...........N.G..ES...G.HPY......--....L...          (141)

chicken   ......A.....N.GMY.SNVT.SPVL.N.V.T--HNHIL.          (139)
```

Figure I.11: Comparison of PTHrP amino acids sequences between species (Wojcik et al., 1998). Comparison of amino acid sequences of mature PTHrP from bovine, human, rat, mouse, canine, and chicken. The top line represents the deduced amino acid sequence of bovine PTHrP derived from a bovine brain cDNA library. Conserved residues in human, rat, mouse, canine, and chicken are designated by a dot.

gland and PTHrP in the homeostasis of lactating mammals was proposed by VanHouten et al. (2004). These authors observed a decrease in milk production and in the milk Ca to protein ratio in mice fed low Ca diets. These authors also demonstrated that both PTHrP production and Ca transport in mammary epithelial cells are regulated by extracellular Ca acting through the CaSR situated on basal surface of mammary epithelial cells. These results have led to a model of the implication of the mammary gland in the regulation of calcemia in lactating animals (Figure I.12).

Recent results also suggest that serotonin (**5HT**), also secreted by the mammary gland, is also involved calcemia regulation (Matsuda et al., 2004, Laporta et al., 2014, Weaver et al., 2016, Hernández-Castellano et al., 2017) but the whole mechanism is less clear. It has been shown that 5HT can affect PTHrP production in lactating animals (Laporta et al., 2015) and

Figure I.12: Role of PTHrP and Ca-sensing receptor (CaSR) within the lactating breast (Kovacs, 2016).The Ca receptor (represented schematically) is expressed by lactating mammary epithelial cells. It monitors the systemic concentration of Ca to control PTHrP synthesis and, thereby, the supply of Ca to the breast. An increase in serum Ca or administration of a calcimimetic inhibits PTHrP expression (A), whereas a decrease in serum Ca or ablation of the Ca receptor from mammary epithelial cells stimulates PTHrP expression (B). The Ca receptor also directly regulates the Ca and fluid composition of milk independent of PTHrP.

that a high 5HT concentration could be associated with a decrease in bone mineral density (Ducy, 2011).

# B) Requirements of Ca and P in dairy cows

Lactation is responsible of high increases in Ca and P exportation in milk, which is an important challenge for Ca and P homeostasis, especially for dairy cows that have been selected for milk production. Thus, to keep healthy and productive dairy cows, a mineral supplementation, relying of the evaluation of the cow's requirement of mineral and evaluation of mineral supply from feeds is preconized.

## 1 Objectives of Ca and P supplementation and consequences of an inadequate supplementation

The first aim of the supplementation of Ca and P is to avoid health problem and suboptimal productive performance of dairy cows. The necessity to specifically supplement

dairy cows in Ca and P has been demonstrated at the beginning of the 20th century (McDowell, 2017). One of the first results justifying this practice was the measurements of the increased daily exportations of Ca during lactation in lactating goats thanks to mineral balances trials (Steenbock and Hart, 1913). This study also demonstrated that a subsequent part of the Ca supplied by the feeds was not absorbed. P deficiency was also identified in sick cattle and sheep craving for bones of dead animals in the South African bush (Suttle, 2010). Osteomalacia in dairy cows due to P deficiency in Norwegian soil was also observed in 1923 (Tuff, 1923). A first real evidence of importance of Ca and P was established in 1936, in a study showing that supplemented bone meal to Jerseys cows increased milk production (+50%) on the whole lactation, with a higher production at the peak of lactation and a better persistence of lactation (Becker et al., 1934, Arnold and Becker, 1936).

Since these pioneered studies, the consequences of low feed supply of Ca and P have been synthetized to several published reviews. As explained earlier, Ca and P stocks are flexible and dairy cows can mobilize up to 25% of their mineral bone mass in case of low dietary supplies of Ca and P (Meschy, 2010). However, symptoms of Ca and P deficiency may appear and they depend on the mineral element considered and the duration of the period of low dietary Ca and/or P supplies (Goff, 2000, Suttle, 2010). Given that calcemia is very finely regulated, notably thanks to bone mobilization, and that bone is an important storage pool for Ca, effects of a chronic dietary Ca deprivation needs long time, i.e. months or even years, to appear (Figure I.13, Suttle, 2010). In that case, the effect of dietary Ca deprivation will be an insufficient bone mineralization (Meschy, 2010). The appearance of clinical symptoms of bone insufficient mineralization will be correlated to the duration of the deprivation (Meschy 2010, Suttle, 2010). On young animals, a chronic dietary Ca deprivation can lead to a failure of bone mineralization and a retarded growth (NRC, 2001). However, when the Ca deprivation is higher and acute, it can happen that the bone cannot be mobilized rapidly enough to allow maintenance of the calcemia and thus the first symptom of Ca deprivation would be a very rapid decrease of calcemia and an associated deterioration of the function of transmission of nerve impulses (Figure I.13, Suttle, 210). An example of these symptoms is milk fever even though it is not related to dietary Ca deprivation at the very beginning of lactation (DeGaris and Lean, 2008). In the first 24 hours after calving, a

cow may lose 23 g of Ca in colostrum whereas the quantity of Ca in the total bloodstream is 3 g (Suttle, 2010). However, it is important to specify that milk fever is above all due to a homeostasis default in postpartum dairy cows and not to a dietary deprivation of Ca. A reduction of Ca supplementation is even proposed as a preventive strategy before calving to favor a quicker bone mobilization after calving by stimulating pre-calving PTH secretion (Goff, 2008).
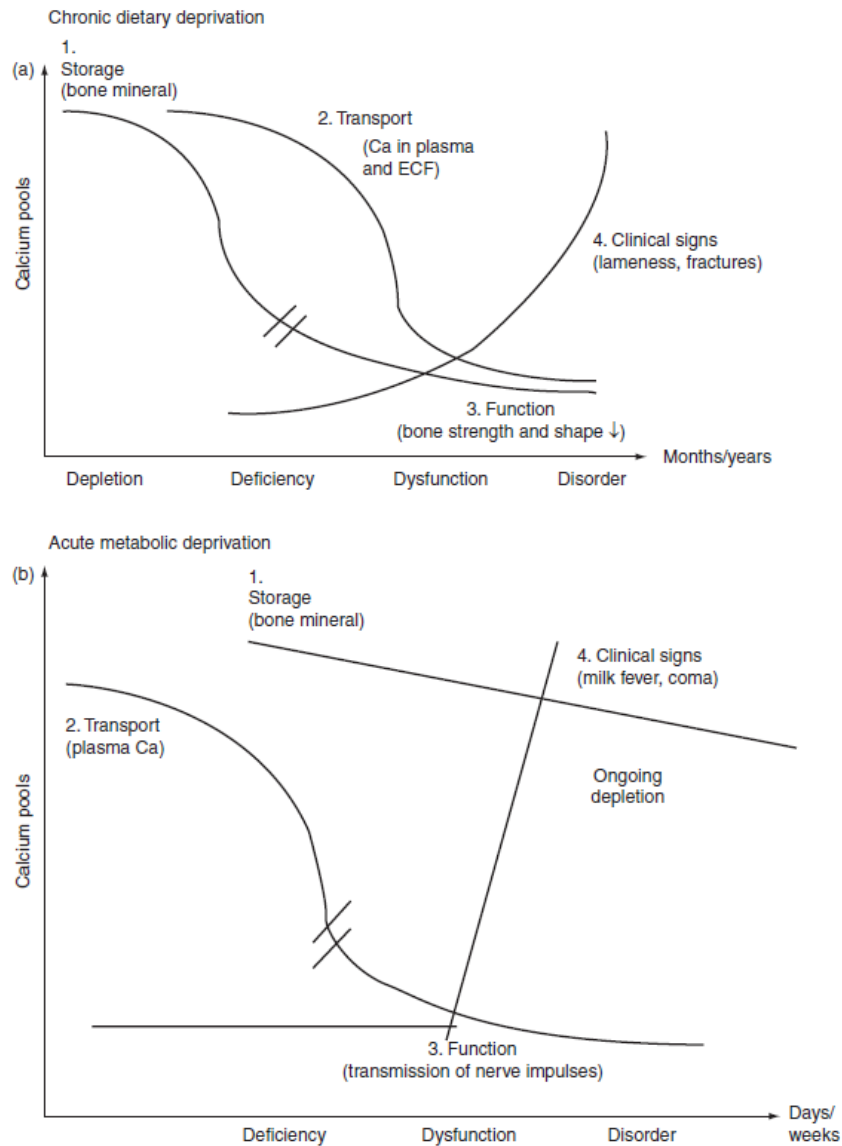


Figure I.13: Sequences of biochemical changes leading to clinical signs in (a) chronic dietary deprivation (i.e. skeletal disorders) and (b) acute metabolic deprivation of Ca, e.g. milk fever (Suttle, 2010)

Given that phosphatemia is more loosely regulated than calcemia, a first consequence of a dietary P deprivation is a decrease in phosphatemia and this decrease occurs very

rapidly even in case of small deprivation (Figure I.14, Suttle, 2010, Anderson et al., 2017, Meschy, 2017). The first symptom of a moderate P deprivation will be a rapid decrease in the amount of dry matter intake (NRC, 2001, INRA, 2010, Meschy, 2010, Suttle, 2010, Puggaard et al., 2014) due to the high dependence of ruminal micro-organism and fiber digestion to P (Meschy 2010, Suttle 2010). According to the importance and the duration of the dietary deprivation, other symptoms such as difficulty to move, growth and milk production decreases could be associated (Meschy, 2010). Pica that can be described as a specific appetence for soil or dead animals is also a sign of dietary P deprivation (Meschy, 2010, Suttle 2010). On a long time, P deprivation may induce insufficient bone mineralization (Bortolussi et al., 1999, Meschy, 2010).
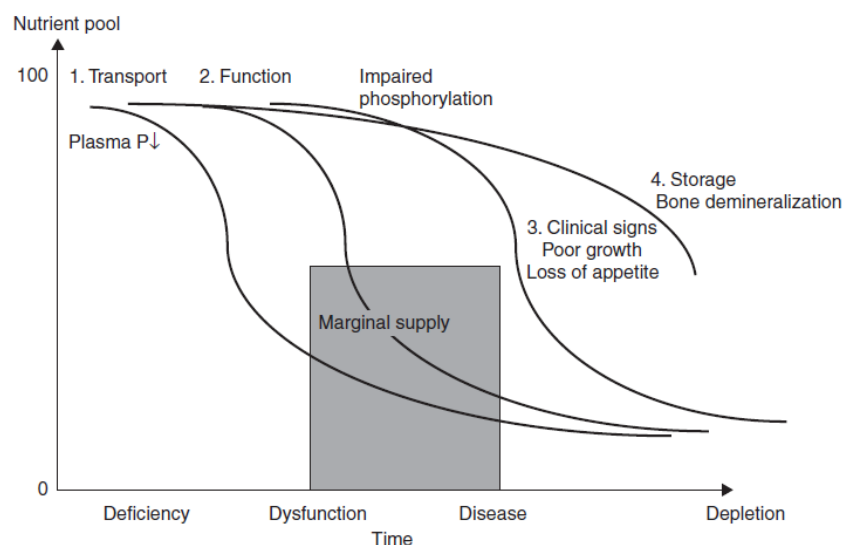


Figure I.14: The sequence of pathophysiological changes that occurs when livestock are given an inadequate dietary P supply. Unlike with most minerals, the transport pool shows an early decline and appetite is lost long before the skeleton becomes clinically affected (Suttle, 2010)

If a Ca and P supplementation can be necessary in dairy cows to avoid health problems and suboptimal productive performances, an excessive supplementation can have deleterious effects, above all from a socio-economic point of view. Indeed, from an animal health point of view, specific pathologies due to excessive dietary supplies of Ca and P are quite uncommon in dairy cows, at least if the very specific period of prepartum is excluded (NRC, 2001). Possible effects of excessive Ca supplies could be a decrease in some trace mineral absorption such as Mn and Zn (INRA, 2018) or the occurrence of urinary stones (NRC, 2001). Ruminants are particularly insensible to P excess thanks to their the capacity of P excretion in saliva and urine (Klop et al., 2014).

There is currently a concern to adjust as much as possible the dietary P supply to the animal requirement 1) because the world P stocks are limited and localized in a very few countries, which represents an economic and political issue (Chen and Graedel, 2016) and 2) because excessive P supplementation leads to an increase of environmental risks of eutrophication (Kebreab et al., 2008).  The question of the management of the world P stocks (Steen, 1998) arose at the end of the 20$^{th}$ century. At this moment, world mineral P stocks were estimated to last from 50 to 120 years (Steen, 1998) but this estimation may not be accurate anymore because global P consumption was multiplied by five in 50 years (Figure I.15, Chen and Graedel, 2016).  It has been estimated that more than 90% of P is used for food production (Gunther, 2005), among which 80% is used as mineral fertilizer and 5% for animal feeding, at least if P used to grow plants to feed animals is not considered. The other issue is that P supplemented in excess to animals is mainly excreted as soluble P from urine and feces and that soluble P has an important potential to increase eutrophication risks of waterways (Dou et al., 2002, Valk et al., 2002, Alvarez-Fuentes et al., 2016).  As a consequence, since the early 2000s, a series of studies was published showing that a slight decrease of the current recommendations of P supply to dairy cows, and thus of P supplementation, is possible without affecting their health and productive capacity (Wu et al., 2000, Odongo et al., 2007, Ferris et al., 2010a, Elizondo Salazar et al., 2013).  However, some of these studies reached a deleterious limit (Puggaard et al., 2014). The P requirements of a lactating dairy cows can be grossly estimated to be around 0.40 g/kg DM (Dry matter) according to the NRC recommendations (2001) that are the most broadly used recommendations in the world.  Some authors suggested that those P requirements could be lowered to 0.35 g/kg DM without affecting performances or health of dairy cows. Wu et al. (2000) observed that total dietary P supply to lactating dairy cows could be lowered from 0.49 to 0.40 g/kg DMI without affecting either milk production, or milk composition or reproduction performance and with a decrease of P fecal losses of about 25%, but they also observed that lowering total dietary P supply to 0.31 g/kg DM can have deleterious effect.  Lopez et al.  (2004) observed no effect of a reduction of daily P supplies from 0.57 to 0.37 g/kg DMI on either milk production or composition. Kebreab et al. (2008) suggested that daily P supplies could be lowered from 0.41 to 0.35 g/kg without impairing cow health or productivity. Ferris et al. (Ferris et al., 2010a, Ferris et al., 2010b)

observed, during a 2-year study, that lowering daily P supply from 0.45 to 0.36 g/kg DM had no effect of milk production and composition and reproduction performance even though body condition score slightly decreased. However, Puggaard et al. (2014) clearly showed that a decrease in daily P supply from 0.34 to 0.23 g/kg DM has very strong and deleterious effects on cow health and productive capacity even though milk production remained possible with 0.28 g/kg DM.
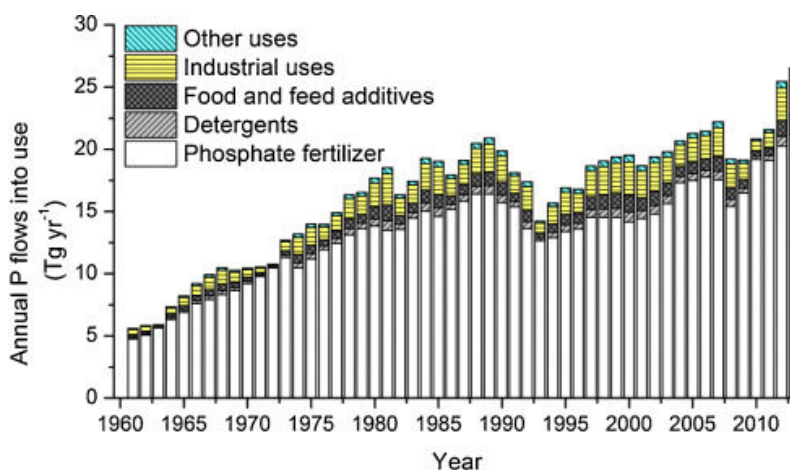


Figure I.15: Evolution of major use of mineral P for the last 50 years (Chen and Graedel, 2016)

No economic and environmental issues around excessive Ca supplementation aroused recent increase in the amount of studies aiming redefining the daily Ca requirement of dairy cows. However, a recent series of studies highlighted that, even in the absence of milk fever, subclinical hypocalcemia after calving can have important effects on cow health and production during the following lactation, with lower reproduction performances (Caixeta et al., 2017), higher risks of acetonemia (Rodríguez et al., 2017) and higher risks of metritis or culling (Wilhelm et al., 2017). Effect of preparation for calving, by decreasing dietary cation-anion difference (**DCAD**, Leno et al., 2017, Neves et al., 2017), or by different strategies of oral Ca supplementation (McArt and Oetzel, 2015) has been investigated to limit subclinical hypocalcemia with contrasted results (Teramura et al., 2015, Amanlou et al., 2016, Miltenburg et al., 2016, Venjakob et al., 2016, Leno et al., 2017, Neves et al., 2017). The link between the risks of subclinical hypocalcemia and the strategies of Ca and P supplementation during previous lactation, that could have affected bone metabolism have not been studied.

## 2 Estimations of Ca and P requirements and supplies, used for optimizing Ca and P supplementation

To ensure that dairy cows received enough Ca and P to cover their requirements for lactation and maintenance, eventually thanks to a specific supplementation, a first step is the estimation of requirements. Requirements are always estimated on a basis of absorbable Ca and P. However, as evocated earlier in this bibliography, a substantial part of dietary Ca and P are not absorbed in the digestive tract. Thus, a second step is the calculation of the absorbable Ca and P supplies from the Ca and P contents of the feed of the diet. Many systems propose estimation of requirements and supplies for Ca and P. AFRC (1991), NRC (2001) and INRA (Meschy, 2010, INRA, 2018) propose estimations of requirements and supplies for both Ca and P whereas the Dutch (Valk et al., 2002) and Danish (Sehested, 2004) systems proposes estimations of requirements and supplies of P. This part of the bibliography will propose a comparison of the AFRC (1991), NRC (2001) and INRA (2010) systems. The NorFor system (Volden, 2011) was discarded from the comparison because it uses NRC estimations of requirements for Ca and P with only minor modifications.

### a Estimations of Ca and P requirements

In the three compared systems (AFRC, 1991, NRC, 2001, Meschy, 2010), estimations of Ca and P requirements are based on the factorial approach that consists, for an adult dairy cow, in summing Ca and P requirements for maintenance, gestation and lactation. Maintenance requirements consist in the minimal daily amount of fecal and urine losses of Ca and P, able to sustain the productive level of the cow. Gestation requirements consist in the daily amount of Ca and P retained in the fetus, whereas lactation requirements consist in the daily amount of Ca and P secreted in milk. Thus, Ca and P requirements are supposed to be the amount of Ca and P to be supplied to replace the daily losses of Ca and P from the organism. The three parts of mineral requirements previously cited, i.e. maintenance, gestation and lactation, are considered as independent. For a growing animal, such as a primiparous dairy cow, growth requirements would also have to be considered. Estimations according to systems are in table I.1. The milk P content used for the estimation requirements of lactation is the same for the 3 systems, i.e. 0.9 g/L whereas the considered milk Ca contents vary according to the system and NRC even considered a different milk

Ca content according to the breed of the cow. It is interesting to notice also, that the NRC is the only system that does not consider the effect of DMI on maintenance requirements of Ca, whereas it considers it for maintenance requirements of P. For none of the 3 systems, the effect of the stage of lactation on requirements is considered and it is assumed that it is described by DMI and milk composition. Ca and P requirements for gestation are negligible before the last three months of gestation in the three systems.

| | Ca requirements | | |
| --- | --- | --- | --- |
| | Maintenance | Lactation | Gestation |
| AFRC | -0.74+0.0079 BW+0.66 DMI | 1.2 MP | $0.0399\ e^{-0.003DG+11.952+13.161\times e^{-0.003DG}}$ |
| INRA | 0.663 DMI + 0.008 BW | 1.25 MP | $\dfrac{23.5}{1+e^{18.8-5.03\times\ln(WG)}}$ |
| NRC | 0.031 BW | $\beta$ MP | $0.02456(e^{(C-H\times DG)DG}-e^{(C-H(DG-1))(DG-1)})$ |
| | P requirements | | |
| AFRC | 1.6×(-0.06+0.693 DMI) | 0.9 MP | $0.433\ e^{-0.003DG + 10.73 -12.750\times e^{-0.003DG}}$ |
| INRA | 0.83 DMI+0.002 BW | 0.9 MP | $\dfrac{7.38}{1+e^{19.1*-5.46\times\ln(WG)}}$ |
| NRC | DMI + 0.002 BW | 0.9 MP | $0.02743(e^{(C-(H\times DG))DG}-e^{(C-H(DG-1))(DG-1)})$ |

Table I.1: Estimation of Ca and P requirements according to AFRC (1991), INRA (2010) and NRC (2001). BW: Body Weight; DMI: Dry Matter Intake; MP: Milk Production; DG: Days of gestation; WG: Week of gestation. $\beta$ value depends on the breed (1.22 g/kg for Holstein, 1.45 g/kg for Jersey and 1.37 g/kg for other breeds). $C$ equals 0.05581 for Ca and 0.05527 for P. $H$ equals 0.00007 for Ca and 0.000075 for P.

### b    Estimations of Ca and P supplies

As cow's Ca and P requirements are estimated on absorbable Ca and P, the 3 compared systems also proposed an estimation of the absorbability of Ca and P from the feeds. The absorbability of Ca and P is defined as part of total Ca and P contents of the feed that can be absorbed throughout digestive tract by the cows. The absorbability is generally approximated by the Real Coefficient of Absorption (**RCA**) that is, for either Ca or P, the ratio between the element intake minus its fecal excretion of the non-absorbed form and the element intake. The fecal excretion of the non-absorbed form is calculated as the total fecal excretion minus the endogenous fecal excretion for the considered element. Thus, the

estimation of the RCA, as well as that of maintenance requirements of Ca and P, requires an estimation of the endogenous part of the fecal excretion of Ca and P. The Apparent Coefficient of Absorption (**ACA**), i.e. the ratio between the element intake minus its total fecal excretion and the element intake, is easier to measure but estimating the absorbability of Ca and P by their ACA would not allow to sum the amounts of Ca and P provided by the feeds that constitutes the diet. Indeed, as we saw previously, endogenous fecal losses are very dependent of the total amount of DMI and for P, of the total amount of P provided by the diet. For Ca particularly, the RCA has to be measured in situation in which the amount of Ca offered to the animal is close or lower to the total animal requirements of Ca.

The three compared systems based the estimation of the absorbability of Ca and P requirements on data basis of experimental measurements of Ca and P flows at the scale of bovine animal. In some cases, the experimental results also include a measurement of endogenous fecal loss thanks to radio-isotopes. The number of coefficients of absorbability to compare the variety of feed that a cow can consume differed greatly between the 3 compared systems. AFRC (1991) uses only one coefficient per element for all feeds, 0.68 for Ca and 0.58 for P. NRC (2001) considers 3 categories of feed, i.e. forages, non-forages and mineral, which coefficients are 0.3, 0.6 and 0.7, respectively for Ca and 0.64, 0.7 and 0.7 respectively for P. INRA considers about 20 categories of feed which contrasted absorbability of Ca and P given in tables I.2 and I.3. The pinciples of the calculation of those RCA for Ca are given in box 1.

| | | RCA of P | | | RCA of Ca |
| --- | --- | --- | --- | --- | --- |
| | | Fresh | Silage | Hay | Fresh or conserved |
| Permanent grasslands | | 0.70 | 0.60 | 0.65 | 0.35 |
| Grasses | Rye grass | 0.60 | 0.60 | 0.65 | 0.40 |
| | Other grasses | 0.70 | 0.60 | 0.65 | 0.40 |
| Cereal forages | Maize | 0.70 | 0.70 | - | 0.40 |
| | Other cereals | 0.66 | 0.66 | - | 0.40 |
| Legumes | Lucerne | 0.70 | 0.65 | 0.60 | 0.30 |
| | Clover | 0.70 | 0.65 | 0.60 | 0.30 |
| | Other legumes | 0.70 | - | - | 0.30 |
| Protein crops | | 0.70 | 0.65 | - | 0.30 |
| Asteraceae (sunflower. ...) | | 0.70 | 0.65 | - | 0.30 |
| Crucicfers | | 0.70 | 0.65 | - | 0.30 |

Table I.2: Real Coefficent of Absorption (RCA) of P and Ca of the forages in the INRA system (INRA, 2018)

**Box 1:** **Estimation of RCA of Ca in the three feeding systems**

**AFRC:** To estimate the RCA of Ca retained in the system, i.e. 0.68, a first database, constituted from data resulting from of studies using radio-isotopes of Ca in the diet, was used to established that RCA and ACA could be linked by a linear relationship,RCA = ACA + 0.016BW. A second database, constituted from data of balance trials (about 600 data for Ca), was used to calculate RCA with the assumption that the amount of absorbed Ca was either equal to the requirement (R) when the intake (I) of absorbable Ca mineral, RCA×I, is sufficient to cover the requirements, or to RCA×I when the intake of absorbable Ca is not sufficient to cover the requirement (ARC, 1980). Within that frame, ACA of Ca and P retained in the system were estimated as the maximum ACA measured from the groups of data with the highest requirement to intake ratios. Then, RCA of Ca was obtained thanks to the relationship evocated above. No differences between feedstuffs are considered in this system.

**NRC:** The selection of the studies used for the determination of the RCA of Ca relied on the principles that the cows needed to be fed under their requirement to activate all the mechanisms of Ca absorption (cf. case where R > RCA×I for AFRC). RCA of Ca for mineral feed were derivate from solubility measurements, considering that the RCA of Ca in $CaCl_2$ is 0.95 (study with six one month old calves from Hansard et al., 1954) and that $CaCl_2$ is 1.2 to 1.32 more soluble than $CaCO_3$ which RCA has been estimated to 0.75 (0.95/1.26). Because it was assumed to be less available than Ca from pure $CaCO_3$, the RCA of Ca from limestone was decreased to 0.70. RCA of Ca for forages was based on estimation of RCA from alfalfa because alfalfa is a major contributor of Ca in dairy rations in US, obtained as an average RCA obtained from 3 studies, i.e. 0.30 (Ward et al., 1972, Hibbs and Conrad, 1983, Martz et al., 1990). RCA of Ca for non-forage feedstuffs was estimated at 0.60 without use of experimental data, which is slightly lower than the RCA of $CaCO_3$.

**INRA:** For the 20 categories of feed (11 for forage, and 9 for concentrates and animal feed), RCA were estimated from data issued from 77 balance trials with lactating cows considering 117 diets and 424 observations (Meschy, 2002, Meschy and Corris, 2005). Trials in which Ca intake represented more than 150% of Ca requirements were discarded to avoid an under-estimation of RCA, except if a specific feedstuff, with natural high Ca content and that is not a mineral feed, was included in the diet. For trials that only measured ACA, RCA was estimated considering that fecal endogenous losses were equal to maintenance requirement (0.008 BW + 0.663 DMI for Ca). Estimation of RCA of Ca of concentrates may not be as good as that of forages because they do not have a high Ca content and they only marginally contributed to the amount of absorbable Ca intake.

**35**

| | | RCA of P | Rca of CA |
|---|---|---|---|
| Cereals | | 0.75* | 0.55* |
| | Wheat | 0.72 | |
| | Barley | 0.76 | |
| Meals | | 0.68* | 0.55* |
| | Groundnut | 0.65 | |
| | Rapeseed | 0.71 | |
| | Cotton | 0.63 | |
| | Linseed | 0.67 | |
| | Soy bean | 0.70 | |
| | Sunflower | 0.65 | |
| Others | Dehydrated lucerne | 0.70 | 0.30 |
| | Rice bran | 0.64 | 0.55 |
| | Brewers' drierd grain | 0.78 | 0.55 |
| | Gluten corn feed | 0.68 | 0.55 |
| | Sugar beet or citrus pulp | 0.90 | 0.20 |
| | Cotton seed | 0.74 | 0.55 |
| Mineral | | 0.70[1] | 0.40[1] |

Table I.3: Real Coefficent of Absorption (RCA) of P and Ca of the main deed materials and minerals in the INRA system (INRA, 2018). * = mean of the category; [1]: may variate according to considered mineral

## c  A focus on the estimation of the fecal endogenous losses of Ca and P

The estimation of the endogenous fecal losses of Ca and of P is important in the feeding system to estimate the maintenance requirements of those elements and their RCA. For years, the best methods to measure endogenous fecal losses of Ca and of P have been based on the use of radio-isotopes that allow the concomitant measurement of endogenous fecal loss and non-absorbed mineral in fecal loss (Hansard et al., 1957, Martz et al., 1990, Vitti and Kebreab, 2010). However, the higher legal requirement in radio-protection makes these methods extremely difficult to use on ruminants nowadays. Alternative consisting in using Ca or P free diets for a limited time has already been used also in the past but this method extremely limits the panel of diets that can be tested (Stein et al., 2006). A slope-ratio method may also be considered to compare feed (Vitti and Kebreab, 2010). By increasing proportions of a considered feed included in the diet, the estimations of the endogenous loss would be obtained by the extrapolation of the regression between fecal losses (response variable) and element intake (explanatory variable) with a null value of element intake. However, this method is not much used for ruminants, as it would require a large number of animals and it would not be relevant for P given the importance of excretion of P in

endogenous fecal losses when P intake increases.

### d Data used for comparisons of feeding systems providing an estimation of Ca and P requirements and supplies

Even though differences between systems for daily estimations of Ca requirements can be estimated as quite low when looking at the equation, the accumulation of these differences during several months of lactation may lead to significant differences of supplementation. To evaluate this, data from an experiment involving multiparous dairy cows during 220 days of lactation have been used to compare the cumulative estimation of requirements of Ca and P according to three systems over several months of lactation. An average cow was estimated from these data. She was fed with two diets given alternatively during the first 70 days of lactation and after this period (Table I.4) and the diets were calculated to cover the Ca and P requirements according to the French system (INRA).

| Stage of lactation | Diet composition (%) | | Mineral composition | |
| --- | --- | --- | --- | --- |
| | 0-70 days | 71-220 days | Ca (g/kg DM) | P (g/kg DM) |
| Corn silage | 70.2 | 72.9 | 2.3 | 1.8 |
| Energetic concentrate | 15.3 | 10.4 | 3.71 | 4.6 |
| Tanned meal | 10.2 | 0.0 | 4.1 | 8.4 |
| Soybean meal | 0.0 | 13.5 | 4.1 | 7.8 |
| Urea | 1.3 | 0.7 | 0.0 | 0.0 |
| Mineral (0-70d) | 3.0 | 0.0 | 200.8 | 44.4 |
| Mineral (71-220d) | 0.0 | 2.5 | 212.7 | 45.6 |

Table I.4: Cows' diet composition and characteristics for the systems comparisons.

The milk production (**MP**), milk protein content, body weight (**BW**) and DMI of the average cow are given in figure I.16. The cow was in early stage of lactation during the experiment (< 220 days), and thus no estimation of gestation requirement was included in the comparisons because the daily gestation requirements for Ca and P were never above 0.5 and 0.6 g/d respectively. As the cow was multiparous, no estimation of growth requirement was included either.

### e Comparisons of Ca and P requirements, supplies and differences between both according to AFRC, INRA and NRC

Estimations of daily absorbable Ca and P requirements over 220 days of lactation are resumed in figure I.17. The differences between the cumulated daily estimations over 220
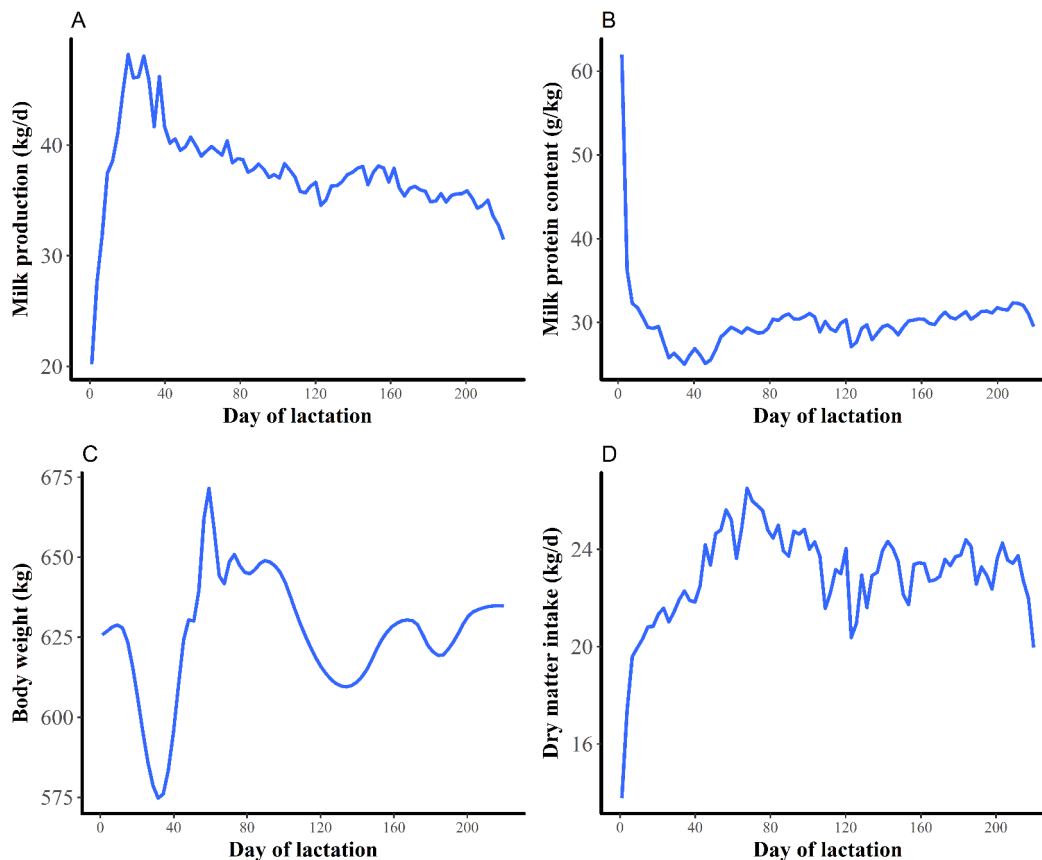
Figure I.16: Zootechnical characteristic of the cow for the comparison of feeding systems on A) milk production, B) milk protein content, C) body weight and D) dry matter intake

days of lactation were extremely low, i.e. less than 0.6 kg for absorbable Ca and 0.8 for absorbable P, for an average cumulated requirement of 14.2 kg of Ca and 12.1 for P over 220 days of lactation. Differences in estimations of absorbable Ca and P requirements used to be more important between systems in the past, varying up to 100% for Ca between NRC (1988) and ARC (1980) (Martz et al., 1990).

Estimated supplies of absorbed Ca and P are presented in figure I.18. Differences between systems, in the estimations of cumulated daily supplies of absorbed Ca and P over 220 days of lactationwere largely higher than those of requirements. With identical DMI, estimation of cumulated daily supplies of absorbed Ca was lower for INRA than for AFRC and NRC. The maximal difference was 8.3 kg over 220 days of lactation for an average cumulated supply of 22.2 kg for the 3 systems (16.7, 24.8 and 25.0 kg for INRA, NRC and AFRC, respectively). Differences in estimation of cumulated daily supplies of absorbed P were not as important as for Ca. Estimations from INRA and NRC were very similar whereas AFRC had lower estimations. The difference in cumulated supplies was less than
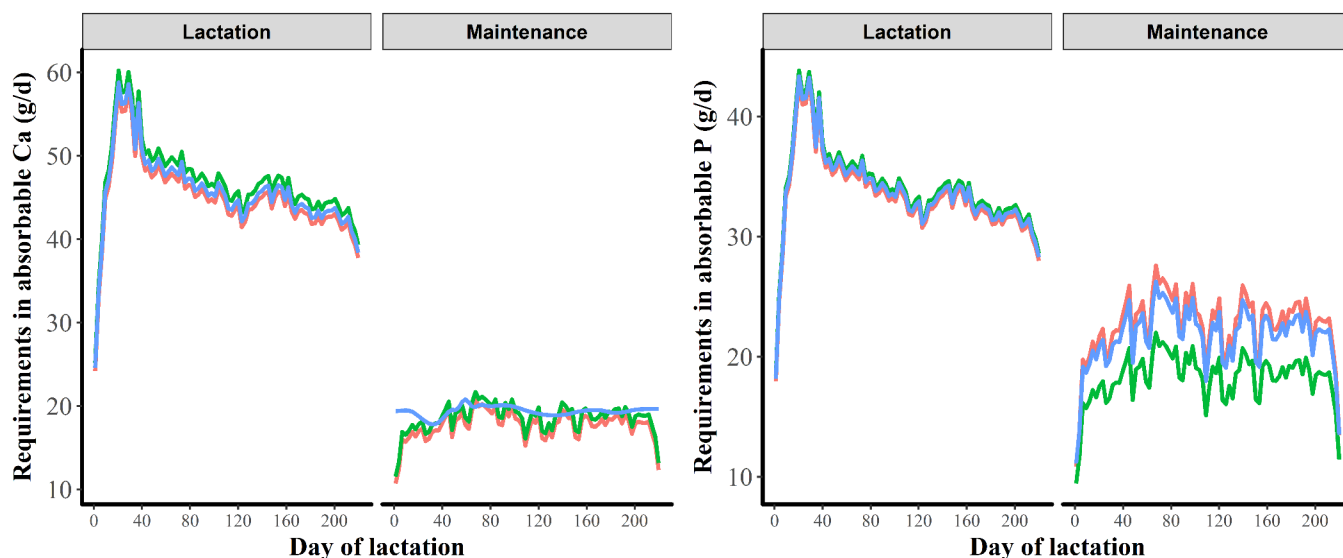
Figure I.17: Comparison of systems for the estimation of absorbable Ca P requirements for a dairy cow during lactation (AFRC, 1991, NRC, 2001, INRA, 2010). AFRC = red, INRA = green, NRC= blue

3 kg between the two extreme systems, for an average of 13.5 kg (14.3, 13.9 and 11.8 kg for INRA, NRC and AFRC, respectively). This result may due to increase in estimation of P absorbability in the literature during the last years (McDowell, 2017), and the fact that AFRC is at least 15 years older than the two other systems.
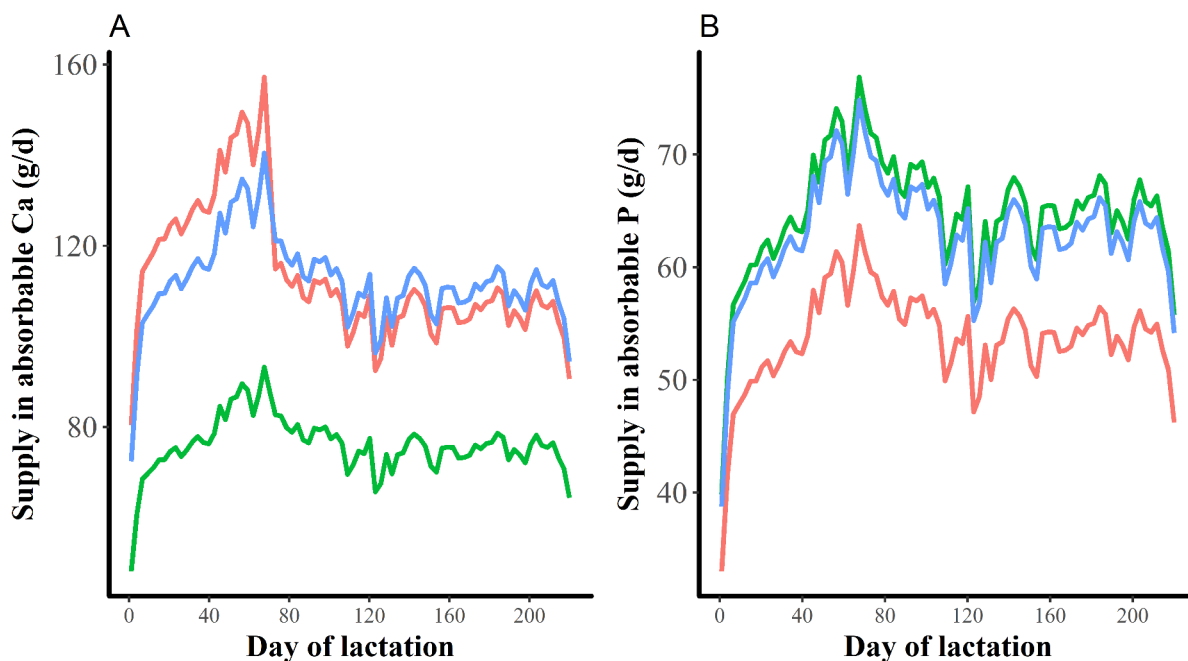


Figure I.18: Comparison of A) absorbed Ca and B) absorbed P according to dietary systems during lactatio AFRC = red, INRA = green, NRC= blue

Consequently, the estimations of daily differences between supplies and requirements of Ca and P (Ca and P balance) were quite different (Figure I.19). Proximity to zero, i.e.

equilibrium between requirements and supplies, is not the relevant question here as the cow's ration was established with the INRA system. The oversupply of P, considering the INRA system, has to be related to variation of the feed content of P during the experiment. Differences in cumulated daily balances over 220 days of lactation were far more important for Ca than for P. The maximal difference for Ca was 8.9 kg, with an average cumulated daily balance of 7.9 kg for 220 days of lactation (2.3, 11.1 and 10.4 kg for INRA, AFRC and NRC, respectively). Those differences were lower for P, with a maximal difference of 2.9 kg for an average cumulated daily balance of 1.1 kg for 220 days of lactation (2.3, 1.6 and -0.6 kg for INRA, NRC and AFRC, respectively). The main difference in estimations of cumulated daily Ca balances between systems was related to the differences of diet absorbability of Ca.
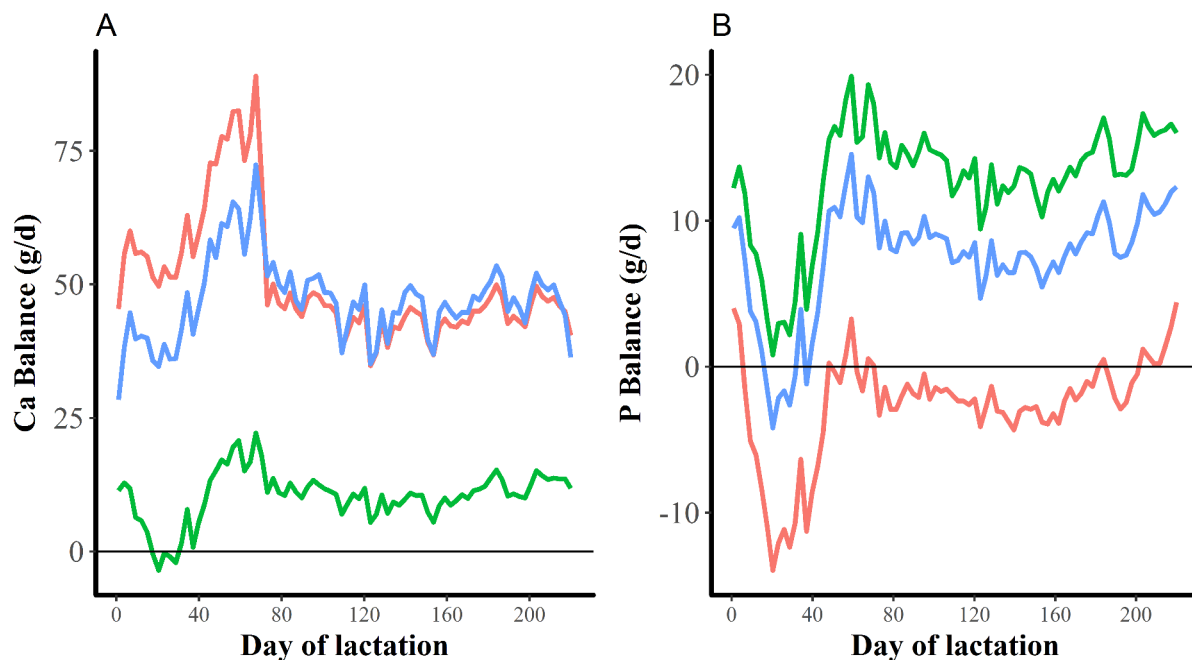


Figure I.19: Comparison of A) Ca balance and B) P balance according to dietary systems during lactation. AFRC = red, INRA = green, NRC= blue

# C) Bone remodeling in relation with regulation of calcemia and phosphatemia and cycle of bone mobilization and reconstitution in dairy cows

As explained earlier in this text, bone can be mobilized in response to insufficient Ca and P supplies or increased requirements and dairy cows are submitted to an important increase of the exportation of Ca, and P in milk, particularly at the beginning of their lactation. Thus, it is important to understand how bone can respond to the fluctuation of supply and requirement of Ca and P by dairy cows during the cycle of lactation-gestation.

## 1 Bone structure and mechanisms of bone remodeling

### a Composition and structure of bones

Bones have several roles in mammals, but the most evident is the protection of organs, with a solid and articulated framework. Even though bone is mainly composed of minerals, it comprises about one third of organic compounds. Rough average composition of bone in mammals is 45% of water, 25% of ashes, 20 % of protein and 10% of fat but it evolves with growing and aging (Bullough, 2010). The mineral content of bone is lower in young mammals (AFRC, 1991) and increases with growing to improve the load bearing capacity of bones as well as its storage capacity of Ca and P (Bonjour et al., 2014). Bone water content decreases while growing (Bonjour et al., 2014). Ca and P are present with a constant mass ratio of 2.15, as they are linked together in mineral bones, more specifically in hydroxyapatite. Ca and P respectively represent around 39 and 17% of bone mineral composition (Keene et al., 2004). Even though hydroxyapatite is highly predominant in bone (Jong, 1926), Ca and P can be bound with others molecules, essentially at the surface of mineral phases of bone, in a hydrated layer (Rey et al., 2009), such as Ca phosphate $Ca_3(PO_4)_2$ but these molecules are very labile and quantitatively less importan,t and their formation is irreversible for some of them (Wu et al., 2001).

Bone matter can be split in two parts, the organic matrix and the mineral phase. The organic matrix gives to the bone its ability to resist to tension (Buckwalter et al., 1996). Initially, bone matter is only constituted of this organic matrix, which is established before

the mineral phase (Buckwalter et al., 1996). This organic matrix is composed of more than 90% of type I collagen, even though others forms such as type IV and V collagens are present. The organic matrix also comprises non-collagenous glycoproteins and bone-specific proteoglycans that assemble collagens together (Bullough, 2010). Mineral phase is accumulated around organic matrix. It allows resistance of bone to compression (Buckwalter et al., 1996) and gives the bone its capacity to store Ca and P. It is mostly composed of hydroxyapatite, but others forms like apatite are present (Buckwalter et al., 1996). The mineral phase of the bone constitutes important Ca and P stocks, with respectively 95 and 70% for Ca and P of the organism being located in bones. For Ca, bone is the only stock in the organism, whereas for P, plasma may also be considered as a stock (Hill et al., 2008).

Even with similar matrix composition, bones can be categorized into two categories, with specific characteristics and functions, i.e. cortical and trabecular bones (McDowell, 2003, Durand and Beaudeux, 2011). Cortical bone is a thin and dense layer of calcified tissue. It is rigid and constitutes tubular bones such as femur or humerus. Trabecular bone is spongy, balancing strength and elasticity. It constitutes the major portion of axial skeleton, i.e. vertebrae and rib inter alia. All bones are not equally sensible to Ca deprivation and for instance, axial skeleton, and particularly the vertebrae between crane and pelvis, is less sensible, to avoid lower protection of organs (Benzie et al., 1955).

### b   Two cell lineages are involved in bone remodeling

Bone mineral phase is always renewed, even in adult animals, due to the activity of two types of cells, the osteoblasts and the osteoclasts. Those two types of cells are permanently active in the cycle of bone remodeling. Osteoblasts are involved in bone accretion that consists in synthesis of the protein extra-cellular matrix, i.e. the organic matrix, and its mineralization. They are derived from mesenchymal stem cells in bone marrow (Durand and Beaudeux, 2011) and lined up along the osteoid marrow, which is the limit of the non-mineralized part of the organic matrix. During the mineralization, they can "brick themselves up" in the mineralized part of the organic matrix, becoming then osteocytes. Osteoclasts are involved in bone resorption that consists in lysis of the mineralized part of the organic matrix of the bone. They are derived from hematopoietic stem cells and contained several nuclei. They are linked to bone matrix by integrins (Durand and

Beaudeux, 2011). In adult organism without deficiency of Ca or P, there is an equilibrium between bone accretion and resorption (Horst, 1986), but accretion is more intense in young growing organisms and resorption is more intense in aging organisms leading to pathology such as osteoporosis.

### c The cycle of bone remodeling

Activity linked to osteoblasts and osteoclasts can be resumed in a cycle decomposed in six steps (Bonjour et al., 2014), that is resumed in figure I.20. During the first step, called quiescence, the surface of the mineralized bone matrix is covered with osteoblasts in terminal phase of differentiation. During the second step, called activation, the osteoclastic precursors are differentiated from the hematopoietic stem cells into mature osteoclasts. During the third step, called resorption, strictly speaking, osteoclasts start lysing the mineralized part of the organic matrix, bone lacunae appears and osteoclasts disappear. During the fourth step, called reversal, osteoblast precursors colonize the lacunae, proliferate and differentiate. During the fifth step, called formation, proteins of the organic matrix, and specially type I collagen, are synthetized by the osteoblasts. During the sixth step, called mineralization strictly speaking, the organic protein matrix is mineralized by osteoblasts, using Ca and P to form hydroxyapatite. These cycles occurs simultaneously in the structure of all bones, whatever the age or the activity of the animal.

The activity of osteoblasts and osteoclasts can be modulated by several hormones or other molecules, allowing the integration of the bone in the regulation of calcemia and phosphatemia as evocated in part A of this chapter. Specifically, it is known that osteoblasts have receptor for both PTH and $1,25\text{-}(OH)_2$-vitamin D (Vitti and Kebreab, 2010), modulating the activity of an alkaline phosphatase and Ca transport. The activity of osteoclasts is also inhibited by calcitonin (Mundy and Guise, 1999). Recently, a system of interactions between energy and Ca metabolisms, relying in relationship between activities osteoblasts, beta cells of the pancreas and adipocytes have been highlighted in mice (Figure I.21, Lean et al., 2014). This system would induce concomitant decrease in energy storage in adipose tissue and Ca storage in bone. Link between P and energy metabolism is also possible but it does not involve bone. It has been shown that 25-OH-vitamin D depletion leads to a secretion of insulin, through $1,25\text{-}(OH)_2$-vitamin D receptor in beta cells of pancreas (Bouillon et al., 1995, Henry, 2011) and that insulin secretion could stimulate P uptake in
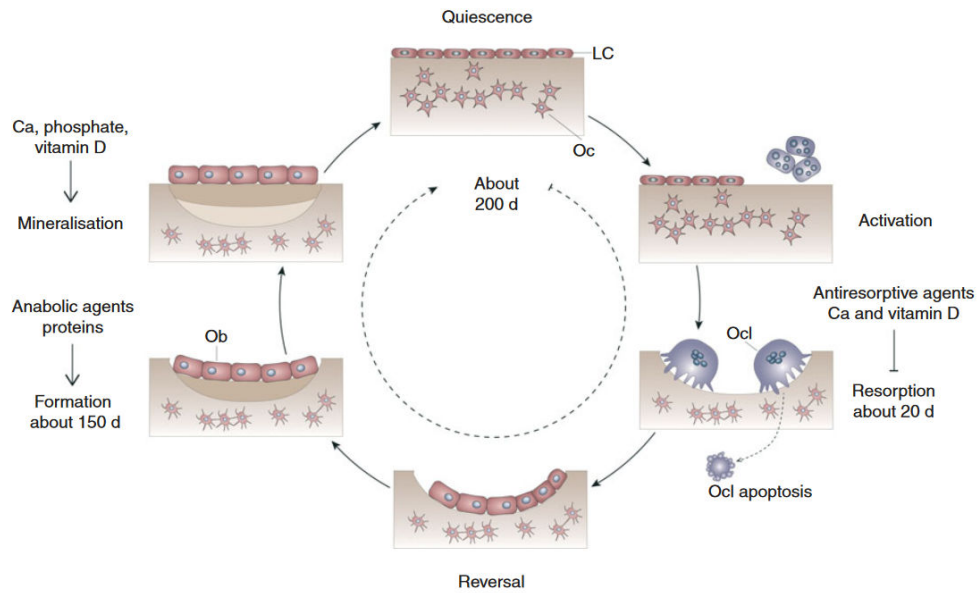
Figure I.20: Bone remodeling cycle. Bone turnover follows a sequence of events that includes activation, recruitment of osteoclasts (Ocl) to begin resorption, degradation and removal of bone, reversal, and formation of new bone by osteoblasts (Ob). After this phase a quiescent or resting period occurs. LC, lining cell; Oc, osteocyte (Bonjour et al., 2014)

mammary epithelial cells to increase milk P content (Rillema, 2002).

## 2 Methods for the evaluation of dynamics of bone mobilization and reconstitution during cycles of lactation and gestation in dairy cows

Bones are submitted to permanent remodeling processes but accretion and resorption are not always in a perfect balance which can induce either net bone mobilization or reconstitution, which may be especially the case during the lactation-gestation cycle of dairy cows. Several methods have been used to estimate the dynamics of bone mobilization and reconstitution that can occur after various events in animals but the methods often determine the kind of information that will be provided. Thus a good strategy could be to combine several methods (Ekelund et al., 2006, Taylor et al., 2009).

### a    Use of Ca and P radio-isotopes

The use of radioactive isotopes of Ca and P, i.e. $^{45}$Ca and $^{32}$P, allowed quantifying daily Ca and P flows flows within body and more specifically between plasma/serum, bones, digestive tract, kidneys and mammary gland. The method consists in incorporating radio-isotopes in cow's diet, or injecting them directly into blood, and following the
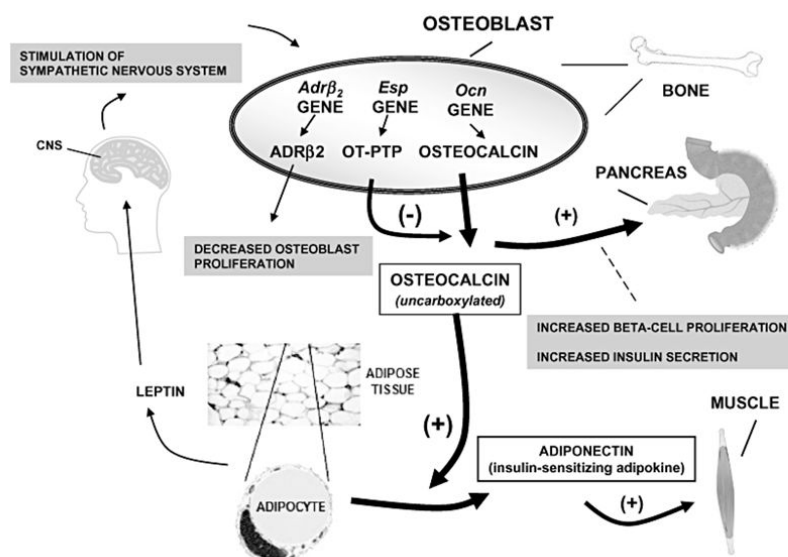
Figure I.21: Relation between Ca and energy metabolisms (Wolf, 2008). Adipocytes secrete the adipokine leptin that influences bone metabolism. Leptin binds to leptin receptors in the brain. The leptin signal causes stimulation of the sympathetic nervous system and activation of the $\beta$2-adrenergic receptor gene (Adr$\beta$2) in bone, which decreases osteoblast proliferation and bone formation. The osteoblast, in turn, influences energy metabolism by expressing osteotesticular protein tyrosine phosphatase (OT-PTP), a product of the Esp gene. OT-PTP apparently influences the vitamin K-dependent $\gamma$-carboxylation of osteocalcin, an osteoblast-specific protein that acts in a hormone-like manner to affect adipocytes and $\beta$ cells in the pancreas. Uncarboxylated osteocalcin increases $\beta$ cell proliferation and insulin secretion in the pancreas, and further influences energy metabolism by affecting adipocyte secretion of adiponectin, an insulin-sensitizing adipokine.

evolution of the radioactivity in several kinds of samples from animal (blood, urine, feces, milk for instance). In early lactating ewes, this method highlighted the importance of bone mobilization in late pregnancy or early lactation and the necessity of a bone reconstitution during the end of lactation and the beginning of gestation (Braithwaite, 1983a). This method allows a very specific estimation of equilibrium between bone accretion and bone resorption via estimation of mineral flows between bones and serum/plasma. However, due to a better knowledge of environmental risks and more severe radioprotection regulation, this method became very difficult to apply even on small ruminants and last results obtained by these methods on dairy cows date from 1970 (Ramberg et al., 1970).

### b    Measurement of input-output balance of Ca and P

The principle of this method is very simpler as it consists in quantifying all the input of Ca and P, i.e. mainly dietary intake and eventually Ca and P ingested with free water, and all the output, i.e. excretion of Ca and P in milk, urine and feces. The difference between daily input and output can be defined as the retention and, as Ca and P are mainly stored

in bones, it can constitute an indirect measurement of Ca and P mobilized from bones or Ca and P retained in bone (Ender et al., 1971). However, this method may be less precise for P than for Ca given that blood also has a role of P storage in the organism (Ekelund et al., 2006). These measurements also allow the estimation of ACA of a diet. A major limit remains that daily total collection of urine and feces is time consuming and impossible to apply on an important number of animals in the case of dairy cows.

### c   Bone biopsy, DXA or X-ray photometry measurements

Those methods, based on direct measurements on bones, allow the quantification of the bone status of the animal that can be considered as its density, its mineral content or more specifically its histological evaluation. Direct measurements of bone composition have been performed on slaughtered animal and more specifically on cows (Benzie et al., 1955, Beighle, 1999, Taylor et al., 2009). They allowed comparison of bones and their capacity to be mobilized (Benzie et al., 1955) but they do not allow the dynamics measurement of bone evolution for a given animal. Bone biopsy, and more specifically rib biopsy have been used in cows (Dixon et al., 2017). However, for obvious reason of animal health and welfare, bone biopsies cannot be repeated with a high frequency. The estimation of bone density could be an interesting alternative to increase the frequency of measurements for evaluation of the bone status. A method to estimate bone density was developed on metacarpe of alive horse using dual-energy x-ray absorptiometry (**DXA**) with a limited number of animals (Donabedian et al., 2005). However, DXA remained hard to applicate in dairy cows because of its cost and the maximal animal weight allowed by the apparatus. This method needs to be improve,d as it was highly operator dependent and only applicable to trained animals, but it gave a good relationship between bone mineral content estimated by DXA and direct measure. However, other studies showed no relation between bone mineral content and DXA or radiographic photometry in dairy cows (Keene et al., 2004).

### d   Blood biomarkers of bone accretion and resorption

Activity of osteoblasts and osteoclasts can be estimated with the serum/plasma concentration of some molecules, called biomarkers of bone accretion and resorption (Liesegang et al., 2000, Seibel, 2000). Four biomarkers of accretion are more currently used to estimate bone accretion. The bone specific alkaline phosphatase (**BALP**) is a membrane-bound protein specific to the bone, but non-bone-specific isoforms exist (Allen,

2003). The osteocalcin (**OC**), also known as serum bone-Gla protein is synthetized in osteoblast and megakaryocytes, which are the cells of bone marrow producing blood thrombocytes. Serum/plasma OC concentration reflects osteoblast activity (Allen, 2003) even though its role is still not yet really well understood. OC is suspected to have a role in synthesis of organic matrix of bone (Buckwalter et al., 1996). The PICP and the PINP, which are the carboxy- and amino-terminal of propeptides of collagen types I, are synthetized by osteoblasts in a late stage of the formation of a new bone (Figure I.22). Their concentrations reflect synthesis of collagen of type I but their use remain uncertain in dairy cows (Allen, 2003).
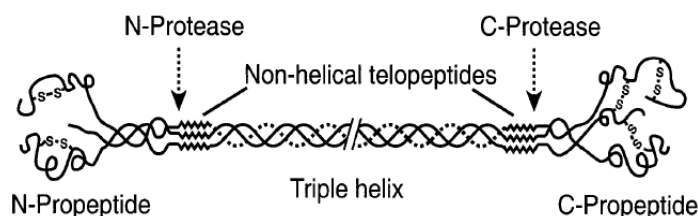


Figure I.22: Molecular basisof PICP and PINP (Seibel, 2000). The carboxy- (PICP) and amino-terminal propeptides (PINP) are cleaved by specific propetidases are partly released into the circulation

Four bone biomarkers of resorption have been described in the literature and most of them are products of degradation of the organic matrix of bone by osteoclasts (Figure I.23). CTX and NTX are carboxy- and amino-terminal cross-linking telopeptides of collagen type I and are issued from the destruction of the collagen type I (Seibel, 2000). Pyridinoline (**PYD**) and deoxypuridinoline (**DPD**) are residues of molecules binding collagen type I together. PYD is more related to amino-terminal and DPD to carboxy-terminal collagen (Allen, 2003). All the cited biomarkers, for accretion and resorption, can be analyzed in serum and sometimes in plasma but only 2 biomarkers of resorption, PYD and DPD, can be analyzed in urine with a correction by creatinine excretion (Seibel, 2000).

The serum concentrations of biomarkers allow measuring relative dynamics of bone accretion and resorption over time but they hardly allow a quantification of the flows. It can even be stated, that because the basal concentrations of these biomarkers vary between individuals, they must be used for dynamics measurements, especially in lactating animals (Liesegang et al., 2000). A limit in the use of these bone biomarkers, beside the cost, is the factors affecting their concentration independently of the bone accretion and resorption
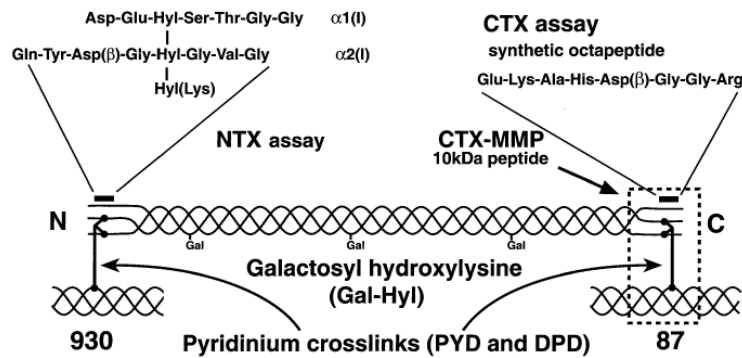
Figure I.23: Molecular basis of the used markers of collagen degradation (Seibel, 2000)

dynamics. Effects of physical activity and diurnal variations have been quantified in human (Hannon and Eastell, 2000). Effect of sex and ethnicity have been highlighted in humans (Allen, 2003). To avoid these biases, plasma has to be sampled with a strict protocol, at a given time of day notably. Some biomarkers, DPD and PYD, were inadequate to detect variation of bone mobilization at the beginning of lactation (Liesegang et al., 2000). Samples must be stored frozen at -20°C or they may degrade. This is particularly true for OC than is recommended to be stored at -80°C. OC, associated with CTX, are the biomarkers the most commonly used in dairy cows to estimate bone accretion and resorption dynamics (Liesegang et al., 2000).

## 3   Quantification of bone mobilization and reconstitution during cycles of lactation and gestation in dairy cows

It has been clearly established that a net bone mobilization occurs at the beginning of lactation of cows (or ewes or goats) and that a net bone reconstitution occurs at the end of the lactation or during gestation. This was illustrated thanks to several methods evocated before, like radio-isotope (Ramberg et al., 1970, Braithwaite, 1983a), bone biomarkers (Liesegang et al., 2000, Ekelund et al., 2006), mineral balance (Taylor et al., 2009) or bone biopsy (Beighle, 1999, Keene et al., 2004). The coexistence of both net bone mobilization at the beginning of lactation, and net bone reconstitution at the end of lactation, has been firstly highlighted with radio-isotopes of Ca and P in dairy cows (Ramberg et al., 1970) and ewes (Braithwaite, 1983a, Braithwaite, 1983b). In these experiments, the model used to adjust the decrease in radioactivity in the followed pools allowed estimated both flows of bone accretion and resorption, and thus net bone mobilization or reconstitution resulting

from the differences between accretion and resorption. In the study of Ramberg et al. (1970), a net mobilization was observed at one month of lactation and a net reconstitution was observed in non-lactating cow. Braithwaite (1983) illustrated the dynamics of bone mobilization and reconstitution during the whole cycle of lactation and gestation in ewes. The application of blood biomarkers of bone accretion and resorption to ruminants in the 90s allowed several publications indirectly quantifying the shape of the dynamics of bone accretion and resorption during lactation and gestation in lactating ruminantss and more specifically cows, in a more continuous way. Relative dynamics of bone biomarkers of accretion and resorption during lactation are quite different. Most studies observed, with Holstein cows, a very transient decrease of plasma/serum concentrations of biomarker of bone accretion, mostly osteocalcin, during the first and sometimes the second month of lactation and a more or less steady plateau after (Liesegang et al., 2000, Iwama et al., 2004, Liesegang et al., 2007, Taylor et al., 2009, Sato et al., 2011). Only, Holtenius et al. (2005) and Ekelund et al. (2006) observed an important decrease after calving and a slow increase all over lactation, which can be attributed to either a different breed, i.e. Swedish Red and White, or maybe a different kit for OC analysis. At the contrary, plasma/serum concentrations of biomarker of bone resorption (CTX) increased more or less sharply at the beginning of the lactation, with a significant variability between studies, but always decreased slowly and regularly after few months of lactation until next caving (Liesegang et al., 2000, Iwama et al., 2004, Holtenius and Ekelund, 2005, Ekelund et al., 2006, Taylor et al., 2009, Puggaard et al., 2014).

Even though blood biomarkers allow quantification of the shape of the dynamics of bone accretion and resorption, they do not allow the quantification of the net flows of bone mobilization and reconstitution. Taylor et al. (2009), measured with repeated measurement of Ca input-output balance in cows that both daily flows of net mobilization and reconstitution could represent between 10 and 30% of the daily flow of Ca intake in cows producing 30 kg milk/d on average over 20 weeks of lactation. In the study of Taylor et al. (2009), both input-output measurement during lactation and bone biopsies (ribs) confirmed the occurrence of a net bone mobilization at the beginning of lactation, until 5 weeks of lactation with adapted dietary Ca supply, and a net reconstitution after. Direct quantifications of bone mobilization and reconstitution are difficult to establish from

studies consisting in analyzing bones. A first reason is that there is big variation in the bone composition and its evolution during lactation according to the considered bone (Beighle et al., 1993). A second reason is that Ca and P contents are often not very variable when expressed in proportion of mineral mater because bone is constituted mainly of hydroxyapatite (Jong, 1926). Despite this, a small decrease of ash Ca and P contents could be observed during the first three months of lactation in cows (Beighle, 1999, Keene et al., 2004). Ca and P densities in fresh bone, expressed in $mg/cm^3$, are more variable but more barely reported (Little, 1972). Beighle et al. (1999) observed that cortical bone Ca content (ribs biopsy) could decrease of 13% between 0 and 2 months of lactation.

A variability of either the dynamics of bone accretion and resorption during lactation or the bone status of the cows has been described according to the parity or the age of the cows and their milk production. A concomitant increase in plasma/serum concentrations of bone biomarkers of accretion and resorption has been observed in younger cows at the beginning of their lactation (Iwama et al., 2004, Kurosaki et al., 2007, Taylor et al., 2008, Sato et al., 2011, Sato et al., 2014), indicating a higher bone remodeling in those cows. However, no data reported differences of the amplitude of bone mobilization at the beginnig of lactation with age and parity. It has been shown also that the bone P content (Taylor et al., 2009), or bone Ca content (Keene et al., 2004) can also be lower in primiparous than in second-lactation cows, the effect depending on the considered bone. These results are coherent with the fact that younger cows are still growing and that the mineral phase of their bone is not totally set up (Bonjour et al., 2014). When cows get older, the results of Keene et al. (2004) illustrated that bone Ca content (caudal vertebra) decreased with parity after the 2nd lactation. It has also been observed that the amplitude of variation of serum concentration of biomarkers of bone resorption (CTX) is higher in cows producing high quantity of milk (Liesegang et al., 2000), which could means that those cow have higher amplitude of bone mobilization during lactation.

Low dietary Ca and P content can induce higher bone resorption and likely more bone mobilization. It has been observed that decreasing dietary Ca content, in a range of variation between 1.0 and 0.5% DM of Ca, increased the blood concentration of biomarker of bone resorption without effecting those of bone accretion (Moreira et al., 2009) and decreased the body Ca retention (or increased the Ca mobilization, input - output balance

measurement, Taylor et al., 2009). If the decreased dietary Ca content is applied during the whole lactation, Taylor et al. (2009) observed that the Ca body retention remained negative for a longer time at the beginning of lactation, this time varying between 2 weeks and 3 months. The effects of decreasing dietary P content on bone mobilization has been far more studied than those of decreasing dietary Ca content (Wu et al., 2001, Ekelund et al., 2006, Moreira et al., 2009, Puggaard et al., 2011, Elizondo Salazar et al., 2013). In lactating dairy cows, the bone mobilization dynamics seems to be affected when dietary P content decreased below 0.4% DM. With dietary P content between 0.4 and 0.3% DM, it has been observed a decrease in both P body retention (Wu et al., 2000) and bone contents of either P or mineral matter (Wu et al., 2001). Similarly to Taylor et al. (2009) with a dietary Ca restriction, Wu et al. (2000) observed that, when a decreased dietary P content is applied during the whole lactation, (0.4 vs. 0.3% DM), the P body retention remained negative for a longer time at the beginning of lactation, this time varying between 2 weeks for a dietary P content of 0.4% DM and 8 weeks for a dietary P content of 0.3% DM. Few studies highlighted an effect of the dietary P content of serum/plasma biomarker of bone resorption or accretion if dietary P content remained above 0.3% DM. Only Puggaard et al. (2014) reported an increase in blood biomarker of bone resorption (CTX) with decreasing P dietary content but the P dietary content was decreased to very low values in this study (0.23% DM). An effect of the DCAD was shown on OC concentration in middle lactation cows, with a decrease of OC with lower DCAD (Boudon et al., 2016), but similar treatment did not result into differences in early lactation (Liesegang et al., 2007).

# D)    Milk Ca and P Contents

## 1    Organisation of the mammary epithelial cells and secretion of Ca and P into milk

Milk production induces huge Ca and P flows into the mammary gland. To avoid toxicity, the cytosol concentration of Ca must not exceed $10^{-5}$M (Horst et al., 1997). Ca is also far more concentrated in milk than in cells or plasma. Consequently, the mammary epithelial cells (**MEC**) insure the function of transfer of Ca from blood to milk with strong constraints which are a strong contrary gradient of concentration and the necessity to keep cytosolic Ca concentration under the toxicity threshold. MEC are less sensible to P excess as they face important quantity of Pi for the energetic metabolism.

The way that Ca enters the MEC is actually not totally understood, even though recent studies increased actual knowledge of this phenomenon. Two channels, TRPV5 and TRPV6 (TRPV for Transient Receptor Potential Voltage) are suspected to be involved in entrance of Ca into MEC (Lee et al., 2006, VanHouten and Wysolmerski, 2007). Ca cannot be transported from basal to apical face of the MEC in its ionic form, because it would exceed toxicity threshold of Ca concentration. Thus, Ca is either transported across the MEC according to two distinct ways, via the Golgi apparatus and the endoplasmic epithelium which was the first way of Ca transport in the MEC that have been described (Horst et al., 1997), or associated to a Ca-binding protein, which has been more recently described (Reinhardt et al., 2004). These ways of Ca transport across the MEC are resumed in figure I.24. Considering the way of Ca transport via the Golgi apparatus and the endoplasmic epithelium, Golgi cisterna has a higher Ca concentration, above 200 M, than cytosol (Lee et al., 2006) and thus Ca is pumped inside by active transporters, SPCA1 and SPCA2 (Secretory Pathway Ca$^{2+}$ ATPase, VanHouten et Wysolmerski, 2007). In the Golgi, Ca starts to bind with other milk components, notably caseins and citrate. In the endoplasmic reticulum, Ca is pumped by Ca-ATPase, like in Golgi apparatus, except that the pump is SERCA2 (Sarco Endoplasmic reticulum Ca$^{2+}$ ATPase). Ca crosses the cell through the endoplasmic reticulum. At the basal face of the MEC, Ca returns to cytosol by ITPR (Inositol 1,4,5-triphosphate receptors) (Neville, 2005). This way of Ca secretion was discovered, suggesting that all Ca transport was totally concomitant with protein

transport (Neville and Peaker, 1979), and leading to the idea that Ca content is determined by protein content (Alais, 1984). The discover of the way of Ca transport by Ca-binding protein questionned this idea. In that case, the considered binding proteins are calbindins-$D_{9K}$ and $D_{28K}$. Non-bonded calbindins to Ca are supposed to increase Ca entry into MEC by TRPV6 (VanHouten and Wysolmerski, 2007). In the continuity with the Golgi apparatus, Ca is secreted into the lumen via an exocytosis (Neville, 2005). The second way of secretion into the lumen via a Ca-ATPase pump, the PMCA2bw (Plasma Membrane $Ca^{2+}$-ATPase) was later described (Reinhardt et al., 2004). About 60% of Ca is secreted by PMCA2bw. Once Ca is secreted into milk, equilibrium between Ca and other milk components, caseins in particular, sets up to determine the part of Ca that will be soluble or colloidal, i.e. associated to casein (Malacarne et al., 2014).
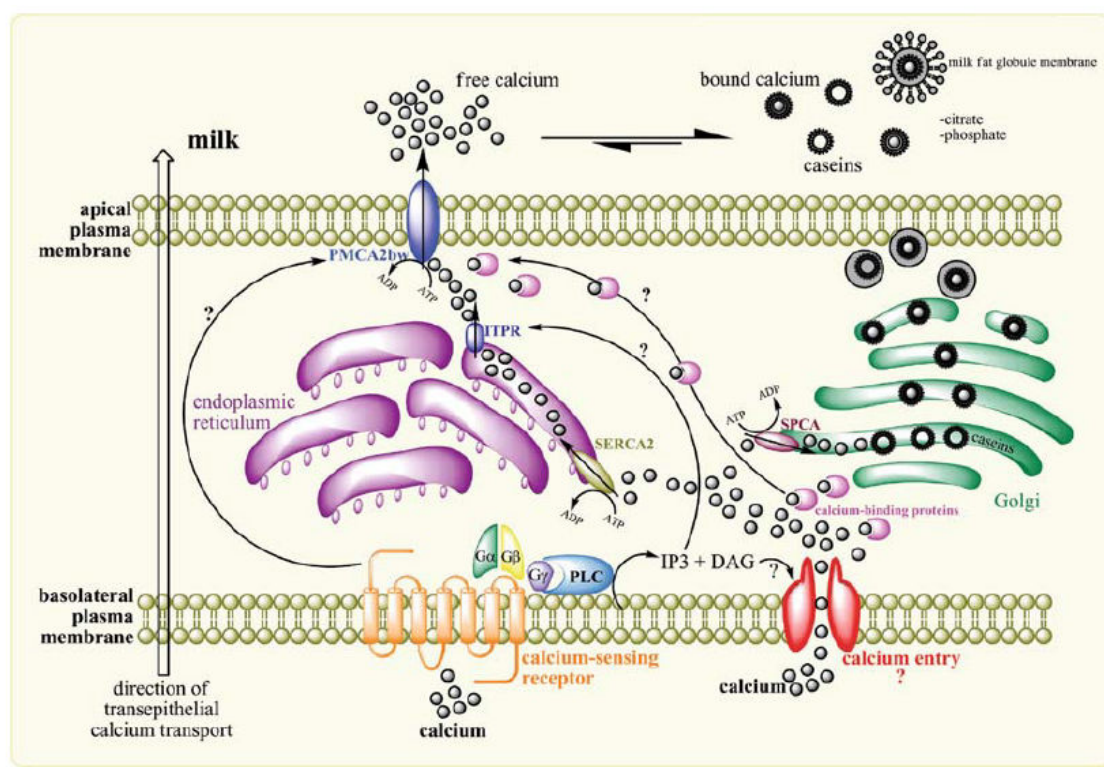


Figure I.24: Ways of Ca secretion into milk by mammary epithelial cells as proposed by VanHouten et al. (2007). After entering the MEC, Ca can be transported through MEC by Ca-binding protein in the cytosol or by entering Golgi apparatus and exocytosis into milk. ITPR: Inositol 1,4,5-triphosphate receptors; PMCA: Plasma Membrane $Ca^{2+}$-ATPase; SERCA: Sarco Endoplasmic reticulum $Ca^{2+}$ ATPase; SPCA: Secretory Pathway $Ca^{2+}$ ATPase

Way of P secretion in MEC has been less investigated than Ca secretion, even though the first description of the way of secretion for these both elements were concomitant (Neville and Peaker, 1979, Shennan and Peaker, 2000). The ways of P transport across the MEC are

resumed in figure I.25.  P can be excreted as Pi or as P integrated in other milk components. For P present in milk components, the way of secretion depends on the component and its specific way of secretion in milk.  For Pi secretion, only one way was described, via exocytosis, but it is very likely that it is the only one because apical MEC membrane has been showed to be impermeable to Pi (Neville and Peaker, 1979).  Pi enters MEC on the basal face with a Na$^+$ co-transporter (Shillingford et al., 1996).  Pi is then incorporated in cell metabolism. It can be included in some cytosolic proteins that will be secreted in milk, or can be bonded in ATP and entered Golgi apparatus.  ATP is hydrolyzed during the formation of lactose and part of resulting Pi remains in Golgi vesicles where it can either remain as Pi or be integrated in proteins like caseins.  In both case, P is then secreted via exocytosis in lumen (Shennan and Peaker, 2000).
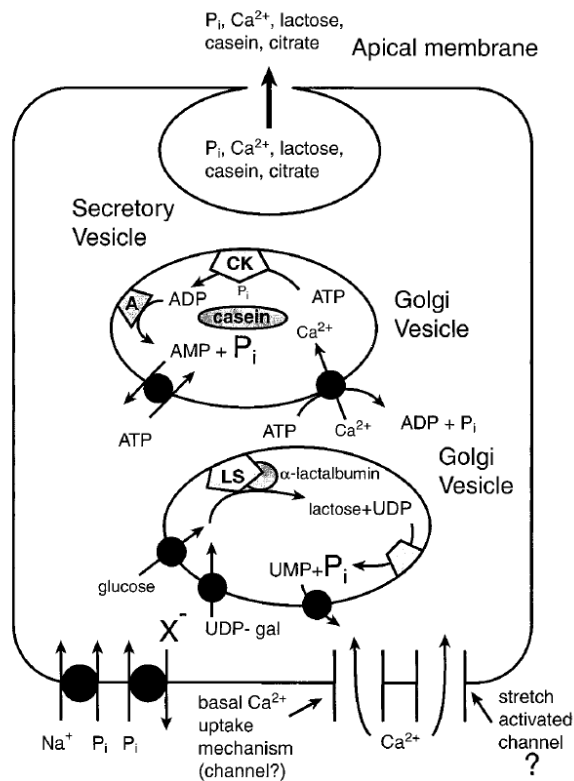


Figure I.25: Pi secretion into milk by mammary epithelial cells as proposed by Shennan and Peaker (2000)

## 2   Forms of Ca and P present in milk

Milk Ca and P contents are quite important in dairy cattle compared to other mammal species (Table I.5). They are far more important in dairy cow's milk than in woman's milk. These important differences are at the origin of the recommendation of the use of dairy

products in westernized diets in human to insure sufficient supply of Ca (Ross et al., 2011). Even though cow's milk Ca and P contents were said to be constant (Alais, 1984), it has now been demonstrated that some variations exist (Kaufmann and Hagemeister, 1987, Kume et al., 1998, Poulsen et al., 2015, Toffanin et al., 2015b, Alvarez-Fuentes et al., 2016). Milk Ca and P contents have been increasing for the last 50 years (Bijl et al., 2013). Ca and P are present in milk in two forms, soluble or colloidal (Neville et al., 1994, Bijl et al., 2013). Around 30-40% of milk Ca is present in the soluble form, and the remainder being bound to organic molecules in the colloidal form (Kaufmann and Hagemeister, 1987, Flynn and Cashman, 1997). Soluble Ca can be either ionic $Ca^{2+}$ or Ca complexed with other non-organic element like citrate or phosphate (Figure I.26, Neves et al., 2017). Milk content of soluble Ca has been shown to be correlated to milk content of soluble citrate (Holt and Muir, 1979). The distribution of P is significantly different with about 45% of soluble P, mainly inorganic, 30% of inorganic colloidal P and 25% organic colloidal P (Walstra, 1999).

|  | Ca | P | Ca/P |
|---|---|---|---|
| Cow | 1,250 | 950 | 1.32 |
| Goat | 1,350 | 1,000 | 1.35 |
| Sheep | 1,900 | 1,500 | 1.27 |
| Woman | 320 | 160 | 2.00 |
| Donkey | 807 | 638 | 1.26 |
| Deer | 2,330 | 1,640 | 1.42 |

Table I.5: Milk mineral composition (g/kg) in different species. Data from (Mahieu et al., 1977, Gallego et al., 2006, Fantuz et al., 2012)
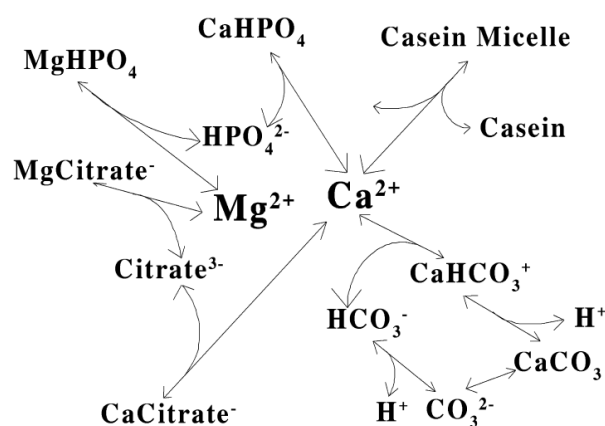


Figure I.26: Equilibrium of Ca forms in milk (Neville et al., 1995)

One role of caseins is to solubilize Ca, thanks to its colloidal form bounded to caseins, to avoid the formation of insoluble precipitate (Farrell et al., 2006). A relationship between

milk Ca content and casein contnthas has been observed when comparing species (Figure I.27). However, variations in the ratio of milk contents of Ca to casein exist in dairy cows as the equilibrium between Ca and casein is always adjusting to physico-chemical variations of milk (Farrell et al., 2006). Repartition and equilibrium of P forms in milk were less studied (Walstra, 1999). P is also linked to caseins as it participates in the elaboration and the stability of casein micelles (Holt, 2004).
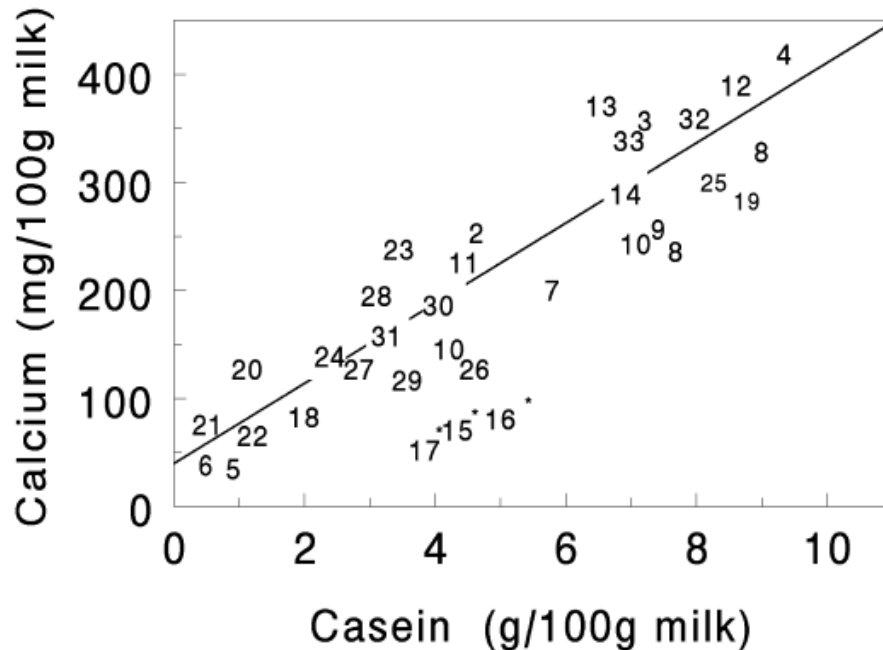


Figure I.27: Relation between Ca and casein concentrations in the milks of different species (Jenness, 1979). 1, Long-tailed bat; 2, little brown bat; 3, tree-tailed bat; 4, rabbit; 5, baboon, 6, human; 7, hamster; 8, rat; 9, mouse; 10, guinea pig; 11, dog; 12; black bear; 13, grizzly bear; 14, polar bear; 15, fur seal; 16, elephant seal; 17, harp seal; 18, Indian elephant; 19, aardvark; 20, horse; 21, burro; 22, rhinoceros; 23, pig; 24, camel; 25, reindeer; 26, giraffe; 27, cow; 28, buffalo; 29, goat, 30, sheep; 31, pygmy sperm whale; 32, fin whale; and 33, blue whale.The best-fitting line had a slope of 8.7 mM/g casein and an intercept of 9.1 mM/kg milk.

## 3    Factors of variations of milk Ca and P contents

The number of studies focusing on the variations of milk Ca and P contents in dairy cows is low, and most results are issued from studies focusing on a unique factor of variation (Forar et al., 1982, Glantz et al., 2009). Few studies allowed a quantification of the relative part of variation explained by several factors (Van Hulzen et al., 2009, Toffanin et al., 2015b) and a confusion, between seasonality and stage of lactation, can be present in those latter studies.

Among the factors that can affect milk Ca content, the most important is the genetics of

the cow. It was first described through a high effect of breed (Cerbulis and Farrell, 1976, Hermansen et al., 2005, Chassaing et al., 2016). Hermansen et al. (2005) showed an average Ca content of 1.23 g/kg for Jersey cows when it was 1.09 g/kg for Holstein cows. Milk Ca content heritability varies from 0.10 (Toffanin et al., 2015b) to 0.57 in Holstein cows (Van Hulzen et al., 2009), and 0.60 whatever the breed (Buitenhuis et al., 2015). These differences may partially due to a less complex model for Buitenhuis et al. (2015) and Toffanin et al. (2015) than for Van Hulzen et al. (2009), increasing the relative importance of residual, and thus decreasing the estimation of the heritability (heritability is defined as the ratio between standard deviation attributed to genetics and sums of standard deviation of genetics and residuals). A second well described factor of variation of the milk Ca content is the stage of lactation of cows, with a sharp decrease in early lactation followed by a smooth increase during the rest of lactation (Hidiroglou and Proulx, 1982, Kaufmann and Hagemeister, 1987, Gaucheron, 2005). Other factors of variation have been suspected. Primiparous cows seem to present higher milk Ca content than multiparous cows but this has been observed only in early lactation (Kume and Tanabe, 1993). Several studies highlighted an effect of the season on milk Ca content (Poulsen et al., 2015, Toffanin et al., 2015a, Chassaing et al., 2016). It is difficult to dissociate the specific effect of the season, i.e. meteorological conditions or light for instance (Boudon et al., 2016), from those related to the stage of lactation, and to the diet of the cows. The nature of the forage, grazing vs. corn silage, is also suspected to affect milk Ca content, with higher contents with diet based on corn silage. Hurtaud et al. (2014) observed higher milk Ca content in winter with feeding system based on corn silage than with feeding system favoring grass whereas no difference was observed in spring and autumn, when both feeding systems used diets based on grazing. This effect was also suspected by Poulsen et al. (2015).

Variations of milk P content have been less studied than those of Ca. A high correlation between milk Ca and P contents, among cow's milk has been illustrated (Poulsen et al., 2015), with the idea of a domain of coexistence between Ca and P contents (Kaufmann and Hagemeister, 1987). However, current knowledge of Ca and P secretion cannot confirm the hypothesis that milk Ca and P contents are dependent on each other. As for Ca, a high effect of the genetics of the cows has been demonstrated. Holstein cows have a milk P content of

985 mg/L when Jerseys had a milk P content of 1,330 mg/L (Cerbulis and Farrell, 1976), and a high heritability, 0.62 (Van Hulzen et al., 2009). Some studies, however, obtained much lower heritability, under 0.15 (Toffanin et al., 2015b, Buitenhuis et al., 2015). This difference may be due to differences in experimental design and models. Buitenhuis et al. (2015) and Toffanin et al. (2015) included only effect of parity and stage of lactation when Van Hulzen et al. (2009) included effect like season and sire, using only primiparous cows. Those differences may explain the difference between the studies, the first ones explaining less variability, and thus have higher residuals and lower heritability. The effect of the stage of lactation on milk P content is unclear. Some studies demonstrated an effect on milk P content similar to htat observed for Ca (Toffanin et al., 2015b), when others studies did observe any significant of effect (Forar et al., 1982, Van Hulzen et al., 2009). It seems that dietary content of P and Ca do no affect milk P content (Forar et al., 1982, Ferris et al., 2010a). Only a meta-analysis (Alvarez-Fuentes et al., 2016) found an increase in milk P content with lower dietary Ca content. Some studies also observed higher milk P content in winter (Forar et al., 1982), when other studies observed higher contents in summer (Glantz et al., 2009). Milk P content seems to be lower with high ambient temperature (Kamiya et al., 2010).

# 4   A suspicion of a link between milk Ca content and bone mobilization in case of low calcemia

As written previously in section A, it has been shown in mice that a low Ca intake induces several responses in early lactation such as a higher PTHrP secretion, a lower milk Ca to content ratio and a lower milk production measured by the evolution of pups weight (VanHouten et al., 2004), suggesting that the mammary gland can both adapt milk Ca content and induce bone mobilization to regulate calcemia. This response is mediated by CaSR that are present on the basal face of MEC. Their activation depends on calcemia, hypocalcemia leading to a lower activation of those receptors. Van Houten et al. (2004) showed that activation of CaSR inhibits PTHrP synthesis and activates Ca transport into milk. It was shown later that CaSR also participates in the regulation of water transport into milk (Kovacs, 2016). However, no differences were found in the premature evolution of mammary gland, thus this decrease in milk production did not seem to be due to difference

of MEC proliferation (VanHouten et al., 2004).

A question remains on how these results could be extrapolated to dairy cows. It has been shown in dairy cows milk a negative correlation between milk Ca content and milk PTHrP concentration in the first week of lactation (Kocabagli et al., 1995), but this was contradicted by other studies correlation over lactation (Law et al., 1991, Onda et al., 2006), suggesting that the decrease in milk secretion of Ca is not a regulation response concomitant with that of PTHrP secretion all over lactation, but maybe only during early lactation. However, the conclusion cannot be firmly stated given that the mechanism of transfer of PTHrP into milk is unknown and that a specific effect of the stage of lactation on milk contents of both Ca and PTHrP is possible.

The organism of lactating dairy cows faces important Ca and P flows due to milk secretion. For that reason, most of the lactating dairy cows receive specific dietary supplementation of Ca and P formulated by evaluating the daily input and output of Ca and P thanks to models elaborating in published feeding systems. Current recommendations of those feeding systems are to replace day-to-day cows' losses, i.e. fecal and urinal excretions and milk secretion by adequate dietary supply. However, it is likely that lactating cows develop the ability to mobilize Ca and P from their bones at the beginning of their lactation and that they restore their pools of Ca and P at the end of their lactation. Then, it could be considered that it would be better to reason mineral supplementation at the scale of the lactation, taking into account the cycles of bone mobilization and reconstitution during lactation and gestation. To achieve this, a first step would be to dispose of methods allowing the quantification of those cycles during long period of time on representative numbers of cows.

Because it has been shown in mice that the mammary gland can, at the same time decrease the amount of Ca exported in milk and increase the bone resorption in case of hypocalcemia, the hypothesis of this PhD thesis is that milk Ca content could be a biomarker of bone mobilization. Thus, the aim of this work was to study if milk Ca content could reflect bone mobilization in dairy cows along lactation. As genetics is known be a major determinant of milk Ca content, it is highly foreseeable that milk Ca content could only use as a dynamic biomarker. **Thus, the main question of this thesis was to**

**determine if the dynamics of milk Ca content over lactation could allow predicting those of biomarkers of bone resorption, or maybe a ratio between resorption and accretion.** Three sub-questions were considered.

**The first sub-question was to determine if milk Ca content was variable in dairy cows, when excluding the genetics effect, and to quantify the relative importance of the factors of variation of milk Ca content.** Thus, the first step of this thesis was to characterize the non-genetics factors of variation of milk Ca content in dairy cows. This characterization was realized using results from a large enquiry realized in the 3 majors French regions of milk production, involving about 1,000 farms. The data obtained from this allowed studying the non-genetic factors of variations of milk Ca content at a large scale, and thus identifying factors leading to low variations of milk Ca content. The existence of a variability of milk Ca content explained by factor of variation other than those related to the breed and the genetics was a first condition allowing to identifying milk Ca content as a biomarker of bone resorption. The analysis of the enquiry was realized in collaboration with IDELE that allowed us the calculation of milk Ca content from MIR spectra.

**The second sub-question was to determine if a relationship could be identified between dynamics of plasma biomarkers of bone accretion and resorption during lactation and those of milk Ca content.** Thus, the second step was an experimental work consisting in measuring concomitantly both dynamics during lactation in various conditions in dairy cows. The main factors of variation considered were the parity, the stage of lactation and the individual. A first experiment was in the Méjusseaume farm (INRA, Brittany). It consisted in measuring both dynamics in a herd of 33 Holstein cows with half primiparous and half multiparous, all fed with a unique total mixed ration. A second experiment was run at the experimental farm of Le Pin (INRA, Normandy). It consisted in measuring both dynamics in a herd of 13 Holstein and 17 Normande cows fed with two feeding strategies, with high or low energy density diets. Dynamics of bone accretion and resorption were measured thanks to plasma biomarkers. These experiment were initially conceived for two projects that were not directly related to my thesis.

**The last sub-question was to determine if an enhanced bone mobilization in early lactation by lower Ca intake and/or low DCAD would lead to a decrease in**

**milk Ca content.** It consisted in inducing an enhanced bone mobilization in 10 cows thanks to either low dietary Ca content or both dietary Ca content and DCAD and to compare dynamics of bone accretion and resorption and milk contents during lactation to those of 5 control cows. A second question of this experiment was to determine how cows replenish their bones after an enhanced bone mobilization at the beginning of lactation. This experiment was designed specifically for my thesis and run at the experimental farm of Méjusseaume. Bone mobilization was measured with two methods, plasma bone biomarkers and cow's mineral input-output balance along lactation.

# Non-genetic factors of variation of milk Ca content

# Characterization of the nongenetic causes of variation in the calcium content of bovine milk on French farms

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## ABSTRACT

Milk is an important source of Ca in Western diets. Milk Ca is important for the cheesemaking process and could be a useful biomarker of Ca regulation in cows. The objective of this study was to identify and quantify nongenetic factors affecting the variation of Ca content in bovine milk. During the PhénoFinLait program, a survey was performed in 3 major areas of milk production in France. This survey consisted of collecting milk samples, together with information about herd management and cow nutrition, from 924 commercial farms. More than 200,000 individual milk samples were collected, and Ca content was measured by mid-infrared spectroscopy. Each farm was surveyed several times during the year, and 3 to 6 milk samples were collected from each cow. An equation to predict milk Ca content from mid-infrared spectra was developed based on the Ca contents of 292 milk samples, and the milk Ca contents of the 200,000 samples were then predicted. Milk Ca content was lowest in Holstein cows, intermediate in Montbéliarde cows, and highest in Normande cows. For all 3 breeds, milk Ca decreased during the first month of lactation and increased after the fourth month of lactation, with the range between minimum and maximum values largest in Holsteins, intermediate in Montbéliardes, and smallest in Normandes. Milk Ca content also decreased with parity in all 3 breeds. By using multiple factorial analysis, 6 major feeding strategies employed on French dairy farms were characterized based on the data from the survey. Calendar month and cow feeding strategy affected milk Ca content, which dropped in the spring during grazing turnout and was lower when cows were fed fresh and conserved grass rather than corn silage. In conclusion, environmental factors induce variations in milk Ca content in addition to the genetics of the cows, which to date have been identified as a main factor of variation of milk Ca content in dairy cows. In several of the tested conditions, increases in milk production and in the amount of Ca daily secreted in milk were associated with a decrease in milk Ca content as though the mammary gland operated to limit the exportation of Ca when milk production rapidly increased. This result would suggest that milk Ca content could be a biomarker of Ca regulation in dairy cows.
**Key words:** dairy cow, calcium, milk

## INTRODUCTION

Calcium is the major mineral contained in bovine milk, with a mean content of 1.25 g/kg (Alais, 1984). Bovine milk and dairy products are the main sources of Ca in the diets of many countries, especially Western diets, and account for 75% of human Ca needs in the Netherlands (Flynn and Cashman, 1997). Milk Ca content is also an important determinant of milk coagulation and cheesemaking capability (Malacarne et al., 2014). However, large variations of milk Ca content exist around the cited average of 1.25 g/kg, with contents ranging between 0.9 and 1.4 g/kg (van Hulzen et al., 2009; Hurtaud et al., 2014; Poulsen et al., 2015; Chassaing et al., 2016). A better quantification and understanding of milk Ca content variation is necessary to evaluate the consequences of these variations on the amount of Ca contained in milk in human diets or on cheese production process. It would also allow exploration of the possibility of using milk Ca content as a biomarker of bone accretion and resorption in dairy cows.

Several studies showed that Ca secretion by mammary glands could be dependent on Ca regulation in mammals, and more specifically in cows (Horst et al., 1997; VanHouten et al., 2004). VanHouten et al. (2004) described in mice that a decrease in blood Ca, caused by decreasing diet Ca content, increased the expression and secretion of parathyroid hormone-related protein

# A). INTRODUCTION

(**PTHrP**) and decreased the secretion of Ca into milk, resulting in a 50% decrease in milk Ca content. These effects were mediated by the Ca-sensing receptor of the mammary epithelial cell. This suggests a possibility that milk Ca could be a biomarker of some events involved in Ca regulation in cows, such as postpartum hypocalcemia or bone accretion and resorption dynamics during the lactation and gestation cycle. It is known that lactating cows undergo important cycles of bone resorption and accretion during lactation (Ekelund et al., 2006), but these cycles are difficult to quantify in large numbers of animals. The idea that milk Ca content could be a biomarker of these cycles would allow better understanding of, for instance, the consequences of mineral nutrition on those cycles or of cumulative unbalanced bone accretion and resorption during several lactations on cow longevity.

Variations in milk Ca content have been clearly related to the genetics of the cows, through systematic breed differences or high heritability within a breed (Hidiroglou and Proulx, 1982; van Hulzen et al., 2009; Buitenhuis et al., 2015; Toffanin et al., 2015b). The relationship between milk Ca content and cow diet, its evolution during the year, or its relationships with other effects, such as the stage of lactation or seasonality, have been less studied and have even been considered to be negligible compared with the relationship between milk Ca content and the genetics of the cows (Alais, 1984; Hermansen et al., 2005). Several studies showed an effect of lactation and seasonality on milk Ca content, but with contrasting results (Gaucheron, 2005; van Hulzen et al., 2009; Toffanin et al., 2015b; Chassaing et al., 2016). van Hulzen et al. (2009) described an increase in milk Ca content, of 0.578 mg/kg per day of lactation, throughout lactation, whereas Toffanin et al. (2015b) described a decrease of more than 100 mg/kg during d 5 to 35 and 36 to 65 of lactation, followed by a similar increase until the end of lactation. A possible reason for the discrepancies between these studies is the significant difficulty in dissociating the effects of the stage of lactation, the cows' diet, and the season.

The PhénoFinLait program (Gelé et al., 2014) consisted of surveying 945 farms between November 2009 and October 2010. The aim of our study was to use the samples and the data collected during this program to better quantify and characterize the nongenetic factors affecting variations of Ca content in bovine milk. Our assumption was that the high numbers of participating farms and the resulting diversity of milk production systems would allow for the dissociation and characterization of the effects of the stage of lactation, diet, and season to allow a better understanding of variations in Ca content in bovine milk.

## MATERIALS AND METHODS

### Study Design

The data used in our study were collected through the PhénoFinLait program, which consisted of a survey performed in the major areas of milk production in France (i.e., Alsace, Brittany, Franche-Comté, Normandy, and Pays de Loire). Between November 2009 and October 2010, 945 farms were surveyed. During this period, several visits (between 2 and 8, averaging 5) were performed at each farm to follow the evolution of the herd and cow diets over the course of a complete year. During each visit, interviewers collected data about the dairy cows (breed, parity, stage of lactation, stage of gestation, age of first calving, milk production) and their diet (description of the composition of the diets by using 54 variables). They also collected individual milk samples, and mid-infrared (**MIR**) spectra of the samples of each cow were measured in the laboratory. The survey resulted in 252,519 milk spectra, 9,180 frozen milk samples, 4,825 visits, and 63,818 dairy cows divided among the 3 main breeds in France (i.e., Holstein, Montbéliarde, and Normande) spread over 5 regions. The initial aim of the project was to characterize the effect of genomics and feeding on milk fatty acid and protein composition across the diversity of French dairy farms for 3 species (i.e., cattle, sheep, and goats; Sanchez et al., 2016). The PhénoFinLait program has been fully described by Gelé et al. (2014).

### Prediction of Milk Calcium Content Using MIR Spectra

A prediction equation specific to our study was developed to predict milk Ca content from MIR spectra. To achieve this, the milk Ca contents of 292 frozen milk samples taken from the bank of samples of the PhénoFinLait program were analyzed by atomic absorption spectrometry after mineralization and dilution of the samples with nitric acid (AFNOR NF ISO 8070, 2007). Those samples were chosen to maximize the diversity of the potential factors affecting variations in Ca content (i.e., parity, lactation stage, breed, localization, cow diet, milk yield, and protein yield). The samples were split into 2 groups: the first group contained 205 samples for calibrating the prediction equation, whereas the second group contained 87 samples used as external data to validate the equation.

### Characterization of Feeding Strategies

For each visit to each farm, the mean diet was estimated by averaging the proportions of each feed in the

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diet. To characterize the effect of seasonality, the feeding strategies of the farms were characterized over 3 periods: the winter period, from November 15 to the end of March; early summer period, from April 1 to June 15; and late summer period, from June 16 to October 15. Only farms that were investigated every period were used to characterize feeding strategies. A multiple factor analysis (**MFA**) was therefore performed to characterize feeding strategies (Escofier and Pagès, 1994) with R (R Development Core Team, 2013) and the package *FactoMineR*. An MFA is a generalization of principal component analysis for the comparison of multiple data tables (Abdi et al., 2013). For our study, each table was a group of variables describing the diet for a period and each individual was a farm. There were 3 groups for the analysis, 1 per period, each with the same 54 variables, with each variable being the proportion of a feed in the diet. As all variables had the same unit, the data were not reduced. An ascending hierarchical classification was then performed on the factor scores, using Ward's criterion to select the number of clusters. The best number of clusters was given by a high ratio of the loss of inertia between n + 1 clusters and n clusters. To confirm the results, the pseudo-$T^2$ test was also used to select the final clusters (Nakache and Confais, 2004). Another restriction was to produce several clusters between 5 and 15. The hypothesis was that fewer than 5 clusters was too few to represent the diversity of feeding strategies and that more than 15 clusters was too many to be characterized and differentiated. A consolidation was performed based on the results of hierarchical classification using k-means clustering and virtual centers of clusters as initial individuals.

For farms that were only represented in 2 periods, missing factor scores for the third period were input with R and the package *missMDA*, using an iterative MFA (Husson and Josse, 2013). Those farms were used as supplementary individuals in the MFA, so they were not included in the characterization of feeding strategies, but it was possible to use those data for the analysis of Ca content. Supplementary farms (i.e., illustrative individuals) were affected by the nearest feeding strategy after the classification and the consolidation and before analyses of the Ca content variations. The data were checked to ensure that feeding strategy characteristics were not affected by the implemented data. Farms that were investigated for only 1 period were removed from all analyses.

### Statistical Analysis

The prediction of milk Ca contents from MIR spectra was performed with a partial least square regression using the PLS procedure in SAS (SAS Institute, 2013) with the data of the calibration group (Ferrand-Calmels et al., 2014). The selection of the number of latent variables was performed using the root mean squared error of prediction with the objective of achieving the smallest possible value. This method has already been used several times to predict different minerals in milk (Soyeurt et al., 2009; Toffanin et al., 2015a). Several statistical parameters of the prediction regression were used to estimate the accuracy of the prediction: the coefficient of determination ($\mathbf{R^2cv}$), the validation coefficient of determination ($\mathbf{R^2v}$), the root mean squared error (**RMSE**), and the ratio of the standard error of cross-validation to standard deviation.

A mixed-model ANOVA was performed using PROC MIXED in SAS to characterize the factors affecting variations in Ca content. The selected model, which was the same for the 3 breeds, was

$$\begin{aligned} Y_{ijklm} = {} & \mu + \text{Month of Lactation}_i + \text{Parity}_j \\ & + \text{Calendar Month}_k + \text{Feeding Strategy}_l \\ & + (\text{Calendar Month} \times \text{Feeding Strategy})_{kl} \\ & + \text{Herd}_m + \varepsilon_{ijklm}, \end{aligned}$$

where $Y_{ijklm}$ was a dependent variable of a cow in the herd $m$, with parity $j$ during month of lactation $i$, during the calendar month $k$, in the feeding strategy $l$, and $\varepsilon_{ijklm}$ was the residual error. With the exception of the herd factor, which was a random factor, all other factors were fixed. For each breed, some feeding strategies were removed from the analyses, as they did not have enough data and were too unbalanced throughout the year. Data from d 1 to 8 of lactation were removed to avoid the effect of colostrum in the analysis. Data after d 360 of lactation were also removed. Parities of 5 or greater were regrouped. Cows with only 1 milk sample were removed from the analyses. The analyses were performed independently for each breed. The same model was used to characterize the effects of the same factors on daily milk production, protein content, amount of Ca secreted daily in milk (i.e., Ca content × daily milk production), and the ratio of Ca content to protein content.

Because of the large amount of data gathered in the data basis, *P*-values could easily be low (<0.001). Thus, the effect size (**ES**) of each simple fixed factor included in the ANOVA model for all explicated variables was also estimated according to the formula given by Cohen (1988):

$$\text{ES} = \frac{\sigma_m}{\sigma},$$

where $\sigma$ was the standard deviation of the overall breed for the considered character, and $\sigma_m$ was the standard deviation due to the considered effect, and defined as

$$\sigma_m = \sqrt{\sum_{i=1}^{I} p_i \left(y_i - \overline{y}\right)^2},$$

where $I$ was the number of levels of the considered factor $m$, $p_i$ the proportion of the total population characterized with the $i$th level of factor $m$, $y_i$ the mean for the $i$-th level and $\overline{y}$ the corrected mean of overall population. The ES of the interaction between calendar month and feeding strategy and of the combined effects of feeding strategy, calendar month, and the interaction between both were also estimated according to the modifications of the formulae suggested by Cohen (1988). The combined effects of feeding strategy, calendar month, and the interaction between those factors are described as combined effects in the following sections.

## RESULTS

### *Prediction of Milk Calcium Content Using MIR Spectra*

The partial least squares regression resulted in the use of 11 partial least squares. The quality of prediction was good, with high coefficients of determination for both calibration and validation. It resulted in an $R^2cv$ of 0.75 for the calibration and an $R^2v$ of 0.80 for the validation (Table 1). The slope of the regression between the measured and predicted values was close to 1 in both cases, with values between 0.96 and 1.00. The RMSE was 56.0 for the calibration and 44.5 for the validation. The ratio of the standard error of cross-validation to standard deviation was always above 2.00, meaning that the accuracy of prediction was good.

### *Effects of Stage of Lactation and Parity*

The numbers of data points involved in the mixed model we used were 75,120, 59,595, and 77,443 for the Holstein, Montbéliarde, and Normande breeds, respectively. The 3 breeds have very similar distributions of dates of calving during the survey period, with a maximum frequency of calving between September and January (Figure 1). The difference in the mean stages of lactation at each visit between the 3 breeds only exceeded 15 d in November, with a difference of 25 d between Holsteins and Montbéliardes for that month (Figure 2). For the 3 breeds, the mean stage of lactation increased between January and July, from d 110 to 205 of lactation. The correlation between stage of lactation (in days) and visit calendar date was low ($R^2$ <0.14 within every breed), and these variables were considered as independent. This was also illustrated by the low differences between minimum and maximum average stages of lactation between calendar months (less than 100 d, Figure 2); therefore, both variables were included in the mixed models.

Within each breed, Ca content was affected by the stage of lactation (ES = 0.33 for Holstein, 0.35 for Montbéliarde, and 0.21 for Normande; $P < 0.0001$; Figure 3), with an important range of variation between months of lactation. For the 3 breeds, Ca content decreased between the first and second months of lactation, with the greatest decrease, 80.3 ± 1.8 mg/kg ($-6.6\%$ of the mean of the first month), in Ca content in the Montbéliardes and the smallest decrease, 39.3 ± 1.7 mg/kg ($-5.7\%$ of the mean of first month), in the Normandes. The lowest Ca contents were observed between mo 2 and 4 of lactation; Ca content then increased from mo 5 of lactation until the end of lactation, reaching values as high as or higher than those observed during mo 1 of lactation. The range of variation of Ca content in milk during lactation was greater for Holsteins (110.7 ± 2.6 mg/kg between mo 3 and 12 of lactation; i.e., 9% variation of Holstein milk Ca content mean) than for

**Table 1**. Results of Ca content (mg/kg) prediction from mid-infrared spectra with partial least squares regression

| Item | 1st group (calibration) | 2nd group (validation) |
|---|---|---|
| N | 205 | 87 |
| Mean (mg/kg) | 1,235.2 | 1,239.1 |
| SD | 112.5 | 99.4 |
| Predicted mean (mg/kg) | 1,235.2 | 1,231.0 |
| $R^2$ | 0.75 | 0.80 |
| Root mean squared error | 56.0 | 45.5 |
| RPD[1] | 2.00 | 2.22 |

[1]RPD = ratio of the standard error of cross-validation to standard deviation.

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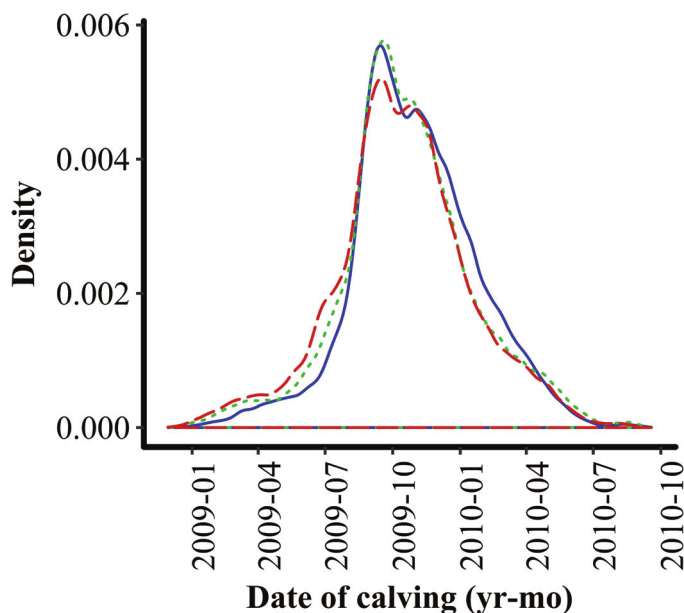


**Figure 1**. Frequency distribution of the dates of calving during the survey period for Holstein (dashed line), Montbéliarde (solid line), and Normande (dotted line). Color version available online.

the 2 other breeds ($66.6 \pm 2.9$ mg/kg, 5.1% variation of breed mean, between mo 3 and 12 of lactation for Normandes; $98.9 \pm 1.7$ mg/kg, 8.0% variation of breed mean, between mo 1 and 4 of lactation for Montbéliardes). In contrast to milk Ca content, the amount of Ca secreted daily in milk decreased throughout lacta-



**Figure 2**. Evolution of the mean stage of lactation of cows during the visits for Holstein (dashed line), Montbéliarde (solid line), and Normande (dotted line). Color version available online.

tion regardless of the breed (ES = 0.47 for Holstein, 0.56 for Montbéliarde, and 0.59 for Normande; Figure 3B) and was always highest in Holsteins and lowest in Normandes, with intermediate values in Montbéliardes. These results were linked to milk production, which was highest in Holsteins, with a peak at 33.7 kg/d, whereas Normandes and Montbéliardes had maximum milk productions of 27.3 and 30.2 kg/d, respectively. For Holsteins and Montbéliardes, milk production increased between mo 1 and 2 of lactation and then decreased throughout the rest of the lactation period, whereas milk production decreased throughout the entire lactation period in Normandes (ES = 0.54 for Holstein, 0.59 for Montbéliarde, 0.62 for Normande; Figure 3A).

The milk Ca content decreased with parity for all breeds (ES = 0.10 for Holstein and Montbéliarde, 0.16 for Normande; $P < 0.0001$; Table 2). The difference between lactation 1 and $\geq 5$ was approximately 30 mg/kg, which represents 2.3 to 2.4% of the average milk Ca content, depending on the breed. In Normandes, the maximum difference between lactation 1 and $\geq 5$ was $36.8 \pm 1.3$ mg/kg, which represents 2.8% of the average milk Ca content. Milk production and the amount of Ca secreted daily in milk increased between parities, with the sharpest increase occurring between parities 1 and 2 for the 3 breeds (data not shown). In contrast, the decrease in milk Ca content was not consistently greater during this period.

### Characterization of Feeding Strategies

Of the 945 farms involved in the survey, 627 were visited at least once during each of the 3 defined periods and then retained in the analysis used to characterize the feeding strategies. Two hundred forty-seven farms were visited at least once during 2 of the 3 defined periods and were used as supplementary individuals after missing factors had been assessed, whereas 40 farms were removed completely because they were visited during only 1 of the 3 defined periods. Some variables and, more specifically, some feed proportions in the diet were removed for specific periods because they did not vary between farms and were null for those periods.

The first 3 dimensions of the MFA, respectively, explained 50.6, 13.0, and 8.6% of the inertia. After the 10th dimension, each factorial axis explained less than 1% of the inertia. The first dimension contrasted corn silage for the 3 periods with hay during the winter period and pasture during the early and late summer periods. The second dimension contrasted mixed hay in the winter period and grass pasture in the early summer period with winter corn silage and mixed pasture in the early summer period. The third dimension contrasted 2 kinds
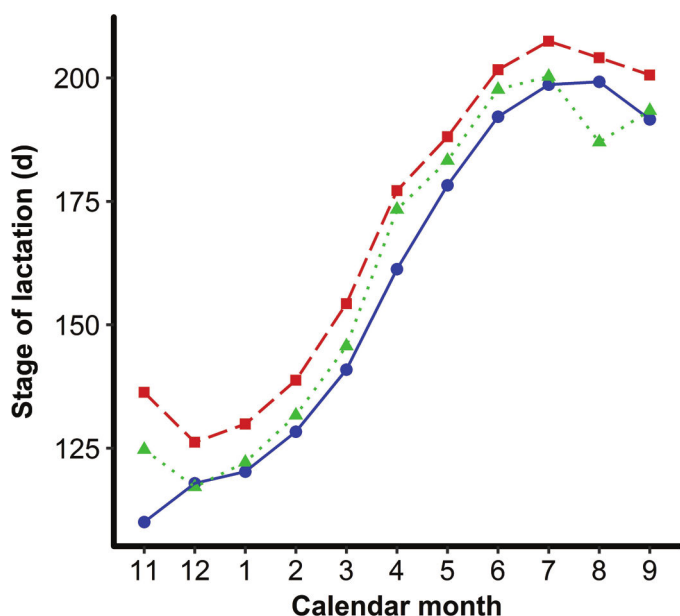
**71**
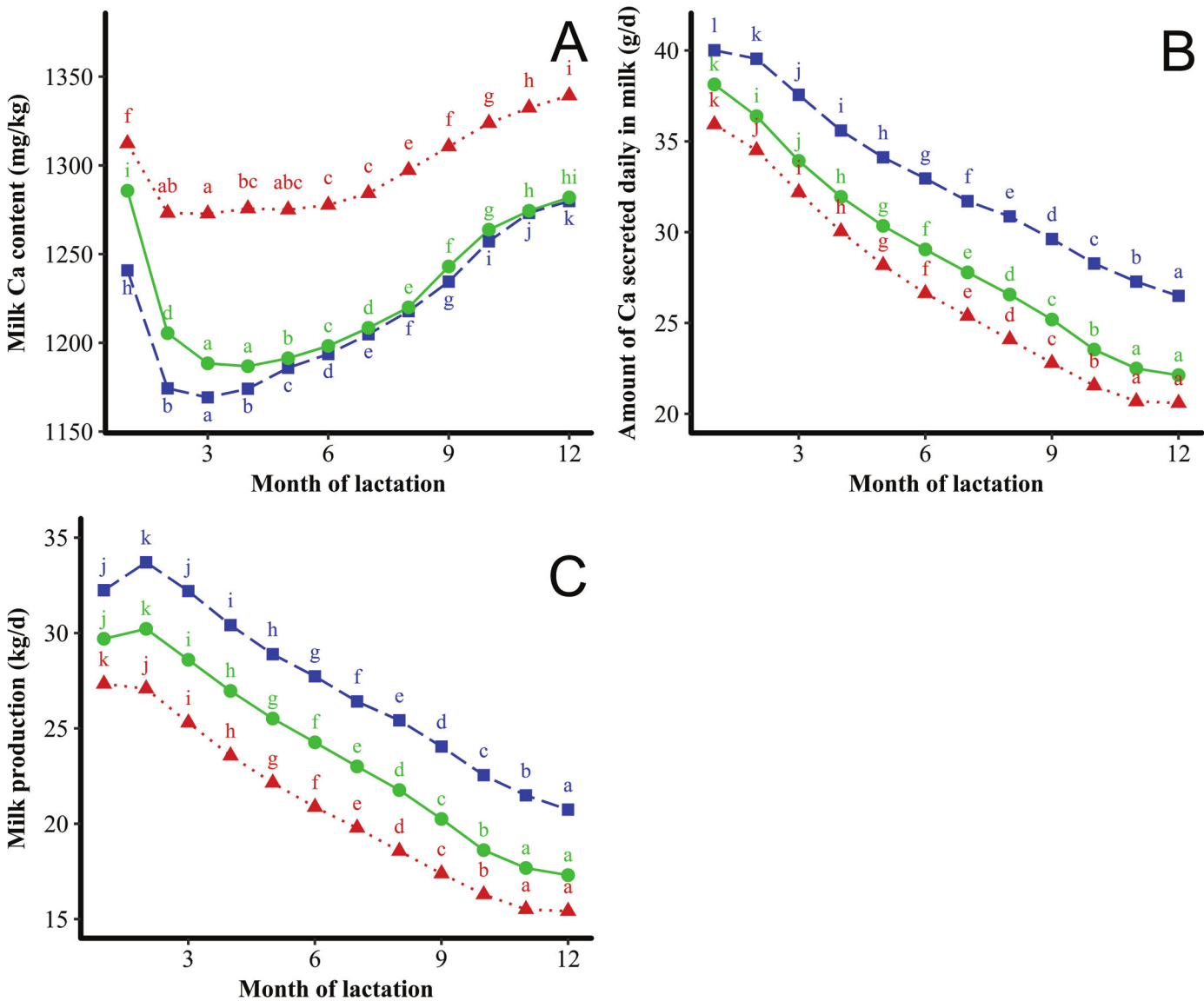
**Figure 3**. Effect of the stage of lactation, within each breed, on (A) milk Ca content, (B) milk production, and (C) amount of Ca secreted daily in milk for Holstein (■), Montbéliarde (●), and Normande (▲). Letters (a–l) indicate the results of comparison between the stages of lactation within a breed; different letters indicate significant differences in Ca content ($P < 0.001$). Color version available online.

**Table 2**. Effect of parity ($P < 0.0001$) on milk Ca content (mg/kg) for each breed

| Parity | Holstein | Montbéliarde | Normande |
|---|---|---|---|
| 1 | 1,230.9 ± 1.7[e] | 1,243.6 ± 2.3[d] | 1,316.1 ± 1.6[d] |
| 2 | 1,223.6 ± 1.7[d] | 1,230.8 ± 2.4[c] | 1,316.0 ± 1.7[d] |
| 3 | 1,216.8 ± 1.8[c] | 1,224.6 ± 2.4[b] | 1,293.4 ± 1.7[c] |
| 4 | 1,211.0 ± 1.9[b] | 1,224.5 ± 2.5[b] | 1,284.6 ± 1.8[b] |
| 5+ | 1,203.5 ± 2.0[a] | 1,221.4 ± 2.4[a] | 1,279.3 ± 1.8[a] |

[a–e]Letters indicate the results of comparison between parities within a breed. Different letters indicate significant differences in Ca content ($P < 0.001$).

of hay in the winter period, field-cured and barn-dried. The construction of the principal components was mostly affected by the variables corresponding to the proportions of forage included in the diet. One reason for this was that the data were not scaled to the unit and forages were the components of the diet with the highest proportion. Corn silage was important in the construction of principal components for the 3 periods, but hay was only important for the winter period and pasture for the early and late summer periods. Most of the proportions of feed that were less than 1% of the diets (as feed basis) did not contribute to the construction of principal components.

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Classification was performed on the first 40 principal components of the MFA, corresponding to more than 99% of the initial inertia. Classification resulted in 6 feeding strategies. The ratio of relative loss of inertia for n + 1 clusters on n clusters was the highest for 6 clusters. At the same time, the pseudo-$T^2$ test showed an increase of 50% from 6 to 7 feeding strategies, the highest possible in our range of restriction. Thus, there was a local minimum with a large increase afterward, which confirmed the first results for the number of feeding strategies. They were mostly distinguished by the evolution of forage in the diet throughout the 3 periods. Descriptions of the feeding strategies over the survey period are summarized in Figure 4.

Strategies were named according to the relative importance of the main forages and the distribution of their contribution to the diet during the year or according to specific use of forages during a given period. The strategies "grazing and FC hay" and "grazing and BD hay" represented feed systems based on grazed pasture during the early and late summer periods and on field-cured (**FC**) or barn-dried (**BD**) hay during the winter period. More than 90% of farms described by these strategies were in the northeastern part of France, more precisely in Franche-Comté, with almost exclusively Montbéliardes. In this area, most dairy farms produce Protected Designation of Origin cheeses with specifications prohibiting corn silage. Additionally, more than 75% of all organic farms involved in the survey were described by these 2 feeding strategies. The proportion of organic farms that used these 2 strategies was 15%, whereas for all the farms, it was only approximately 5%. The strategy "maximum grazing" consisted of a maximal use of grazing in early and late summer periods followed by diets based on corn silage during the winter period. This strategy is under-represented in Franche-Comté, but at least 4,000 milk samples were classified in this strategy for each of the 3 breeds. "Maximum grazing" was the counterpart of the 2 previous feeding strategies for other regions of France, with corn silage instead of hay in winter. The strategies "grazing and FC hay," "grazing and BD hay," and "maximum grazing" included a large proportion of grazing as forage in the summer periods, but 80% of farms in the "grazing and FC hay" and "grazing and BD" hay strategies were in Franche-Comté whereas 89% of farms in the "maximum grazing" were in western regions. The strategy grazing and corn silage was based on corn silage, but with lower contributions from pasture than for the maximum grazing strategy (28 and 61% of pasture in late summer period for strategies "grazing and corn silage" and "maximum grazing," respectively). The strategy "grazing and corn silage" was more represented in north-

western France, but this strategy was well represented in each breed. The strategy "corn silage" was based on this forage for all periods. It was highly represented in the extreme northeast of France, in Alsace, where at least 65% of farms were classified in this strategy, even though this strategy was represented in all regions and was well represented in each breed. A gradient in the diet compositions, from pasture-based diets to corn silage-based diets in the early and late summer periods, was discernable for the 3 strategies "maximum grazing," "grazing and corn silage," and "corn silage." The strategy "grazed temporary pasture" was based on non-permanent pasture during the early and late summer periods and on corn silage and a substantial amount of grass silage in the winter. It was associated with a use of temporary pasture, which was over-represented in western regions of France (21% of farms in those regions characterized by the strategy versus 6% on average for all studied regions). It was under-represented in Montbéliarde cows, with only 0.71% of the samples collected from Montbéliarde cows classified in this strategy (Table 3).

### Effect of Feeding Strategy and Calendar Month on Milk Calcium Content

For the mixed-model analysis within breed, under-represented feeding strategies (fewer than 2,000 milk samples, Table 3) were removed. Thus, the strategies "grazing and FC hay" and "grazing and BD hay" were not included for Holsteins and Normandes, and the strategy "grazed temporary pasture" was not included for Montbéliardes. Feeding strategy, calendar month, and their interaction clearly affected milk Ca content ($P < 0.0001$; Figure 5), but, more generally, all effects included in the mixed model were highly significant ($P < 0.0001$; Table 2, Figure 3) for every predicted variable. Feeding strategy had a higher $P$-value in Holsteins (ES = 0.05; $P = 0.0012$). $P$-values were only given if they were higher than 0.0001, but ES were given because they brought information about the variability due to each effect. For the 3 breeds, model milk Ca content estimations were more accurate between December and July with low mean standard errors, whereas at the beginning and the end of the investigation the mean standard errors were higher due to smaller amounts of data. For instance, the mean standard error of the mean milk Ca content for all the feeding strategies was 9.07 and 10.18 mg/kg for Holsteins in August and September, respectively, whereas it did not exceed 6 mg/kg for the other months. The results from August and September will be less discussed, as the adjustment of the models was not as good as for the other calendar
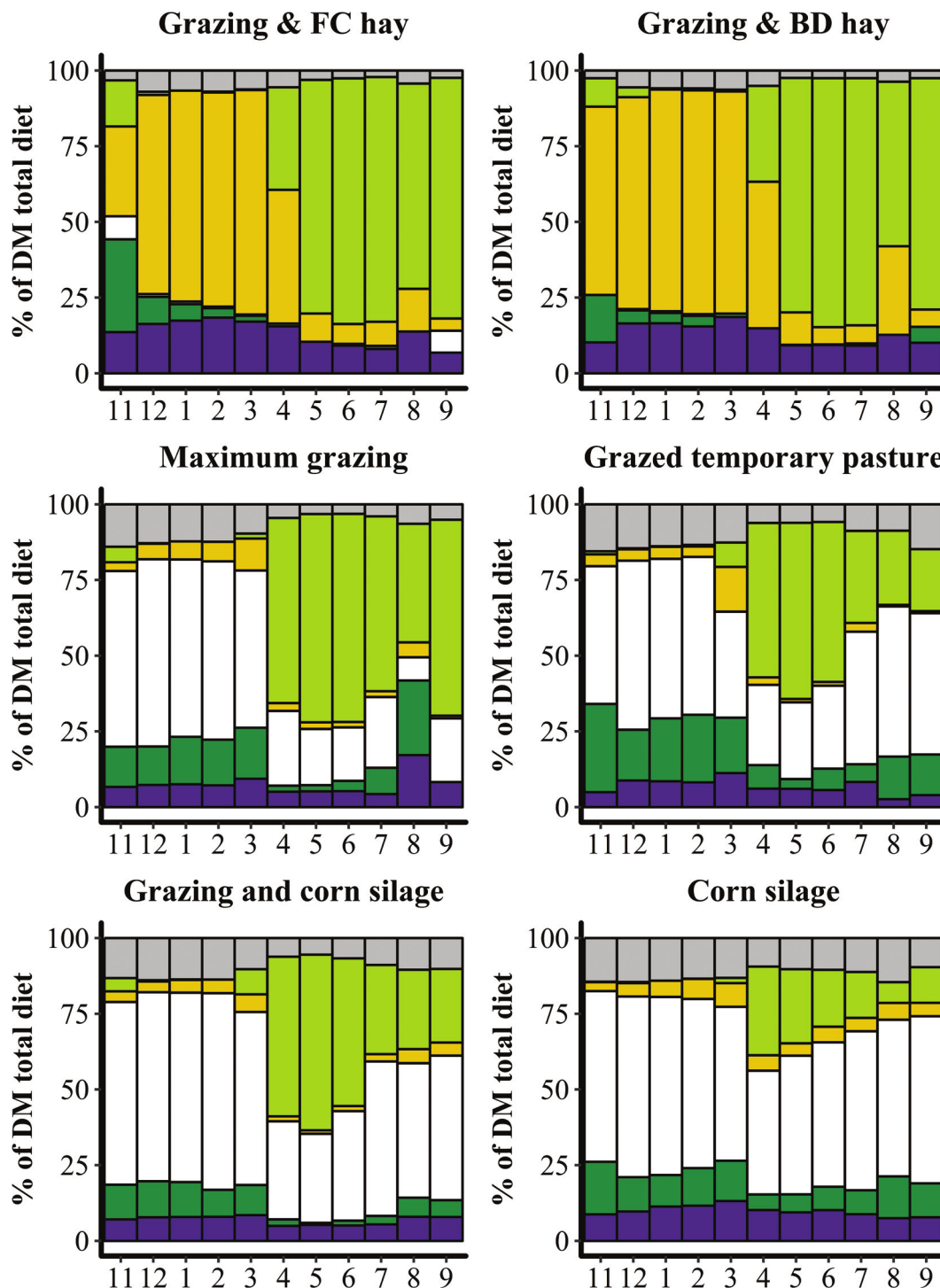
**Figure 4**. Evolution of the mean diet during the survey period for the 6 feeding strategies (gray/purple = feed concentrate and minerals; black/dark green = grass silage; white = corn silage; gray with black stripes/yellow = hay; black with white stripes/light green = grass; white with black stripes/gray = oil meal and oleaginous seeds). FC = field-cured; BD = barn-dried. Color version available online.

months. For each breed, the differences in the mean stages of lactation between the feeding strategies and within each calendar month were low (data not shown).

For the 3 breeds, Ca content clearly decreased between March and May, regardless of feeding strategy, and started increasing from July onwards (ES of calendar

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**Table 3**. Distribution of milk samples among breeds and feeding strategies

| Feeding strategy | Breed | | |
|---|---|---|---|
| | Holstein | Montbéliarde | Normande |
| Grazing and field-cured hay | 1,529 | 23,042 | 1,270 |
| Grazing and barn-dried hay | 387 | 14,363 | 1,674 |
| Maximum grazing | 9,857 | 4,318 | 22,443 |
| Grazing and corn silage | 30,592 | 7,443 | 40,251 |
| Corn silage | 27,538 | 10,429 | 7,496 |
| Grazed temporary pasture | 7,409 | 430 | 7,253 |

month = 0.24 for Holstein and Montbéliarde, 0.16 for Normande; Figure 5). The period between March and May corresponded to pasture turn-out for all feeding strategies (Figure 4), whereas the proportion of grazed pasture started to decline after July for all strategies except those combining grazing and hay ("grazing and FC hay" and "grazing and BD hay").

For Holsteins, milk Ca content in the winter (i.e., mainly January and February) was higher with the strategy "grazed temporary pasture" compared with the other strategies, whereas in the summer (i.e., in June and July) it was higher with the strategy "corn silage" compared with the other strategies (ES of feeding strategy = 0.05; Figure 5). The drop in Ca content between March and June was higher for strategies based on higher proportions of pasture. The largest decrease was for "maximum grazing," with a decrease of $86.1 \pm 5.3$ mg/kg ($-6.9\%$ of the mean of the feeding strategy in March) over this period, whereas "grazed temporary pasture" showed a decrease of only $22.7 \pm 2.4$ mg/kg ($-1.9\%$; ES of combined effects = 0.25).

For Montbéliardes, the strategies "grazing and FC hay" and "grazing and BD hay" resulted in lower milk Ca content from November to April compared with the 3 other strategies (i.e., "corn silage," "grazing and corn silage," and "maximum grazing"). The difference between these 2 specific feeding strategies and the others was approximately 50 mg/kg (4.1% of the breed mean) for this period. In winter, these 2 strategies were based on hay, whereas the 3 other feeding strategies were based on corn silage (ES of feeding strategy = 0.16; Figure 4). The decrease between the winter period and the early summer period was less important for the strategies "grazing and FC hay" and "grazing and BD hay" than for the other strategies (ES of interaction = 0.13, combined effects = 0.31; Figure 5). After turn-out to pasture, in April, these 2 strategies were not different from the others. Between April and June, higher values were obtained for strategies based on a higher proportion of corn silage ("grazing and corn silage" and "corn silage") rather than grazed pasture ("grazing and BD hay," "grazing and FC hay," and "maximum grazing").

For Normandes, differences between feeding strategies were less important than for the other 2 breeds (ES = 0.05 for feeding strategy and interaction, ES = 0.18 for combined effects). Only the strategy "grazed temporary pasture" resulted in lower milk Ca content from November to June compared with other strategies. Differences between the 3 other strategies were rarely more than 30 mg/kg (2% of breed mean) within a calendar month and were rarely significant, except during the diet transition in March and April. For Holsteins and Montbéliardes, higher Ca content was obtained with a higher proportion of corn silage rather than pasture, and these effects were greater for Holstein and Montbéliarde than for Normande.

### Effect of Feeding Strategy and Calendar Month on Milk Production and Amount of Calcium Secreted Daily in Milk

For Holsteins, milk production increased from November to April, regardless of feeding strategy, and then started decreasing until August (ES of calendar month = 0.13; Figure 6A). Milk production was higher with the feeding strategy "corn silage" from December to July, except for April, and was lower with the feeding strategy "maximum grazing" (ES of feeding strategy = 0.15, interaction = 0.05, and combined effects = 0.20). The higher the proportion of corn silage in the diet was, the higher the milk production. The amount of Ca secreted daily in milk increased from November to April, decreased until August, and then increased as the proportion of corn silage in the diet increased (Figure 6B). However, the relative increase in the amount of Ca secreted daily in milk between January and April was less important than the increase in milk production. For instance, for the strategy "maximum grazing," milk production showed an increase of 12.3%, whereas the amount of Ca secreted daily in milk showed an increase of 7%, which was concomitant with an important drop in milk Ca content. The same trends were observed for the other strategies but with lower amplitudes. Similar trends were also observed for Montbéliardes and Normandes (data not shown; ES of combined effects = 0.24 for both breeds).

### Effect of Feeding Strategies on Milk Protein Content and Ca Content-to-Protein Content Ratio

Milk protein content was significantly affected by the feeding strategy, but the differences did not exceed 1 g/kg in Holstein cows in a given month (ES of feeding strategy = 0.03; Figure 7). For Montbéliardes and Normandes, the differences between feeding strategies in a given month exceeded 1 g/kg only in August, Septem-
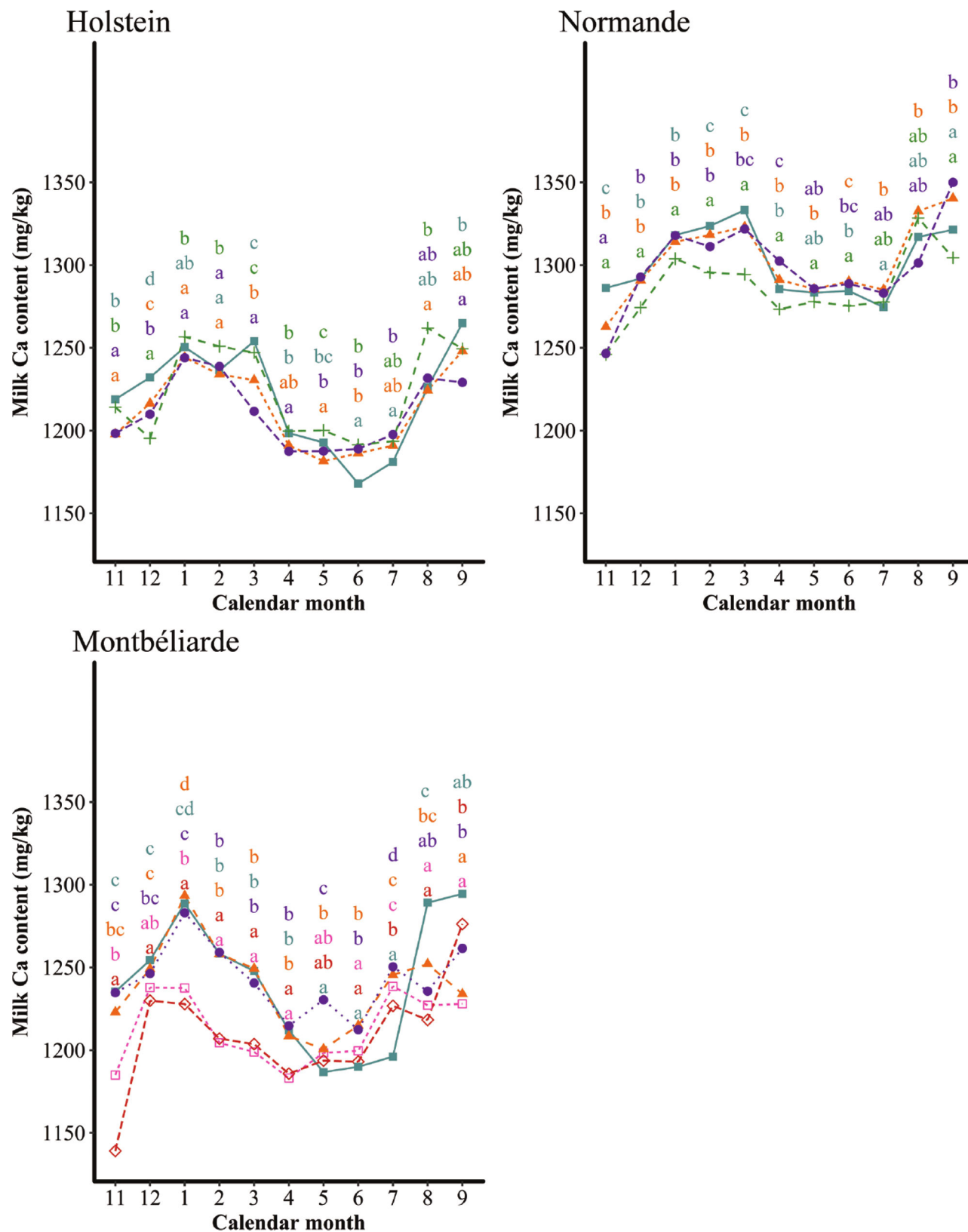
**Figure 5**. Effect of feeding strategy and calendar month on milk Ca content for the 3 breeds. Letters (a–d) represent each feeding strategy over a side-by-side comparison within a month ($P < 0.0001$): grazing and field-cured hay (◇), grazing barn-dried hay (□), maximum grazing (■), grazing and corn silage (▲), corn silage (●), grazed temporary pasture (+). Color version available online.

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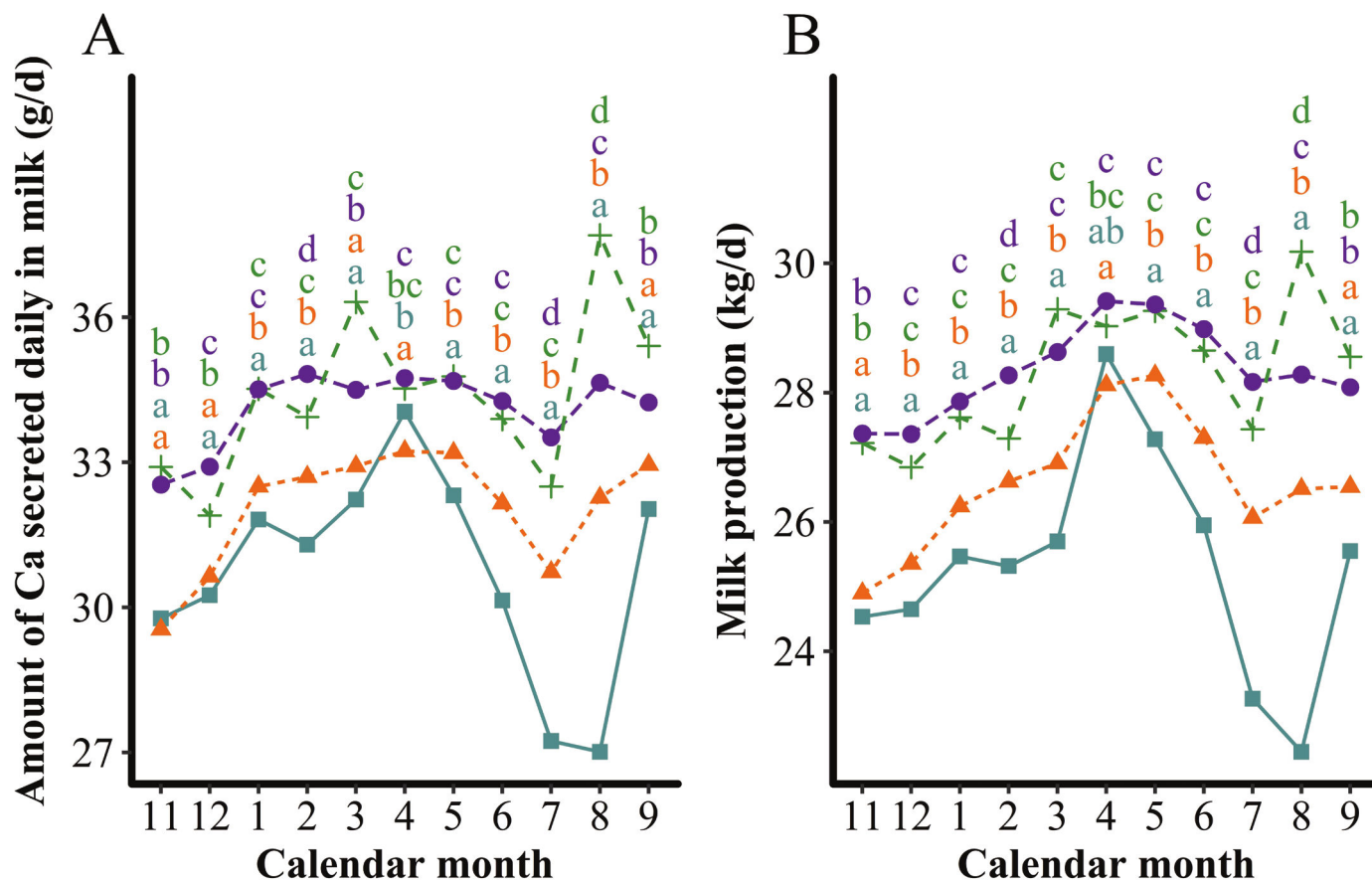


**Figure 6**. Effect of calendar month and feeding strategy in Holstein on (A) amount of Ca secreted in milk and (B) milk production. Letters (a–d) represent each feeding strategy over a side-by-side comparison within a month ($P < 0.0001$): maximum grazing (■), grazing and corn silage (▲), corn silage (●), grazed temporary pasture (+). Color version available online.

ber, and November, when estimations were less accurate (data not shown; ES of feeding strategy = 0.08 for Normande and 0.09 for Montbéliarde). Protein content decreased from November to July and then increased until the end of the survey period. The ratio of Ca content to protein content was clearly affected by the calendar month, with variations greater than 2 mg/g between months (ES of calendar month = 0.18 for Holstein, 0.24 for Montbéliarde, and 0.20 for Normande). In Holsteins, it increased slightly between December and March and decreased sharply between March and April due to the Ca content in milk. After April, the ratio started decreasing again until July, when protein content was lowest for every feeding strategy.

The feeding strategy always affected the dynamics of the ratio of milk Ca content to protein content and of the milk protein content for the 3 breeds (ES for combined effect = 0.19 for Holstein, 0.32 for Montbéliarde, 0.22 for Normande; Figure 7 for Holsteins, data not shown for Montbéliardes and Normandes). For Normandes, "maximum grazing" had the lowest protein content for the majority of the survey period, leading to the highest ratio of milk Ca content to protein content compared with the other feeding strategies; however, variations due to calendar month were still more important than those due to feeding strategy. For Montbéliardes, the trends were the same as they were for Holsteins, but the variation range was greater, between 35.2 and 40.5 mg/g (14% of variation of breed mean) from December to July. "Grazing and BD hay" and "grazing and FC hay" had lower ratios in winter than the other feeding strategies due to lower milk Ca content, with a difference of at least 1 mg/g for that season.

## DISCUSSION

### Quality of MIR Prediction and Relevance of the PhénoFinLait Program

The quality of prediction of the Ca content in milk was similar to those that were performed previously

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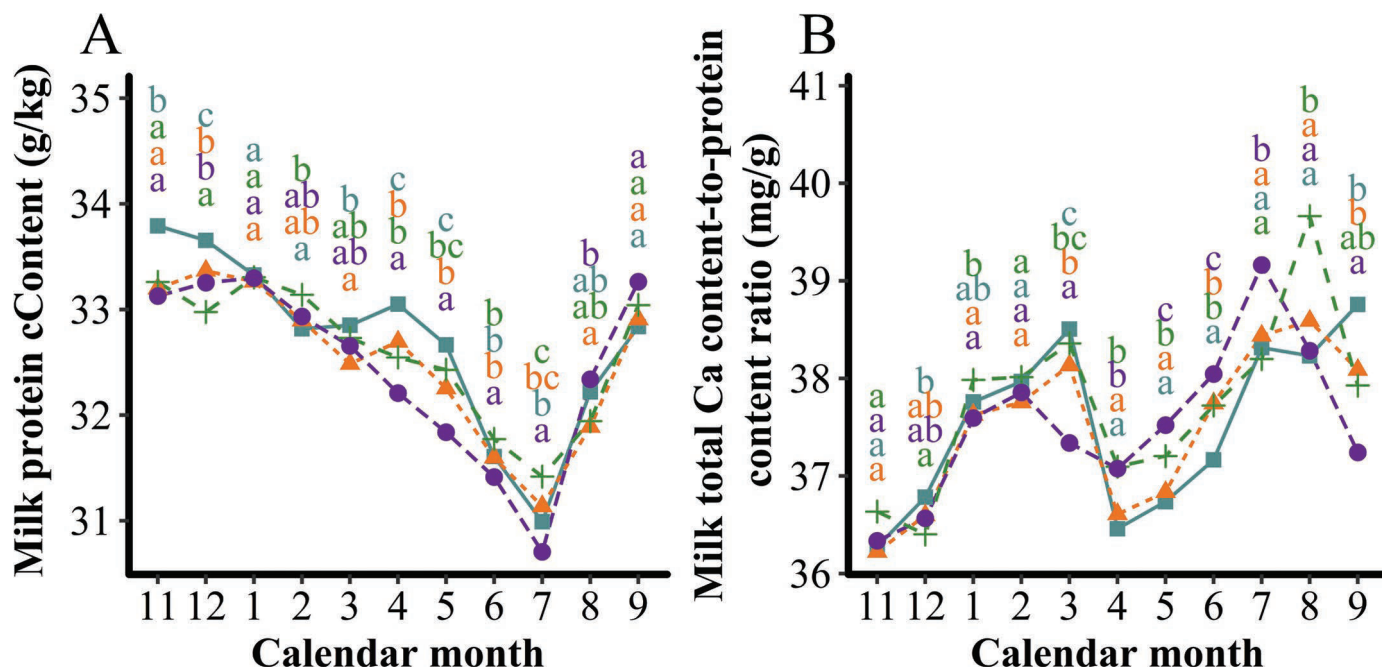
**Figure 7**. Effect of calendar month and feeding strategy in Holsteins on (A) protein content in milk and (B) the ratio of Ca content to protein content in milk: maximum grazing (■), grazing and corn silage (▲), corn silage (●), grazed temporary pasture (+). Different letters (a–c) indicate significant differences between feeding strategies within a calendar month ($P < 0.001$). Color version available online.

(Soyeurt et al., 2009; Toffanin et al., 2015a). The mean milk Ca content we observed in this study (i.e., 1,230 mg/kg) was in the same order of magnitude as those obtained by Soyeurt et al. (2009) and Toffanin et al. (2015a) in the databases they used to develop MIR prediction equations. The $R^2$cv we obtained was similar to that of Toffanin et al. (2015a; 0.75), but was lower than that obtained by Soyeurt et al. (2009; 0.97). The better $R^2$cv of Soyeurt et al. (2009) is likely linked with their validation data that were split into 2 distinct and extreme groups, with a range of approximately 400 mg/kg without data. This should have increased the $R^2$cv of the regression with data that were not normally distributed, according to the hypothesis of the generalized linear model; RMSE was not reported by Soyeurt et al. (2009). Toffanin et al. (2015a) had an RMSE of approximately 85, depending on the software used, whereas ours did not exceed 56. The large number of samples used in our study, 292 vs. 203 for Toffanin et al. (2015a) and 87 for Soyeurt et al. (2009), did not seem to increase the quality of prediction but was necessary to obtain a large panel for the source of variability in milk Ca content. It may have increased the inference in the prediction equation.

The PhénoFinLait program constituted a very good opportunity to study nongenetic factors affecting variations of Ca content in milk. Compared with previously published studies that aimed to characterize the variability of milk Ca content (van Hulzen et al., 2009; Poulsen et al., 2015; Chassaing et al., 2016), our study had the disadvantage of using MIR predictions of milk Ca content instead of direct measurements, as in Toffanin et al. (2015b), but had the advantage of relying on individual milk sampling, several times during the year, from a large number of cows and with a high diversity of diets with relatively well-described compositions. van Hulzen et al. (2009) and Toffanin et al. (2015b) both worked on individual samples of milk, but their studies did not involve more than 2,500 cows, and each cow was only sampled once per study. In contrast, the PhénoFinLait program involved over 50,000 cows with several samples during the same lactation. Toffanin et al. (2015b) did not give any information about cow diet, and Van Hulzen et al. (2009) had only 1 diet for all cows in the study. van Hulzen et al. (2009) only had 1 sampling date, whereas the sampling lasted almost 1 yr for Toffanin et al. (2015b), although no effect of season was included in the latter study. Only Chassaing et al. (2016) included the effect of seasonality, with 5 periods of sampling, and included diet, but it was less well described than in PhénoFinLait. However, that study was done on tank milk and it was not possible to assess the effects of the stage of lactation or parity on milk Ca content.

### Effects of Breed, Stage of Lactation, and Parity on Milk Ca Content

The effect of breed, and more generally of the genetics of cows, on milk Ca content is well known and has been described several times (Hermansen et al., 2005; van Hulzen et al., 2009; Chassaing et al., 2016). For instance, Jersey cows have a higher milk Ca content than Holstein cows (Hermansen et al., 2005), and high heritability of milk Ca content was found in Holsteins with a value of 0.57 (van Hulzen et al., 2009). However, as milks were sampled on the same date, from only primiparous cows, and as cows were fed the same ration, this heritability may have been overestimated. In our study, we observed large differences in milk Ca content between Holstein, Montbéliarde, and Normande cows, with higher values for Normandes.

The effect of the stage of lactation on milk Ca content had already been described in dairy cows (Kaufmaan and Hagemeister, 1987; Gaucheron, 2005; Hermansen et al., 2005). A sharp decrease between the first 2 mo of lactation has often been described, followed by a smooth increase until the end of lactation (Kaufmaan and Hagemeister, 1987). Our results confirmed these dynamics but showed different orders of magnitude between breeds, with Holstein cows having the largest decrease in milk Ca content during the beginning of lactation, Normande cows the smallest decrease, and Montbéliarde cows an intermediate decrease. It can be noted from our results that the magnitude of milk Ca content variation during lactation was negatively related between breeds to the amount of Ca secreted daily in milk. To explain this, we hypothesized that the larger drop in Ca content at the beginning of lactation in breeds with higher amounts of Ca secreted daily in milk could be explained by a greater solicitation of operating organs involved in the regulation of calcemia, including the mammary gland. Indeed, it has been observed in mice (VanHouten et al., 2004) that the milk Ca content could decrease to increase calcemia in cases of hypocalcemia. If we assume that such mechanisms exist in dairy cows, a reduction in milk Ca content could be a mechanism for cows with high milk production to maintain their calcemia. This reduction of Ca content would result from the detection of an insufficient Ca supply by the Ca receptor of the mammary epithelial cells that would induce a secretion of PTHrP by the mammary gland. The PTHrP could then increase bone Ca mobilization (VanHouten et al., 2004; Kovacs, 2005). After mo 2 of lactation, we can assume that the decrease in milk production and amount of Ca secreted daily in milk would likely result in a lower need for Ca mobilization from bone and possibly in a lower reduction of Ca secretion by the mammary gland, which might explain the limitation of the Ca content drop. In this scenario, the increase of Ca content after mo 4 of lactation, which occurred at the same time for every breed, could also be related to the continuous decrease in the total amount of Ca secreted daily in milk during lactation.

A decrease in milk Ca content with the parity of the cow has been described by Kume and Tanabe (1993) in colostrum. In our experiment, milk Ca content decreased with parity regardless of the stage of lactation. This result may not be dissociated from the fact that older cows are known to have greater difficulty in mobilizing Ca from bone and in absorbing Ca from their digestive tract and are therefore more susceptible to postpartum hypocalcemia (Horst, 1986; Reinhardt et al., 2011). It might be assumed that in those cows a decrease in Ca secretion by the mammary gland could be a more important mechanism to conserve Ca and to regulate calcemia than in younger cows, according to the mechanism previously described in mice by VanHouten et al. (2004); this mechanism would even be amplified if we considered that the amount of Ca secreted daily in milk is higher in older cows.

### Effect of Feeding Strategy and Seasonality on Milk Ca Content

The data obtained from the PhénoFinLait program were very useful in our study to characterize the effects of season, stage of lactation, and cow diets on milk Ca content and to dissociate them from each other as much as possible. Our objective was to explain the discrepancy observed between studies by characterizing the effect of the stage of lactation or season on milk Ca content that was likely linked to a partial confusion between these 2 parameters (Toffanin et al., 2015b; Chassaing et al., 2016). In our case, seasonality was sufficiently uncorrelated with the stage of lactation to consider that the variations in milk Ca content according to the stage of lactation that we observed were independent of the season and may also be independent of the feeding strategy, as calving dates were distributed equally between strategies.

However, we also showed that, at least in the context of milk production in France, the effects of the feeding strategy and seasonality on milk production parameters could not be totally dissociated. Indeed, our study confirmed that the cow diets are very specific to season and region in relation to climate parameters controlling the supply of certain forage types (Figure 4). We made a choice to characterize the effect of annual feeding strategy on milk Ca content rather than that of individual cow diets, as they were described previously in the PhénoFinLait program (Gelé et al.,

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2014). The main advantage of that choice was to include potential delayed and remnant effects of diets on the phosphocalcic metabolism of cows, and thus milk Ca content, which could be inferred given the duration of the bone accretion or resorption cycles during lactation and gestation in cows (Ekelund et al., 2006; Taylor et al., 2009). Another advantage was the ability to use a complete block design between feeding strategy and calendar month.

We clearly showed an effect of the feeding strategy on milk Ca content that has been rarely described in the literature. Milk Ca content was often lower with diets based on pasture or conserved grass, such as silage or hay, than with diets based on corn silage. This was observable in our study in the winter and the spring in Montbéliarde cows and in the summer in Normande and Holstein cows. The difference in milk Ca content between diets could be high, with a difference between corn silage and hay of approximately 50 mg/kg in Montbéliardes. Hurtaud et al. (2014) also reported lower Ca content in cows grazing or fed conserved grass than in cows fed corn silage, although the difference was not significant. Poulsen et al. (2015) suspected an effect of cow diet on the milk Ca content, but did not have records of their diet. Chassaing et al. (2016) noticed milk Ca content variations possibly linked to the effect of the diet, but the effect of the diet was confused with a lot of other environmental effects in our study.

Because March to April is a major period of pasture turnout between winter diets and grazing, the drop in milk Ca content systematically observed during that period for the 3 breeds could also be linked, at least partly, to the nature of the diets fed to the cows. This would confirm the idea that milk Ca content is lower when cows are fed fresh or conserved grass rather than corn silage. Such an effect of the month of the year has been described previously, with higher milk Ca content in winter than in summer (Poulsen et al., 2015; Chassaing et al., 2016). It is impossible to confirm that it is not also linked at least partially to seasonal specificities, such as climatic conditions or day length. However, Boudon et al. (2016) found that increased day length increased Ca content in milk, but the increase they measured could only explain a small part of the drop observed between March and April. The drop in milk Ca content between March and April could also be explained by the increase in milk yield, and thus the increased demand for Ca secretion in milk, that occurred between February and April (data shown in Holstein cows; Figure 6). The concomitance of these 2 phenomena, the decrease of milk Ca content and the increase of the amount of Ca secreted daily in milk, is consistent with the hypothesis that Ca content could be a mechanism to regulate calcemia by decreasing the amount of Ca secreted in a given milk yield (VanHouten et al., 2004). A maximal milk yield in March is understandable, given that, in the French dairy system, March is a period of high nutrient supply due to the association of good quality of grazed herbage and supplementation with winter diets. If a decrease in milk Ca content can occur quickly to regulate calcemia when the milk yield increases, a delay in the increase in milk Ca content occurs when the milk yield decreases. Indeed, this is illustrated by the fact that the decrease in milk yield in July occurred with all feeding strategies, and the amount of Ca secreted daily in milk (Figure 6) was thus only accompanied by a delayed increase in milk Ca content in August.

The fact that one of the chief functions of casein micelles in milk could be to solubilize the Ca and phosphate and avoid the formation of insoluble precipitates (Farrell et al., 2006) implies that the milk micellar Ca-to-soluble Ca ratio and the milk total Ca content-to-protein content ratio should vary within a very narrow range (Alais, 1984; Gaucheron, 2005). Our results showed that the milk total Ca content-to-protein content ratio can vary according to the diet of the cows, the season, or both, from 35 to 40.2 mg/g. These results tempered the idea that the milk total Ca content-to-protein content ratio is constant and that the main determinant of milk Ca content is the casein content. The reasons for the variation in the milk total Ca content-to-protein content ratio are unclear. It would have been very useful to know the micellar and soluble Ca contents in the milk in our study, but MIR equations to predict micellar and soluble Ca were not developed. These results raised the question of the interaction of the systemic regulation and secretion of Ca in milk via the Ca receptor and the regulation of the partition of Ca in the mammary epithelial cells between the Golgi apparatus and the cytoplasm (Kovacs, 2016). We did not observe any effect of the breed on the milk total Ca content-to-protein content (data not shown), in contrast to what was previously observed (Alais, 1984; Gaucheron, 2005).

We have shown that, even though genetics is considered the major determinant of Ca milk content in cows, nongenetic factors also affect milk Ca content. In our study, variations in milk Ca contents due to stage of lactation or interacting effects of seasonality and diet could even be higher than differences between breeds. High heritability of milk Ca content has been shown in the literature by van Hulzen et al. (2009) and Buitenhuis et al. (2015). The heritabilities they obtained differed between breeds, with a higher heritability for Holsteins (0.72) than for Jerseys (0.63; Buitenhuis et al., 2015), but also differed between studies. Toffanin et al. (2015b) found a low heritability for milk

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Ca content (0.11), but data they used included more environmental sources of variability in milk Ca content with a higher diversity of sampling seasons, parities, or diets. Heritability is always estimated as the ratio between variance due to individual and the sum of variance due to individual and residual variance after correction for nongenetic factors (i.e., environment and physiological status; van Hulzen et al., 2009). Thus, a high heritability of milk Ca content in studies where environmental factors of variation of milk Ca contents are well controlled or described can be associated with a high variability of milk Ca content due to nongenetic factors.

## CONCLUSIONS

This study illustrated that genetics is not the only factor that affects Ca content in bovine milk. The stage of lactation, parity, seasonality, and cow diet, and more specifically the nature of the forage, also explained a significant range of variation in milk Ca content. Variations in milk Ca content within a single lactation can be at least as important, depending on the breed, as the variations between breeds at the same stage of lactation. Cow diet and seasonality had lesser effects on milk Ca content than breed or stage of lactation, but these effects remained non-negligible. However, differences in milk Ca content due to parity were small compared with those explained by the factors previously cited, with a 5 mg/kg decrease in lactation. We also observed that those nongenetic factors affecting milk Ca content may be related to the fact that the mammary gland is also an organ involved in the Ca regulation of lactating cows, as well as bones, the digestive tract, and kidneys, suggesting that milk Ca content may be an easy way to follow the evolution of the Ca status of cows through lactation and between lactations. However, the observed effect of the cow diet and more specifically of the nature of the forages on milk Ca content remains to be explained. A remaining question is to determine whether this effect could be related to the Ca content of the diets, which could not be quantified with the required precision in this study.

## ACKNOWLEDGMENTS

## REFERENCES

Abdi, H., L. J. Williams, and D. Valentin. 2013. Multiple factor analysis: Principal component analysis for multitable and multiblock data sets. Wiley Interdiscip. Rev. Comput. Stat. 5:149–179. https://doi.org/10.1002/wics.1246.

AFNOR NF ISO 8070. 2007. Lait et produits laitiers - Détermination des teneurs en calcium, sodium, potassium et magnésium - Méthode spectrométrie par absorption atomique.

Alais, C. 1984. Science du Lait: Principes des Techniques Laitières. SEPAIC. Société d'Edition et de Promotion Agro-alimentaires, Industrielles et Commerciales. Paris, France.

Boudon, A., M. Johan, A. Narcy, M. Boutinaud, P. Lamberton, and C. Hurtaud. 2016. Dietary cation-annion difference and day length have an effect on milk calcium content and bone accretion of dairy cows. J. Dairy Sci. 99:1527–1538. https://doi.org/10.3168/jds.2015-9664.

Buitenhuis, B., N. A. Poulsen, L. B. Larsen, and J. Sehested. 2015. Estimation of genetic parameters and detection of quantitative trait loci for minerals in Danish Holstein and Danish Jersey milk. BMC Genet. 16:52. https://doi.org/10.1186/s12863-015-0209-9.

Chassaing, C., C. Sibra, J. Verbič, O. M. Harstad, J. Golecký, B. Martin, A. Ferlay, I. Constant, C. Delavaud, C. Hurtaud, V. Ž. Pongrac, and C. Agabriel. 2016. Mineral, vitamin A and fat composition of bulk milk related to European production conditions throughout the year. Dairy Sci. Technol. 96:715–733. https://doi.org/10.1007/s13594-016-0300-7.

Cohen, J. 1988. Statistical Power Analysis for the Behavioral Sciences. 2nd ed. Routledge, Abingdon, UK.

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. https://doi.org/10.1016/j.livprodsci.2005.07.001.

Escofier, B., and J. Pagès. 1994. Multiple factor analysis (AFMULT package). Comput. Stat. Data Anal. 18:121–140. https://doi.org/10.1016/0167-9473(94)90135-X.

Farrell, H. M., E. L. Malin, E. M. Brown, and P. X. Qi. 2006. Casein micelle structure: What can be learned from milk synthesis and structural biology? Curr. Opin. Colloid Interface Sci. 11:135–147. https://doi.org/10.1016/j.cocis.2005.11.005.

Ferrand-Calmels, M., I. Palhière, M. Brochard, O. Leray, J. M. Astruc, M. R. Aurel, S. Barbey, F. Bouvier, P. Brunschwig, H. Caillat, M. Douguet, F. Faucon-Lahalle, M. Gelé, G. Thomas, J. M. Trommenschlager, and H. Larroque. 2014. Prediction of fatty acid profiles in cow, ewe, and goat milk by mid-infrared spectrometry. J. Dairy Sci. 97:17–35. https://doi.org/10.3168/jds.2013-6648.

Flynn, A., and K. Cashman. 1997. Nutritional Aspects of Minerals in Bovine and Human Milks. P.F. Fox, ed. Springer US, New York, NY.

Gaucheron, F. 2005. The minerals of milk. Reprod. Nutr. Dev. 45:473–483. https://doi.org/10.1051/rnd:2005030.

Gelé, M., S. Minery, J.-M. Astruc, P. Brunschwig, M. Ferrand-Calmels, G. Lagriffoul, H. Larroque, J. Legarto, O. Leray, P. Martin, G. Miranda, I. Palhiere, P. Trossat, and M. Brochard. 2014. Phé-

notypage et génotypage à grande échelle de la composition fine des laits dans les filières bovine et caprine. INRA Prod. Anim. 27:255–268.

Hermansen, J. E., J. H. Badsberg, T. Kristensen, and V. Gundersen. 2005. Major and trace elements in organically or conventionally produced milk. J. Dairy Res. 72:362–368. https://doi.org/10.1017/S0022029905000968.

Hidiroglou, M., and J. G. Proulx. 1982. Factors affecting the calcium, magnesium and phosphorus content of beef cow milk. Can. J. Comp. Med. 46:212–214.

Horst, R. L. 1986. Regulation of calcium and phosphorus homeostasis in the dairy cow. J. Dairy Sci. 69:604–616. https://doi.org/10.3168/jds.S0022-0302(86)80445-3.

Horst, R. L., J. P. Goff, and T. A. Reinhardt. 1997. Calcium and vitamin D metabolism during lactation. J. Mammary Gland Biol. Neoplasia 2:253–263.

Hurtaud, C., M. Dutreuil, M. Coppa, C. Agabriel, and B. Martin. 2014. Characterization of milk from feeding systems based on herbage or corn silage with or without flaxseed and authentication through fatty acid profile. Dairy Sci. Technol. 94:103–123. https://doi.org/10.1007/s13594-013-0147-0.

Husson, F., and J. Josse. 2013. Handling missing values in multiple factor analysis. Food Qual. Prefer. 30:77–85. https://doi.org/10.1016/j.foodqual.2013.04.013.

Kaufmaan, W., and H. Hagemeister. 1987. Composition of milk. Chapter 4 in Dairy-Cattle Production. H. O. Gravert, ed. Elsevier, Amsterdam, the Netherlands.

Kovacs, C. S. 2005. Calcium and bone metabolism during pregnancy and lactation. J. Mammary Gland Biol. Neoplasia 10:105–118. https://doi.org/10.1007/s10911-005-5394-0.

Kovacs, C. S. 2016. Maternal mineral and bone metabolism during pregnancy, lactation, and post-weaning recovery. Physiol. Rev. 96:449–547. https://doi.org/10.1152/physrev.00027.2015.

Kume, S., and S. Tanabe. 1993. Effect of parity on colostral mineral concentrations of Holstein cows and value of colostrum as a mineral source for newborn calves. J. Dairy Sci. 76:1654–1660. https://doi.org/10.3168/jds.S0022-0302(93)77499-8.

Malacarne, M., P. Franceschi, P. Formaggioni, S. Sandri, P. Mariani, and A. Summer. 2014. Influence of micellar calcium and phosphorus on rennet coagulation properties of cows milk. J. Dairy Res. 81:129–136. https://doi.org/10.1017/S0022029913000630.

Nakache, J.-P., and J. Confais. 2004. Approche Pragmatique de la Classification: Arbres Hiérarchiques, Partitionnements. Éditions Technip, Paris, France.

Poulsen, N. A., I. Rybicka, H. D. Poulsen, L. B. Larsen, K. K. Andersen, and M. K. Larsen. 2015. Seasonal variation in content of riboflavin and major minerals in bulk milk from three Danish dair-ies. Int. Dairy J. 42:6–11. https://doi.org/10.1016/j.idairyj.2014.10.010.

R Development Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Vet. J. (Lond.) 188:122–124. https://doi.org/10.1016/j.tvjl.2010.03.025.

Sanchez, M. P., A. Govignon-Gion, M. Ferrand, M. Gelé, D. Pourchet, Y. Amigues, S. Fritz, M. Boussaha, A. Capitan, D. Rocha, G. Miranda, P. Martin, M. Brochard, and D. Boichard. 2016. Whole-genome scan to detect quantitative trait loci associated with milk protein composition in 3 French dairy cattle breeds. J. Dairy Sci. 99:8203–8215. https://doi.org/10.3168/jds.2016-11437.

SAS Institute. 2013. SAS Institute. SAS/STAT User's Guide, SAS Institute Inc., Cary, NC.

Soyeurt, H., D. Bruwier, J.-M. Romnee, N. Gengler, C. Bertozzi, D. Veselko, and P. Dardenne. 2009. Potential estimation of major mineral contents in cow milk using mid-infrared spectrometry. J. Dairy Sci. 92:2444–2454. https://doi.org/10.3168/jds.2008-1734.

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. 92:223–237. https://doi.org/10.3168/jds.2008-1345.

Toffanin, V., M. De Marchi, N. Lopez-Villalobos, and M. Cassandro. 2015a. Effectiveness of mid-infrared spectroscopy for prediction of the contents of calcium and phosphorus, and titratable acidity of milk and their relationship with milk quality and coagulation properties. Int. Dairy J. 41:68–73. https://doi.org/10.1016/j.idairyj.2014.10.002.

Toffanin, V., M. Penasa, S. McParland, D. P. Berry, M. Cassandro, and M. De Marchi. 2015b. Genetic parameters for milk mineral content and acidity predicted by mid-infrared spectroscopy in Holstein-Friesian cows. Animal 9:775–780. https://doi.org/10.1017/S1751731114003255.

Van Hulzen, K. J. E., R. C. Sprong, R. van der Meer, and J. M. van Arendonk. 2009. Genetic and nongenetic variation in concentration of selenium, calcium, potassium, zinc, magnesium, and phosphorus in milk of Dutch Holstein-Friesian cows. J. Dairy Sci. 92:5754–5759. https://doi.org/10.3168/jds.2009-2406.

VanHouten, J., P. Dann, G. McGeoch, E. M. Brown, K. Krapcho, M. Neville, and J. J. Wysolmerski. 2004. The calcium-sensing receptor regulates mammary gland parathyroid hormone-related protein production and calcium transport. J. Clin. Invest. 113:598–608. https://doi.org/10.1172/JCI18776.

# Effects of parity and individual variability on bone accretion and resorption and milk calcium and phosphorus content during lactation in dairy cows
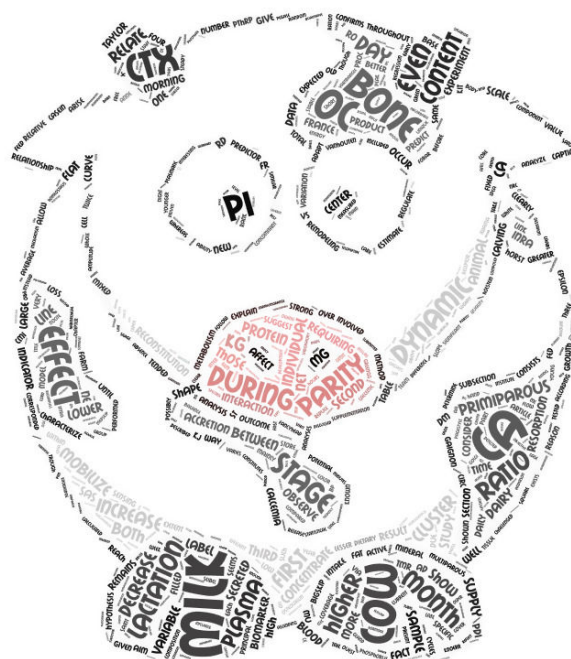
# Effects of parity and individual variability on bone accretion and resorption and milk calcium and phosphorus content during lactation in dairy cows

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Short title: Bone metabolism and milk composition in cows

## Abstract

Current recommendations for Ca and P supplementation in dairy cows do not take into account the dynamics of bone mobilization/reconstitution that occur during lactation. This study aimed to determine if the dynamics of milk Ca content during lactation could allow for the prediction of the dynamics of bone mobilization/reconstitution. It consisted of measuring monthly milk Ca and P contents and the plasma concentrations of biomarkers of bone accretion (**OC**) and resorption (**CTX**) on 33 Holstein cows in their first (n=17), second (n=10), and third or greater (n=6) lactation from 15 days before expected calving to the end of the 9th month of lactation. Cows were fed a unique total mixed ration during the whole experiment. Primiparous cows showed higher plasma OC and CTX concentrations ($P < 0.01$) and a higher increase in CTX during the first months of lactation than that in multiparous cows ($P < 0.05$). They also showed a higher decrease in evening milk Ca content ($P < 0.03$), concomitant to the increase in CTX, suggesting that a reduction in milk Ca secretion could be a way for the animal to regulate its calcemia during the first month of lactation. However, the individual dynamics of milk Ca content did not allow the estimation of the shape of the individual dynamics of bone accretion and resorption. The milk Ca to P ratio seemed to be a promising indicator of the plasma OC to CTX ratio within individuals. The consistency of this indicator remains to be estimated in challenging situations for Ca homeostasis in dairy cows.

**Keywords:** dairy cow, calcium, phosphorus, bone, parity

## Implications

In dairy farms, Ca and P supplementation is at the heart of rising concerns, due to its multiple impacts on dairy cows' health, reproduction and production, as well as the economic or environmental performances of the farms. To increase the efficiency of mineral supplementation, it has been suggested that Ca and P supplementation should not be considered on a day-to-day basis but on a larger time scale that needs better knowledge of the dynamics of bone mobilization on the scale of lactation. The milk Ca to P ratio could be an indirect indicator.

# A) Introduction

Current recommendations for Ca and P supplementation in dairy cows are based on a factorial approach of requirements that consists of replacing day-to-day, unavoidable Ca and P losses, as well as the accumulation of Ca and P in tissues linked to growth or gestation, in an animal with a given production level (AFRC, 1991, NRC, 2001, INRA, 2010). In this approach, there are requirements for maintenance, lactation, gestation and growth. For dairy cows, specific Ca and P supplies that can be drawn from bone mobilization at the beginning of lactation are not included, and neither are the specific Ca and P requirements for bone reconstitution that arise after the first third of lactation (Horst et al., 2005). At the beginning of lactation, all mammal females are submitted to a strong exportation of Ca, and P to a lesser extent, in milk, which is a main reason for the strong bone mobilization observed in dairy cows (Horst et al., 2005). This mobilization is at least partly compensated by a bone reconstitution after the first third of lactation (Horst et al., 2005). Bone mobilization and reconstitution are mainly driven by Ca homeostasis but are also involved in a release of or a P supply because bone is mainly composed of hydroxyapatite, which is a Ca phosphate (Ekelund et al., 2006). This fact highlights the advantage that can arise from considering both minerals together (Moreira et al., 2009, Elizondo Salazar et al., 2013). An expected benefit of taking account of the bone mobilization-reconstitution for both Ca and P requirements during lactation could be a reallocation of both mineral supplies from the beginning of lactation to the end of lactation, with the objective to reduce P rejection in early lactation and to enhance bone

reconstitution in late lactation. However, to achieve this outcome, a better quantification of bone accretion and resorption cycles over lactation is necessary, requiring the development of a fast and efficient method of estimation.

Available methods to follow bone cycles in dairy cows, such as bone biomarkers (Seibel, 2000), biopsies (Beighle, 1999) or input/output balances (Taylor et al., 2009), are efficient but also expensive or too time-consuming to be used in large numbers of cows. It has been described in mice that, in the case of Ca intake deficiency, milk Ca content can decrease in relation to a decrease in blood Ca detected by the Ca-sensing receptor of the mammary epithelial cell (VanHouten et al., 2004). This phenomenon also induces a secretion of parathyroid hormone-related protein (**PTHrP**), which is responsible for bone mobilization. A hypothesis arising from this mechanism is that milk Ca content variations could be a potential indicator of bone metabolism throughout lactation. With the possibility of using medium infrared spectra to determine milk Ca content, it would be a very useful way to follow cows' bone cycles throughout lactation. The aim of this study was to analyze variability in milk Ca and P content dynamics during lactation inherent to cow and the parity in relation with the variability of the dynamics of plasma biomarkers of bone accretion and resorption, i.e., osteocalcin (**OC**) and C-terminal telopeptide (**CTX**).

# B)  Material and methods

## 1  Animals and monitoring

This study involved 33 Holstein cows in their first (n=17), second (n=10), and third or greater (n=6) lactation, from 15 days before expected calving to the end of the 9th month of lactation, from September 2015 to June 2016. Cows were offered a unique total mixed ration (**TMR**) ad libitum throughout the lactation, which was composed of 63% corn silage, 11% dehydrated alfalfa, 11% soybean meal and 15% energy concentrate and minerals. The composition of the TMR was calculated to cover the $NE_L$ (Net Energy for Lactation), protein (**PDI**, protein digestible in the intestine), absorbable Ca and P, and other minerals; the requirements of cows after the lactation peak (INRA, 2010), with a PDI/$NE_L$ of 90 g PDI for 6 726 kJ of $NE_L$, showed absorbable Ca and P contents of 2.86 g/kg and 1.94 g/kg DM, respectively. Cows were housed in a free stall barn with a cubicle covered with rubber carpet.

The TMR was distributed twice a day by an automatic dispenser into an individual trough specific to each animal, owing to RFID identification. Cows had free access to the trough and to water during the day. Straw was added for the first 15 days of lactation (500 g/day). Cows were milked twice a day, at 0630 h and 1630 h. Milk production and DM intake were recorded daily individually. Milk composition (fat and protein contents) and somatic cell count were measured twice a week, upon evening and morning milking.

## 2   Blood and milk samples

Blood was sampled 15 days prior to the estimated date of calving, 15 days after calving and every 4 weeks after. Cows were grouped for sampling according to stage of lactation ($\pm$ 3.5 days), so that all blood samples could be collected every Thursday. Blood was sampled after milking, before cows were fed, by venipuncture of the tail vessels into Vacutainer tubes coated with lithium heparin for Ca and inorganic P (**Pi**) analyses, and EDTA for OC and CTX. Plasma was recovered after centrifugation at 3 000 x g for 12 minutes within 30 minutes of sampling and stored at -80°C for OC analysis and at -20°C for other analyses. Milk was sampled for Ca and P analyses the previous evening and on the morning of blood samples and was stored at -20°C.

## 3   Chemical analyses

Feed samples were dried and ground, and subsamples were mineralized by calcination at 550 °C for 5 hours in a muffle furnace. Feed, plasma and milk were analyzed by atomic absorption spectrophotometry (Spectra-AA20 Varian, Les Ulis, France) for Ca contents (Murthy and Rhea, 1967, Brûlé et al., 1974) and by the Allen method using a KONE PRO multi-parameter analyzer (Thermo Fisher Scientific, Illkirch, France) for P contents (Pien, 1969). Milk fat and protein concentrations were determined by a commercial laboratory using mid-infrared analysis (MyLab, Chateaugiron, France). Plasma CTX and OC concentrations were determined by ELISA with a Crosslaps kit from IDS (Paris, France) for CTX and a kit for OC from Quidel (San Diego, CA).

## 4   Calculation

The differences in absorbable Ca and P (**Caabs** and **Pabs**) supply – requirements were calculated according to absorption coefficient and cow requirements proposed by INRA

(2010), except that the actual Ca and P milk contents were used instead of the default values of 1.25 g/kg for Ca and 0.90 g/kg for P. Morning and evening milk production and DM intake were averaged on plus or minus 3 days to avoid effect of daily fluctuation.

## 5   Statistical analysis

Variables related to milk production and composition, plasma composition for explained variable and mineral requirement coverage were analyzed with a mixed-model ANOVA, using PROC MIXED in SAS (SAS Institute, 2013):

$$Y_{ijk} = \mu + Stage\,of\,Lactation_i + Parity_j +$$
$$Stage\,of\,Lactation : Parity_{ij} + Cow_k + \epsilon_{ijk}$$

where $Y_{ijk}$ was the explained variable, stage of lactation and parity were qualitative fixed effects, and cow was a random effect.

To classify the shapes of individual dynamics of milk Ca content throughout lactation and relate it to those of plasma concentrations of OC and CTX, principal component analysis was performed with R (R Development Core Team, 2008) and the package *FactoMineR*. In the table, the columns corresponded to a combination of a stage of lactation and a time of milk sampling (morning or evening) and the lines corresponded to a cow. As average milk Ca content varies between animals and as our objective was to classify dynamics, the data were scaled to limit the individual effect:

$$x'_{ij} = \frac{x_{ij} - x_i}{x_i}$$

Where $x_{ij}$ is the considered value for cow $i$ at the stage of lactation x time of sampling $j$, $x_i$ is the base value for cow $i$ (i.e., average of the considered data after the 6th month of lactation) and $x'_{ij}$ is the new scaled value. As all the data had the same unit, the data were not scaled for the principal component analysis. An ascending hierarchical classification was then performed on the factor scores using Ward's criterion to select the number of clusters. The best number of clusters was given by a high ratio of the loss of inertia between n+1 clusters and n clusters. Effect of clusters of milk Ca dynamics was analyzed by ANOVA using PROC MIXED in SAS with the following model:

$$Y_{ijk} = \mu + Stage\,of\,Lactation_i +\, of\,milk\,Ca\,Dynamic_j +$$
$$Stage\,of\,Lactation : Cluster\,of\,milk\ \,Dynamic_{ij} + Cow_k + \epsilon_{ijk}$$

where $Y_{ijk}$ was the explained variable, and stage of lactation and cluster of milk Ca dynamic were considered as qualitative fixed effects; cow was a random effect. Finally, regressions were performed between variables related to plasma concentrations of biomarkers, Ca and Pi (plasmatic component related to bone metabolism) and milk related to milk Ca and P contents (potential milk biomarker of bones), using PROC GLM in SAS:

$$Y_{ij} = \mu + x + Cow_i + Cow_i : x + \epsilon_{ij}$$

where $x$ is a milk potential biomarker, $Y$ is a plasmatic component related to bone metabolism, and cow is the fixed effect of the $i$-th animal. Only morning milk contents were kept, as their samples were done at the same time as that of the blood samples.

# C) Results

## 1 Differences in milk quality and Ca metabolism due to parity

Milk production was lower for primiparous cows than for multiparous (Figure III.1A), with an average daily milk production of 29.5 kg for the primiparous cows and 36.8 kg for the multiparous cows during the 9 months of lactation. The primiparous cows had a less differentiated peak of lactation and a better persistency than multiparous cows (Stage of lactation:Parity, P < 0.01). Milk protein content was not affected by parity (Figure III.1B). Milk Ca content varied from 957 to 1,816 mg/kg, with a mean of 1,249 mg/kg and a standard deviation of 113.0 mg/kg. Both morning and evening milk Ca contents decreased in early lactation and increased at the end of lactation (Stage of lactation, P < 0.01) but they were unaffected by the parity (P > 0.05, Figure III.1C). However, evening milk Ca content remained relatively steady throughout the lactation in cows in the third month of lactation or later, whereas it decreased during the 1ˢᵗ month of lactation and continuously increased after in primiparous cows, the dynamics being intermediate for second lactation cows (Stage of Lactation:Parity, P < 0.05). The interaction between parity and stage of lactation was not significant for morning milk Ca content. Morning and evening milk Ca to protein ratios increased during the first month of lactation and decreased after to reach a minimum between the 7ᵗʰ and the 8ᵗʰ month of lactation, and they slightly increased
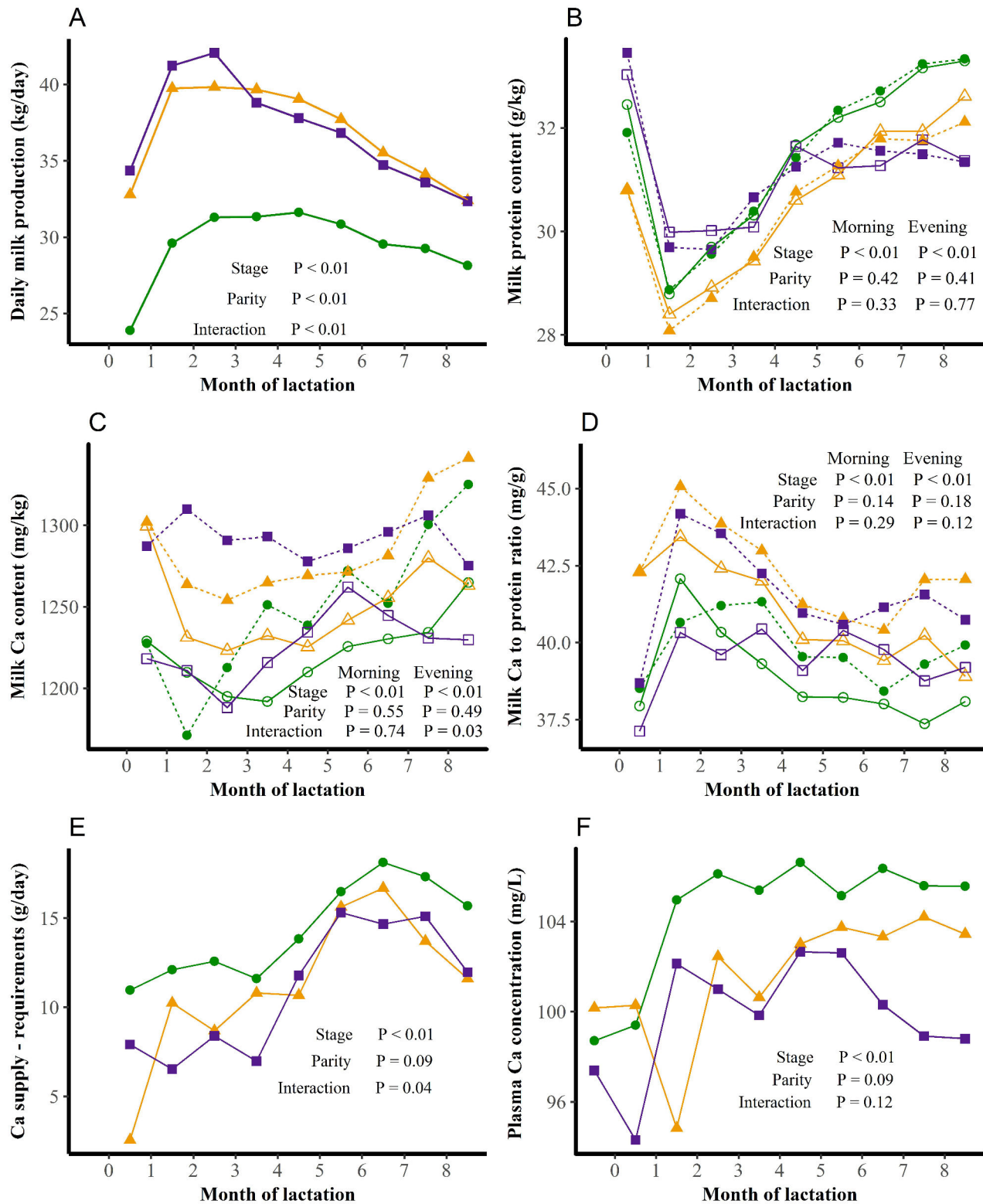
Figure III.1: Effect of parity and stage of lactation on A) daily milk production, B) milk protein content, C) milk Ca content, D) milk Ca to protein ratio, E) Ca supply - requirements, F) plasma Ca concentration. First Lactation ●/○, second lactation: ▲/△, third or more lactation: ■/□. White filled shape and straight line: morning sample, color filled shape and dotted line: evening sample.

afterward, regardless of the parity (Stage of lactation, $P < 0.01$, Figure III.1D). Both milk Ca contents and milk Ca to protein ratio were lower in the morning.

The difference in Caabs supply - requirements varied between -7.7 to 25.9 g/day (Figure III.1E). It increased from calving to the 7th month of lactation and decreased after (Stage of lactation, $P < 0.01$). It tended to be higher for primiparous throughout the lactation (parity, $P < 0.10$), but the discrepancy was above all important during the first 4 months of lactation (Stage of lactation:Parity, $P < 0.05$). Individual plasma Ca concentrations were between 71.4 and 120.8 mg/L, and only one cow had a plasma Ca concentration lower than 80 mg/L, which indicated that no cow was submitted to subclinical hypocalcemia in this trial (Taylor et al., 2008). Lower plasma Ca concentrations were reached during the first or the second month of lactation and then increased to values higher than pre-calving values (Stage of lactation, $P < 0.01$, Figure III.1F). After the second month of lactation, plasma Ca concentrations remained relatively steady. This outcome was clearer in primiparous than in multiparous cows, even though the interaction stage of lactation:parity was not significant. Primiparous cows also tended to have higher plasma Ca concentrations ($P < 0.10$, Figure III.1F).

Morning and evening milk P contents decreased during the first 2 months of lactation and then remained stable until the end of the lactation (Figure III.2A, $P < 0.01$) They were also higher for primiparous than for multiparous ($P < 0.02$). Unlike milk Ca content, milk P content was higher for evening milk. Milk Ca to P ratio slightly increased all over lactation (Figure III.2B, $P < 0.01$) and was lower for primiparous cows ($P < 0.01$). The group average of plasma Pi concentration (Figure III.2C) ranged from 39.8 to 63.5 mg/L. Primiparous had a tendency to have a lower plasma Pi concentration (47.6 vs. 50.8 mg/L average on lactation, $P < 0.10$, Figure III.2C). Plasma Pi was clearly affected by the stage of lactation, with a decrease after calving and then an increase until the end of lactation, regardless of the parity ($P < 0.001$).

Plasma concentrations of both OC (biomarker of bone accretion) and CTX (biomarker of bone resorption) were clearly higher in primiparous cows than those in second parity cows and higher in second parity cows than those in third or higher parity cows (Figures III.3A and B, $P < 0.01$). The plasma OC concentration clearly decreased after calving for all parities and increased during the second month of lactation to reach a plateau specific to each parity (Figure III.3A, stage of lactation, $P < 0.1$, stage of lactation:parity, $P > 0.15$).
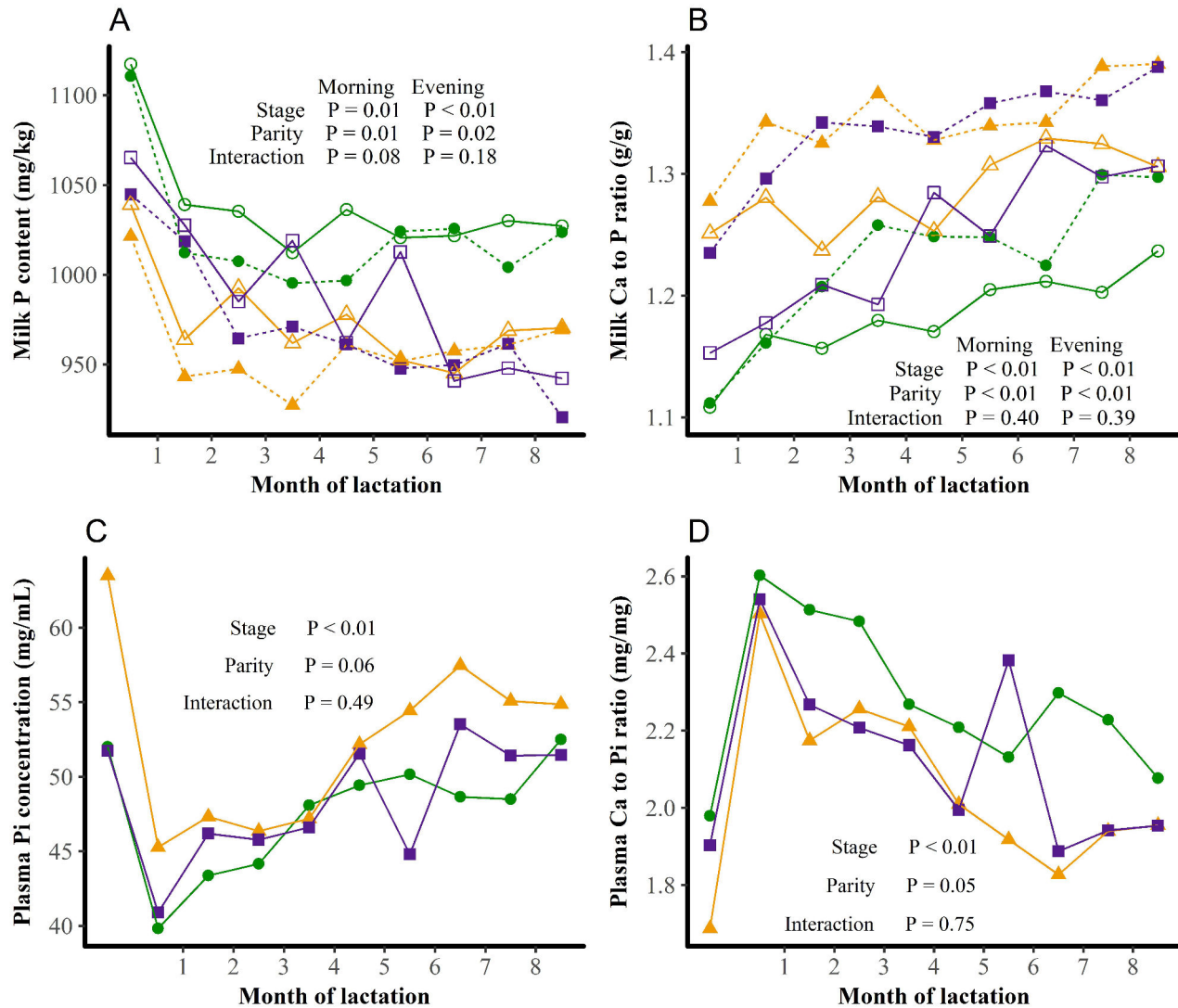
Figure III.2: Effect of parity and stage of lactation on A) milk P content, B) milk Ca to P ratio, C) Plasma Pi concentration, D) Plasma Ca to P ratio. First Lactation ●/○, second lactation: ▲/△, third or more lactation: ■/□. White filled shape and straight line: morning sample, color filled shape and dotted line: evening sample.

Plasma CTX concentration increased after calving to reach a maximum during the second month of lactation, then it decreased in primiparous and second parity cows and remained stable in third and higher parity cows (Figure III.3B, stage of lactation, P < 0.02). The amplitude of the variations of plasma CTX concentrations throughout lactation was higher in primiparous than that in the second lactation cows and higher in the second lactation than that in the third or higher lactation cows (stage of lactation:parity, P < 0.05). When considering only primiparous cows, the 13 2-year-old cows tended to have higher plasma OC concentrations than those of the 2 3-year-old cows (83.8 vs 70.2 ng/mL, on average during lactation, P = 0.06, data not shown, same model as that described to study parity effect,
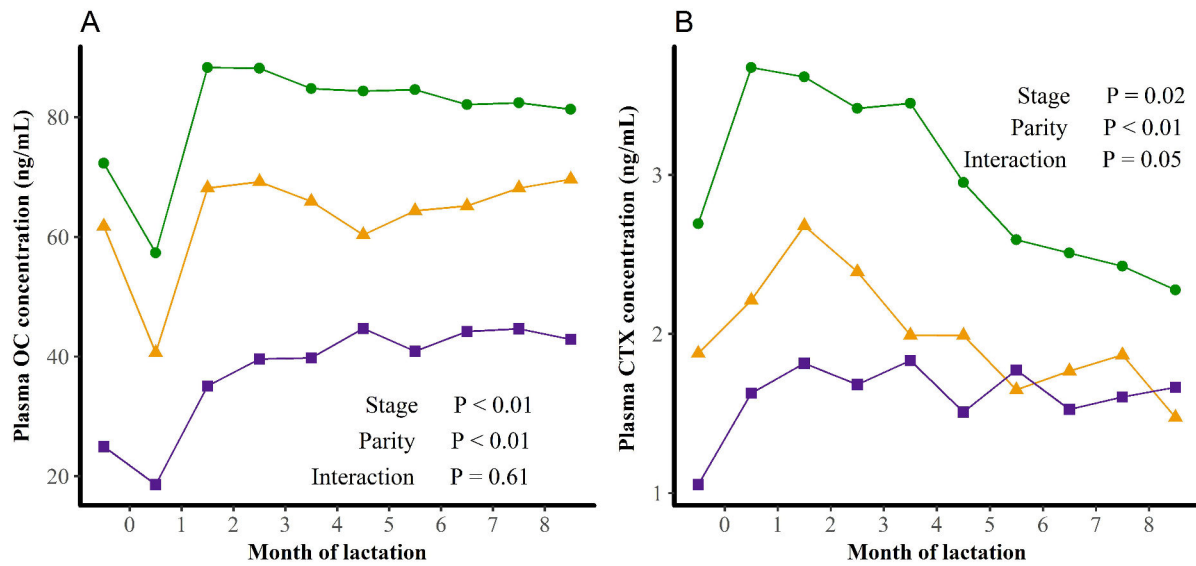
Figure III.3: Effect of parity and stage of lactation on A) plasma osteocalcin concentration and B) plasma CTX concentration. First Lactation ●, second lactation: ▲, third or more lactation: ■

on interaction effect). However, the age of primiparous cows did not affect plasma CTX concentrations.

## 2 Characterization of dynamics of milk Ca content

Three clusters of dynamics of the milk Ca content were characterized from principal component analysis and hierarchical ascendant classification (Figure III.4A). Clusters of dynamics were named according to the form of the milk Ca curves. The cluster *"Increasing"* dynamic was characterized by an increase in milk Ca content throughout lactation. It involved 9 cows, mainly primiparous (Table III.1). The *"Curving"* dynamic was characterized by a decrease in milk Ca content from the beginning of the lactation to 4.5 months of lactation for morning and evening milks and then by an increase until the end of lactation. It involved 8 cows. The *"Flat"* dynamic was characterized by a stable Ca content all over lactation and involved 16 cows with an almost homogenous partition of parity. The milk Ca content was not affected by the cluster of dynamics; this outcome was expected but was clearly affected by the stage of lactation and the interaction dynamic:stage of lactation.

| Milk Ca Dynamic | 1st Lactation | 2nd Lactation | 3rd Lactation or more |
|---|---|---|---|
| *"Increasing"* | 7 | 1 | 1 |
| *"Curving"* | 4 | 3 | 1 |
| *"Flat"* | 6 | 6 | 4 |

Table III.1: Repartition of cows between milk Ca content dynamics according to their parity
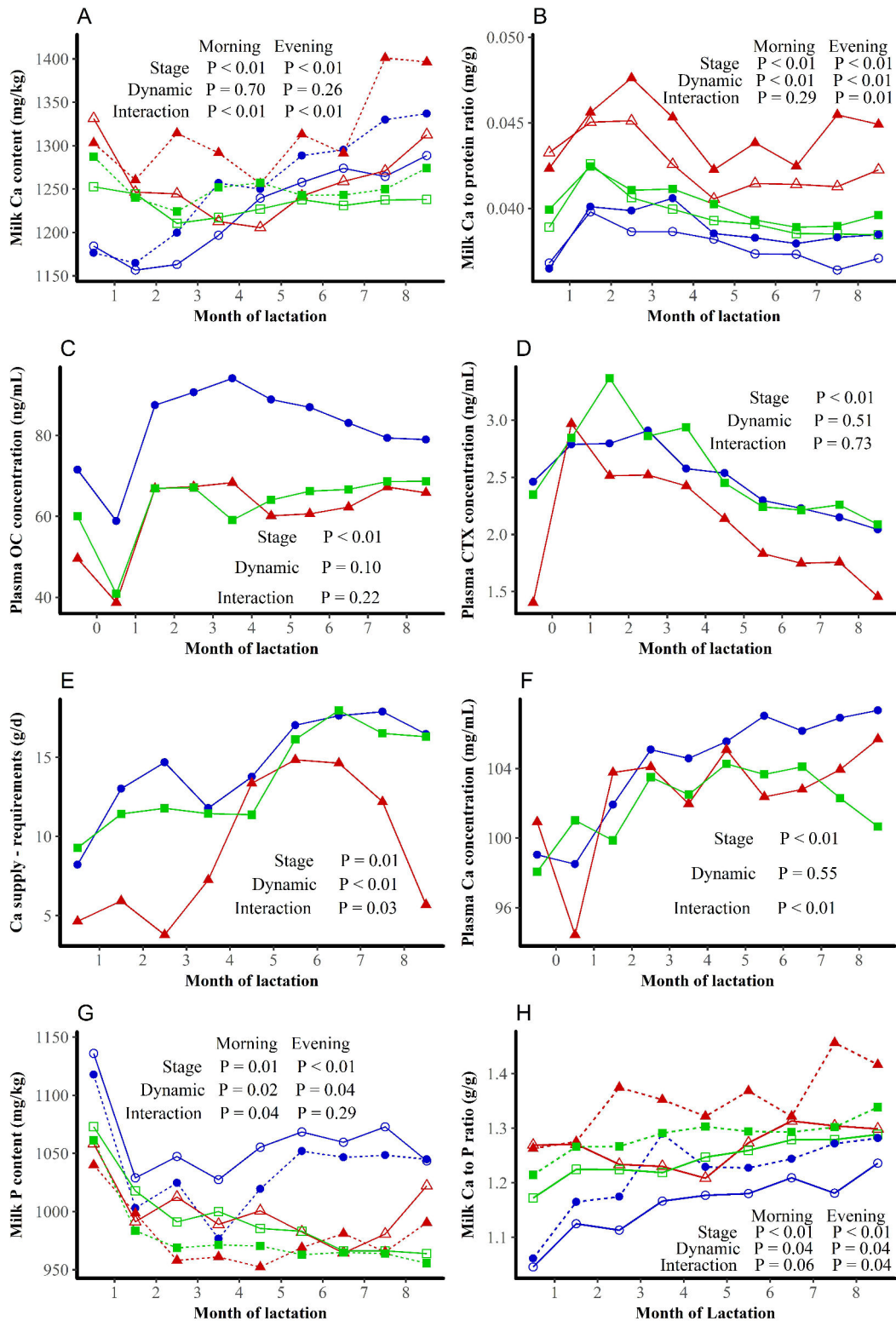
Figure III.4: Effect of cluster of milk Ca dynamic and stage of lactation on A) milk Ca content, B) Plasma Ca concentration, C) Plasma OC concentration, D) Plasma CTX concentration, E) Milk P content, F) Milk Ca to P ratio, G) Ca supply-requirements, H) Milk Ca to protein ratio. *"Increasing"* Dynamic: ●/○, *"Curved"* Dynamic: ▲/△, *"Flat"* Dynamic: ■/□. White filled shape and straight line: morning sample, color filled shape and dotted line: evening sample.

Neither the plasma concentrations of OC and CTX, nor their dynamics throughout lactation, were significantly affected by the three clusters of dynamics of milk Ca contents (Figures III.4C and D, P > 0.10). Milk production was not affected either by these clusters. Milk protein content was lower (P < 0.05, data not shown), and milk Ca to protein ratio was higher for the *"Curving"* dynamic (Figure III.4B, P < 0.001), with a higher variation during the first 5 months of lactation (interaction P < 0.01 in the evening). With the *"Curving"* cluster, the Ca supply – requirement was also clearly lower than those of both other clusters in the first four months of lactation, increased after the fifth month of lactation to reach similar values than those of both other clusters and decreased again after the 6th month of lactation (Interaction, P < 0.03). Plasma Ca concentration was lower after calving with the *"Curving"* than with both other clusters (P < 0.01). Morning and evening milk P contents were higher with the *"Increasing"* cluster (Figure III.4G, P < 0.05). Morning milk P content increased after the 6th month of lactation with the *"Increasing"* dynamic cluster, whereas it remained flat with the *"Curving"* and *"Flat"* dynamics (Figure III.4G, P < 0.05). Morning and evening milk Ca to P ratios were lower for the *"Increasing"* clusters compared with the *"Curving"* and *"Flat"* clusters (Figure III.4H, P < 0.05) and increased more during lactation (Figure III.4F, P < 0.06).

## 3   Relationship between plasmatic components related to bone metabolism and potential milk biomarkers

Four milk variables identified a priori as potential indicators of bone metabolism were tested: Ca content, P content, Ca to P ratio, and amount of Ca daily secreted in milk. They were used as predictive variables in regression to estimate plasma concentrations of Ca, Pi, CTX, OC and OC to CTX and CTX to OC ratios (Table III.2). About half of the tested models were not significant (P > 0.05). Among the significant models, a prediction of the plasma OC to CTX ratio from the milk Ca to P ratio showed a very significant effect of the predictor (P < 0.001), and no effect of either individual intercept or interaction between individual effect and milk ratio. When considering this latter relationship, most of the individual regression had a positive slope between milk Ca to P ratio on the plasma OC to CTX ratio with a similar slope coefficient (Figure III.5). Among the four cows with a negative slope, three had a very bad quality of regression.

| Heading | Tested effect | Milk Ca content | Milk P content | Milk Ca to P ratio | Daily Ca secreted in milk |
|---|---|---|---|---|---|
| Plasma OC | Predictor | 0.217 | < 0.001 | 0.002 | < 0.001 |
| | Individual | < 0.001 | < 0.001 | 0.038 | < 0.001 |
| | Interaction | < 0.001 | < 0.001 | 0.64 | < 0.001 |
| Plasma CTX | Predictor | 0.293 | 0.072 | 0.007 | 0.696 |
| | Individual | < 0.001 | 0.170 | 0.001 | 0.014 |
| | Interaction | < 0.001 | 0.293 | 0.008 | 0.310 |
| Plasma Ca | Predictor | 0.745 | 0.479 | 0.304 | < 0.001 |
| | Individual | < 0.001 | 0.170 | < 0.001 | < 0.001 |
| | Interaction | < 0.001 | 0.293 | < 0.001 | < 0.001 |
| Plasma Pi | Predictor | 0.332 | 0.003 | 0.186 | 0.011 |
| | Individual | 0.229 | 0.067 | 0.137 | 0.089 |
| | Interaction | 0.231 | 0.049 | 0.154 | 0.127 |
| Plasma OC to CTX ratio | Predictor | 0.582 | 0.005 | < 0.001 | 0.093 |
| | Individual | 0.086 | 0.252 | 0.813 | < 0.001 |
| | Interaction | 0.036 | 0.155 | 0.726 | < 0.001 |
| Plasma CTX to OC ratio | Predictor | 0.235 | 0.006 | < 0.001 | < 0.001 |
| | Individual | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | Interaction | < 0.001 | < 0.001 | < 0.001 | < 0.001 |

Table III.2: Prediction of bone metabolism by potential milk biomarker
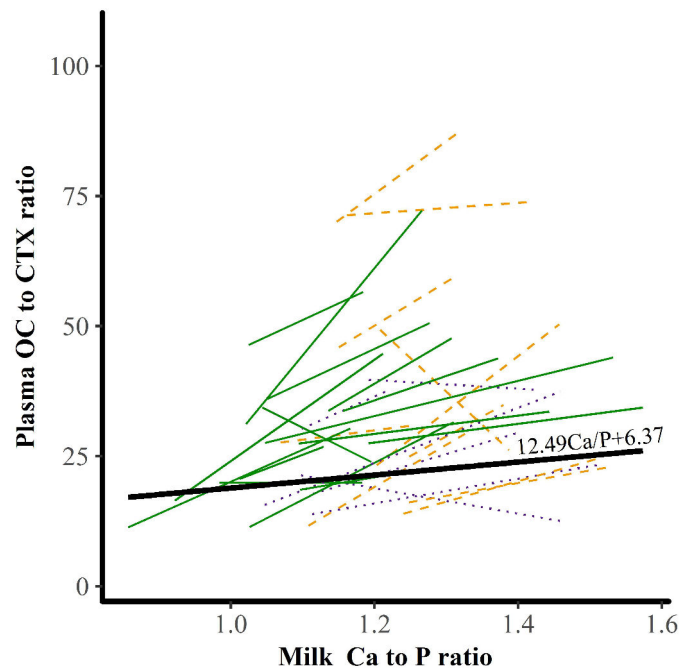


Figure III.5: Relationship between plasma OC to CTX ratio and milk Ca to P ratio. Black bold line = inter-individual regression. Intra-individual regression = green straight line for 1st lactation, orange dashed line for 2nd lactation, purple dotted line for 3rd or more lactation.

# D)   Discussion

## 1   An effect of parity on bone remodelling and on dynamics of bone resorption during lactation related to that of milk content of Ca

An effect of parity on bone remodeling and on the dynamics of bone resorption during lactation related to that of the milk content of Ca. The increasing plasma concentrations of OC and CTX with decreasing parities observed in this experiment can be related to the fact that primiparous cows and, to a less extent, $2^{nd}$ lactation cows are still growing, as well as to the fact that bone turnover is higher in those animals than that in $3^{rd}$ or higher rank lactation cows. OC is a hydroxyapatite-binding protein synthetized by osteoblasts; this protein is suspected to be active in the organization of the extracellular matrix of bone, and thus, it constitutes a plasmatic biomarker of bone accretion that is widely used, especially in cows (Seibel, 2000). In contrast, CTX is a degradation product of bone collagen constituted by a crosslink containing the collagen telopeptide, which constitutes a plasmatic biomarker of bone resorption. The fact that plasma OC concentration increased with parity can be clearly related to the animal growth, and this outcome has been observed at least during the first months of lactation in several studies (Iwama et al., 2004, Taylor et al., 2008, Taylor et al., 2009, Sato et al., 2011, Sato et al., 2013). The fact that plasma CTX also increased is less intuitive, but it illustrates that bone is a metabolically active tissue that undergoes continuous remodeling with concomitant accretion and resorption, and this remodeling is more active in young animals throughout lactation. Similar results have been observed by Iwama et al. (2004) and Taylor et al. (2009) during the first month of lactation, but with deoxypyridinoline instead of CTX.

Monitoring whole lactation in this study allows us to show that the relative increase in plasma CTX at the beginning of lactation was proportionally higher in primiparous cows and, to a lesser extent, in $2^{nd}$ lactation cows compared to higher rank lactation cows. It is known, in particular, owing to the analyses of plasma biomarkers of bone accretion and resorption, that a decrease in bone accretion consistently occurs during the first month of lactation and that an increase in bone resorption occurs during the first months of

lactation (Liesegang et al., 2000, Taylor et al., 2008, Taylor et al., 2009, Puggaard et al., 2014). Our results confirmed this outcome, but the bone resorption increase was likely higher in younger animals when considering CTX measurements. This result would be coherent with the idea that in younger cows, bone $1,25\text{-}(OH)_2\text{-}$ vitamin D receptor activity is higher, which gives younger animals a higher ability than older cows to mobilize Ca from bone store (Horst et al., 1990). Such results have been suggested by Iwama et al. (2004) and Taylor et al. (2009), but as these authors measured plasma biomarkers during 1 to 4.5 months, it was difficult to conclude from their results whether the higher CTX concentration of young animals during the first month of lactation was a higher bone remodeling or an increase of net bone mobilization. In our study, the fact that plasma CTX increased and decreased during the first 4 months of lactation, with a higher amplitude in younger animals, indicates that net bone mobilization should have been higher in those animals. It cannot be considered in our experiment that primiparous cows mobilized their bone more in relation with their specific Ca supply, because their Ca requirements coverage tended to be higher than those of older cows.

An interesting result was that the greater amplitude of the increase in CTX at the beginning of lactation in primiparous cows was accompanied by a greater amplitude of the decrease in milk Ca content during the same period, at least when considering the evening milk sampling. Animals with higher ranks of lactation even showed an almost steady curve of milk Ca content at the evening sampling. This coincides with our hypothesis that the dynamic of milk Ca content could be used as an indicator of dynamic of bone mobilization during lactation. This hypothesis is based on the observation that the extracellular Ca-sensing receptor of the mammary gland would allow sensing of the calcemic status of the cows, in order to maintain or drive Ca in the blood circulation when there are strong Ca demands by organs, by both decreasing Ca secretion in milk and increasing bone resorption by PTHrP secretion (VanHouten et al., 2004). Whether this effect of the interaction parity:stage of lactation is only observed in evening and not in morning milk remained unclear. It is known that melatonin, secreted during the night, can affect bone accretion-resorption balance (Cardinali et al., 2003) and that the higher milking interval during the night is susceptible to affect milk composition because of the higher accumulation of some milk compounds in the udder cisterns. The interaction

parity:stage of lactation did not affect either the milk Ca to protein ratio, even though a part of Ca is secreted in milk with casein via the Golgi apparatus with a necessity of presence of Ca for stability of casein (VanHouten and Wysolmerski, 2007). This outcome shows that the effect of the interaction parity:stage of lactation on milk Ca content is not mediated by the casein secretion in milk, confirming that milk casein content can only explain a part of the milk Ca content (Gaignon et al., 2018b).

We did not observe any effect of the parity on milk Ca content, which contrasts with the results of Gaignon et al. (2018b), who showed that milk Ca content decreased in cows with high parity. However, the effect of parity observed by Gaignon et al. (2018b) remained low, less than 7 mg/kg, between two successive parities of Holstein cows and was negligible compared to individual genetic effect on milk Ca content (Gaignon et al., 2018b). Given the relatively low number of cows involved in our study, it is likely that the absence of parity effect in the present study could be explained by the difference of detection threshold in statistical analysis between our study and that of Gaignon et al. (2018b). The tendency for a higher calcemia in primiparous cows compared with higher parity cows could be related to both results; those cows may have a better ability to mobilize bone for calcemic regulation, and their Ca requirement coverage also tended to be better. However, the calcemia variations in our experiment remained minor, which shows that calcemia was well regulated.

## 2 The shape of the dynamics of milk Ca content does not allow a prediction of the shape of the dynamics of bone mobilization during the lactation but may reflect a lower coverage of Ca requirements

The use of principal component analysis and hierarchical classification shows that a variability of dynamics of milk Ca contents during lactation exists between individuals and that this variability is not entirely explained by the parity, even though one cluster of dynamics of milk Ca contents included mainly primiparous cows. However, the dynamics of plasma concentrations of either OC or CTX were unaffected by the cluster of dynamics of milk contents of Ca during lactation, suggesting that the relationship between the dynamics of plasma CTX and milk Ca that we observed between parities could not be

extrapolated between individuals. Several reasons for this could be evocated. It can be considered that OC and CTX are only biomarkers of bone accretion and resorption and that their measurement at one time during a month may not be precise enough to characterize the variability of dynamics of bone mobilization throughout lactation that could occur between individuals. From this point of view, milk samples present the advantage of giving a relatively integrative image of physiological processes over a period of a few hours. It can also be considered that the time scale at which milk Ca secretion or bone mobilization may be involved to regulate calcemia may not be concomitant during lactation but also between individuals.

It is interesting to notice that the *"Curving"* cluster is the only one in which the dynamics of milk Ca content is totally consistent with those described in the literature (Kaufmann and Hagemeister, 1987, Gaignon et al., 2018b) . This cluster corresponded to cows having a lower coverage of their Ca requirements in the first 4 months of lactation and concomitant decreasing milk Ca contents, as well as lower calcemia 15 days after calving. It cannot be excluded that a decrease in milk Ca secretion at this time did not contribute to the Ca homeostasis and that mammary glands may adapt to a lower Ca supply by decreasing the milk Ca content. This outcome was not concomitant to bone mobilization, possibly because this cluster integrated mainly multiparous cows for which bone mobilization seemed less readily involved.

## 3   The milk Ca to P ratio: an indicator of bone mobilization?

Our initial hypothesis was that the dynamics of milk Ca content during lactation could reflect the dynamics of bone mobilization because a decrease in milk Ca content could be a leverage to reduce milk Ca exportation and to maintain calcemia when the homeostasis is challenged. We observed that this hypothesis may be valid when comparing parities, but not when considering variations between individuals. However, we also observed a clear effect of parity on milk P contents, with higher contents in primiparous cows, which may be related also to the better of ability of these cows to mobilize their bones. A higher plasma Pi concentration in primiparous cows was expected (Forar et al., 1982, Anderson et al., 2017) but not observed in our experiment. Evocated reasons for this expected higher plasma Pi concentration in primiparous cows are both high growth-hormone contents, which can increase renal phosphate reabsorption, and high bone turnover observed in

young animals (Anderson et al., 2017). A reason for the absence of higher plasma Pi concentration in primiparous cows may be the fact that plasma sampling frequency was low in our experiment, whereas an effect of the time of sampling on plasma Pi concentration exists (Forar et al., 1982). However, we observed a clear increase in the milk P contents in primiparous cows, which is also consistent with other studies (Forar et al., 1982). This could suggest that P can be secreted in milk when bone mobilization or remodeling is increased. As more bone remodeling is occurs in first lactation cows, it is possible to consider that greater P flows are present throughout the body. Saliva is known to be a great way to excrete excess P, which could occur during high bone remodeling. It is possible that a part of this excess flow is also transferred into milk. Alvarez-Fuentes et al. (2016) showed that a decrease in Ca intake, likely generating bone resorption, led to an increase in milk P content, likely in relation with more P released from the bone into the organism. However, this mechanism remained a hypothesis, given that the process of P secretion in milk is not well known, although an active mechanism of secretion via Golgi apparatus has been suggested (Neville and Peaker, 1979, Shennan and Peaker, 2000).

Our data suggest that the milk Ca to P ratio seems appropriate to estimate relative variation of plasma OC to CTX ratio at least within individuals. Even though the effects of individual and interaction predictor:individual on the plasma OC to CTX ratio were non-significant, it is likely that the relationship may not be strong enough for an inter-individual prediction, even though it certainly contributed to estimate the variability between primiparous and multiparous cows. Indeed, using the regression intra-stage of lactation to explain the OC to CTX ratio by the milk Ca to P ratio (by including an effect of the stage of lactation, instead of an effect of the cows, in the model) did not give a significant effect on the milk Ca to P ratio (data not shown). The link between those ratios is biologically consistent. On the one hand, the plasma OC to CTX ratio constitutes a relative estimation of net bone reconstitution, because it gives an idea of the equilibrium between OC, a biomarker of bone accretion, and CTX, a biomarker of bone resorption. On the other hand, the milk Ca to P ratio is also expected to increase when net bone reconstitution increases because milk Ca is expected to decrease with bone mobilization according to our hypothesis, and milk P is expected to increase according to the results discussed above. It is interesting to consider that the Ca to Pi ratio has also been

investigated in blood and showed a good relationship with plasma CTX concentration in beef cows (Anderson et al., 2017). However, given the limited knowledge of P regulation and P secretion in milk, notably their link with Ca in the context of lactation, the relevance of the milk Ca to P ratio to predict bone net reconstitution remains to be consolidated. The characterization of clusters of individual dynamics of the milk Ca to P ratio did not allow either to predict the individual dynamic of plasma OC to CTX ratio (data not shown).

# E) Conclusion

This experiment confirms that primiparous cows are better able to mobilize their bone at the beginning of lactation to regulate their calcemia and shows that the increase in bone mobilization in these animals was concomitant to a decrease in milk Ca content. However, individual dynamics of milk contents of Ca during lactation did not allow the prediction of individual dynamics of bone mobilization/reconstitution at the scale of the lactation. The milk Ca to P ratio could be an interesting indicator of bone mobilization within individuals. This indicator would have to be confirmed in a challenging situation for calcemia regulation.

# Acknowledgements

# Ethics statement

Procedures relating to care and use of animals for the experiment were approved by an animal care committee of the French Ministry of Agriculture, in accordance with French regulations (authorization n APAFIS# 3122- 2015112718172611).

# Effects of breed and feeding strategies on bone accretion and resorption and milk calcium and phosphorus content during lactation in dairy cows

# Abstract

Current recommendations for Ca and P supplementation in dairy cows do not take account of the dynamics of bone mobilisation/reconstitution that occurs during lactation. This study aimed to determine if the dynamics of milk Ca content during lactation could allow predicting the dynamics of bone mobilisation/reconstitution for cows fed with two different feeding strategies. It consisted in measuring monthly milk Ca and P contents and plasma concentrations of biomarkers of bone accretion (OC) and resorption (CTX) on 30 Holstein and Normande cows spread between two feeding strategies, based on different energy density, from 15 days before expected calving to the end of lactation. Multiparous cows showed higher plasma OC and CTX concentrations in Normande cows than in Holstein cows($P < 0.01$) but also higher plasma biomarkers concentrations in the high feeding strategy than in the low feeding strategy ($P < 0.01$). This point was related with an important increase in milk production ($P < 0.01$) with the cows having a higher diet energy density. In the same time, primiparous cows only showed effect of feeding strategy on bone accretion ($P = 0.05$), but no differences due to breeds or on bone resorption. However, differences related to breed and feeding strategy on bone accretion and resorption could not be related to variations in milk Ca content over lactation. The possibility to use the milk Ca to P ratio to estimate plasma OC to CTX ratio as it was suggested in the literature was also unsatisfactory, notably for the cows receiving low diet energy density.

**Keywords:** Dairy cows, calcium, diet, bone, breed

## Effects of breed and feeding strategies on bone accretion and resorption and milk calcium and phosphorus content during lactation in dairy cows

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# A)  Introduction

Important flows of Ca and P occur in dairy cows' organism during lactation, mainly because of important flow of Ca and P excretion in milk (Horst et al., 1997). Bones play a major role in the regulation of calcemia and phosphatemia, notably in early lactation when Ca and P supplies cannot cover requirements (Horst et al., 2005). Bones allow then mobilization of their mineral phase, largely composed of Ca and P bound hydroxyapatite molecules (Buckwalter et al., 1996). Bones are also able to store Ca and P again when supply of those mineral exceeds the requirements (Braithwaite, 1983a). Bone tissue is constantly being renewed thanks to combined existence of accretion and bone resorption flows (Ramberg et al., 1970, Braithwaite, 1983a, Seibel, 2000). The difference between these flows of accretion and resorption features either a net bone mobilization or a net bone reconstitution (Ramberg et al., 1970; Braithwaite, 1983). The beginning of lactation in lactating cows is associated with a relatively higher bone resorption than bone accretion (Ekelund et al., 2006; Puggaard et al., 2014) and thus often a net bone mobilization (Ramberg et al., 1970; Taylor et al., 2009). At the contrary, after 3 months of lactation, accretion becomes relatively higher than resorption leading to a bone reconstitution (Ekelund et al., 2006; Taylor et al., 2009; Puggaard et al., 2014). Consequences of the amplitude and completeness of these cycles of bone mobilization and reconstitution during lactation on reproduction and health of cows have been suspected but not demonstrated yet (McNeill et al., 2002, Oetzel and Miller, 2012, Dixon et al., 2017).

Several studies showed that a variability of the amplitude and completeness of cycles of bone mobilization and reconstitution exists in dairy cows but quantifications are too

scarce to allow a characterization of their effects on reproduction performances or health of cows. It has been observed that parity (Gaignon et al., 2018a) or milk yield (Liesegang et al., 2000) affected the amplitude of those cycles, with higher bone resorption variation during lactation in primiparous or high producing cows. Dietary contents of Ca and P have been also shown to clearly affect either the amplitude of those cycles (Braithwaite, 1983; Taylor et al., 2009; Elizondo Salazar et al., 2013; Puggaard et al., 2014) or their completeness (Braithwaite 1983). However, a major limit in the characterization of cycles of bone mobilization and reconstitution is that methods available to quantify them cannot be applied on a high number of animals. The measurement of input and output flows of Ca, and eventually P, at the scale of the animal, eventually coupled to radio-isotope, supplies interesting information about the net daily flow of body retention and mobilization of those elements, and thus on bone mobilization and reconstitution if we consider that more than 99% of Ca is stored in bones (Ramberg et al., 1970; Taylor et al., 2009). However, those methods require blocking animals to daily collect urine and feces, that is difficult to perform at the scale of a whole herd, and regulation about radio-isotope makes their use almost impossible with large animals. The use of blood biomarkers of accretion and resorption allowed numerous studies quantifying the amplitude of the bone cycles in the last 20 years (Liesegang et al., 2000; Ekelund et al., 2006; Puggaard et al., 2014), but the cost of the analyses relatively limits the number of animals involved in those studies. Bone biopsies (Beighle, 1999, Dixon 2017) gives information about either the chemical composition or histomorpholgy of bones but repetitions of those measurements are technically challenging and can be ethically controverted

The possibility to use milk Ca content to reflect variations in bone resorption could be suggested from the results of VanHouten et al. (2004), who showed a decrease in milk Ca content with increased bone resorption in mice. Gaignon et al. (2018a) suggested that the milk Ca to P ratio may better reflect variations in the equilibrium between bone accretion and resorption than milk Ca alone in dairy cows. With the possibility to use MIR spectra to predict milk mineral content (Soyeurt et al., 2009; Gaignon et al., 2018b), this milk ratio could allow the possibility to follow bone mobilization during lactation on a large number of dairy cow. Gaignon et al. (2018a) observed that milk Ca to P ratio was correlated to the ratio between a blood biomarker of bone accretion, i.e. osteocalcin (**OC**),

and a blood biomarker of bone resorption, i.e.  C-terminal telopeptide of type I collagen (**CTX**) when considering both effect of the stage of lactation and parity. Questions remained to determine if this correlation is still significant when considering variability arising from feeding strategies at the scale of the lactation or breeds. Normande cows, in comparison to Holstein cows, are characterized by a lower milk production and higher milk protein and fat contents (Delaby et al., 2009). Even though milk Ca content in Normande is higher than in Holstein, Normande secrete lower amount of Ca in milk daily (Gaignon et al., 2018b). The consequences of those traits on dynamics of bone accretion and resorption during lactation have never been measured. It has also been suggested that bones can be involved in energy systemic regulation (Wolf, 2008), which could suggest that feeding strategies differentiated on the energy density of the diet could affect the dynamics of bone accretion and resorption at the scale of the lactation.  Our hypothesis was that testing the consequences of these factors of variation on the correlation between milk Ca to P ratio and plasma OC to CTX ratio would allow determining if this correlation is generalizable to various conditions of lactation, which would mean that milk Ca to P ratio could be an interesting predictor of dynamics of bone accretion and resorption at the scale of the lactation.

During one year, measurements of blood biomarkers of accretion and resorption and milk contents of Ca and P were added to the multi-annual experimental design described by Bedere et al. (2017).  The first aim was to investigate the effect of breed and energy density in diet on dynamics of bone accretion and resorption along lactation.  The second aim was to test the hypothesis of the milk Ca to P ratio as a biomarker of bone accretion and resorption equilibrium in various conditions of lactation in dairy cows.

# B)   Material and methods

## 1   Animals, diets, management, and experimental design

Thirty cows were involved in this study between 2 months before their expected date of calving in November 2015, and the end of the lactation in December 2016.  These cows were taken from the 60 cows annually involved in a multi-annual experimental design that takes place, since 2006, at the dairy research farm of Le Pin-au-Haras (48.448N, 0.098E, Normandy, France).  The experimental design has been described by Bedere et al. (2017).

Each year, about 60 cows constituted the experimental herd, with half Normande and half Holstein. Within each breed, half cows were managed under a 'High' feeding strategy (**FS**) that consisted in higher energy density of diets enabling higher milk yield while limiting body condition (**BC**) loss; and half cows were managed under a 'Low' FS that consisted in diets with lower energy density, limiting milk yield while inducing a large BC loss (Table IV.1). Cows were randomly assigned to the feeding strategies three weeks before their first calving and remained in it until they were culled due to lack of pregnancy, severe health problem or accidental death.

| | Season | | | |
| | Winter | | Summer | |
| Feeding strategy | High | Low | High | Low |
| Ration Type | TMR[1] | TMR[1] | PMR[2] | Grazing |
| Corn Silage | 57% | - | 0-4 kg DM/d | - |
| Dehydrated Alfalfa | 12% | - | | |
| Energy concentrate[3] | 31% | - | | |
| Grass Silage | - | 51% | | |
| Big Hale Haylage | - | 47% | | |
| Energy concentrate[4] | - | - | 4 kg DM/d | - |
| CMV[5] | - | 2% | | |
| | | | | |
| **Diet Feeding Value**[6] | | | | |
| PDI/UF (g/ 1700 kcal) | 96.3 | 103.4 | | |
| CP (g/kg DM) | 151.7 | 162.1 | | |
| Ca (g/kg DM) | 9.1 | 11.2 | | |
| P (g/kg DM) | 3.6 | 4.7 | | |
| | | | | |
| **Grazing**[7] | | | | |
| Surface (ha/cow) | - | - | 0.33 | 0.55 |
| PDI/UF (g/ 1700 kcal) | | | 108.2 | 106.1 |
| CP (% DM) | | | 17.7 | 16.7 |
| Ca (g/kg DM) | | | 6.5 | 7.1 |
| P (g/kg DM) | | | 3.2 | 3.3 |

Table IV.1: Table of diet composition according to season and feeding strategies. [1]: TMR = Total Mixed Ration. [2]: Partial Mixed Ration. [3]: 12% wheat, 12% corn, 12% barley, 11% beet pulp, 45% soybean meal, 1% soybean oil, 2% melasse, 4% CMV, 1% chloride sodium. [4]: 21%, 21% corn, 21% barley, 21% beet pulp, 12% tanned soybean meal, 2% vegetal fat, 1% melasse, 1% chloride sodium. [5]: 50% lime, 22% monocalcium phosphate, 15% magnesia, 4% chloride sodium, 3% melasse, 2% sodium sulfate, 1% water. [6]: for TMR and non-grazing part of PMR. [7]: for grazing and grazing part of PMR.

The thirty cows involved in our experiment were selected in October 2015, about two months before the average calving date of the herd, with half cows taken from the high

FS and half taken from the low FS. The first criteria of selection was their expected date of calving with the objective to obtain a group with calving date as gathered as possible around a week of similar rank within each month to allow one milk and blood sampling every 4 weeks, gathered at similar stages of lactation among involved cows. Repartition of cows according to breed, FS, parity and month of calving are presented in table IV.2.

Cows started to receive the winter diet, according to the FS they are attributed, three weeks before expected calving. Cows were housed in a free stall barn in winter. A total mixed ration was distributed once a day by an automatic dispenser at 0900 h. Cows had free access to the trough and to water. Orts were recorded daily before the a.m. feedings. Lactating cows were fed *ad libitum* and offered quantities were calculated to allow 10% orts. Cows were milked twice a day, at 0800 h and 1645 h. Milk production was recorded daily individually. Milk composition (fat and protein contents) was measured six times a week on evening and morning milking and somatic cell count was measured once every two weeks. Cows were weighted once a week. From 23$^{rd}$ March to 1$^{st}$ December, cows grazed permanent grassland with rotational grazing, paddock changes being decided according to the decline of milk production of the herd (Hoden et al., 1991).

## 2 Blood and milk samples

Blood was sampled 15 days prior expected calving date, 15 days after actual calving date ($\pm$ 3 days) and every 4 weeks after. Blood was obtained, after milking, before cows were fed, by venepuncture of the tail vessels into Vacutainer tubes coated with lithium heparin for Ca, inorganic P (**P**i) and Non Esterified Fatty Acids (**NEFA**) analyses, and EDTA for OC and CTX. Plasma was recovered after centrifugation at 3 000 x g for 12 minutes within 30 minutes of sampling, and stored at -80°C for OC analysis and at -20°C for other analyses. Milk was sampled for Ca and P analyses on the previous evening and the morning of blood samples and was stored at -20°C.

## 3 Chemical analyses

Plasma and milk were analysed by atomic absorption spectrophotometry (Spectra-AA20 Varian, Les Ulis, France) for Ca content (Murthy and Rhea, 1967; Brûlé et al., 1974) and by the Allen method using a KONE PRO multi-parameter analyzer (Thermo Fisher Scientific, Illkirch, France) for P content (Pien, 1969). Milk was mineralized (550°C, 8h) for analyses of

P contents. Milk fat and protein contents were determined by a commercial laboratory using mid-infrared analysis (Lilano, Saint-Lô, France). Plasma CTX and OC concentrations were determined by ELISA with a Crosslaps kit from IDS (Paris, France) for CTX and a kit for OC from Quidel (San Diego, CA). Plasma NEFA concentrations were determined by enzymatic colorimetry on a multiparameter analyser (Kone Instruments Corporation, Espoo, Finland).

## 4  Statistical Analysis

A generalized linear mixed model was used to analyze the using PROC GLIMMIX in SAS (SAS Institute, 2013):

$$Y_{ijkl} = \mu + Stage\,of\,Lactation_i + Breed_j + Feeding\,Strategy_k +$$
$$Stage\,of\,Lactation : Breed_{ij} + Stage\,of\,Lactation : Feeding\,Strategy_{ik} +$$
$$Breed : Feeding\,Strategy_{jk} +$$
$$Stage\,of\,Lactation : Breed : Feeding\,Strategy_{ijk} + Cow_l + \epsilon_{ijkl}$$

where $Y_{ijkl}$ was the explained variable, stage of lactation (i $\in$ [1,9]), breed (j $\in$ [1,2]) and feeding strategy (k $\in$ [1,2]) were qualitative fixed effects, and cow, within a breed and a feeding strategy, a random effect. Repeated values were on the cow according to stage of lactation. Selection for the best covariance matrix was performed using AIC for every variable. Morning and evening milk mineral contents were analyzed separately. Primiparous and multiparous were analyzed separately, as parity has an important effect on bone mobilization (Iwama et al., 2004; Taylor et al., 2009; Gaignon et al., 2018a). Due to high number of elements in the statistical model, non-given p-values in the figures mean absence of significant effect (P > 0.10). Regressions between plasma OC to CTX ratio and milk Ca to P ratio were also calculated, using the following model

$$Y_{ij} = \mu + x + Cow_i + Cow_i : x + \epsilon_i j$$

where x is the milk Ca to P ratio, Y is the plasma OC to CTX ratio, cow is the fixed effect of the i-th animal. Only morning milk contents were kept for these analysis because their sampling were concomitant to that of blood.

| Month of calving | | January | | February | | March | | Total | | Average lactation |
| Parity | | Primi | Multi | Primi | Multi | Primi | Multi | Primi | Multi | rank* |
| Breed | FS | | | | | | | | | |
| Holstein | High | 2 | 0 | 1 | 1 | 1 | 1 | 4 | 2 | 1.5 (2.5) |
| Holstein | Low | 1 | 2 | 1 | 0 | 1 | 2 | 3 | 4 | 2.8 (4.2) |
| Normande | High | 2 | 1 | 0 | 0 | 0 | 3 | 2 | 4 | 3.3 (4.5) |
| Normande | Low | 4 | 1 | 0 | 4 | 0 | 2 | 4 | 7 | 2.7 (3.7) |

Table IV.2: Repartition of cows between breed, parity, feeding strategy and month of calving. FS: Feeding strategy; *: Rank of lactation between parenthesis is the average rank of lactation without primiparous

# C) Results

Due to differences in reproduction performances between breeds (Bedere et al., 2017) and to constraints on calving date specific to our experiment, the proportion of primiparous and multiparous cows differs between modalities of breed and feeding strategy. The proportion of primiparous cows was higher for Holstein with high feeding strategy compared to the 3 other FS x breed intersections (Table IV.2). The average rank of lactation of Holstein were 1.5 ($\pm$ 0.34) and 2.8 ($\pm$ 0.70) for high and low FS respectively while the average rank of lactation of Normande were 3.3 ($\pm$ 0.95) and 2.7 ($\pm$ 0.57) for high and low FS.

## 1 Milk production, milk protein and fat contents

Milk production was lower for Normande compared with Holstein for both primiparous and multiparous ($P < 0.01$ for both, Figure IV.1A and B). On average over the 40 weeks of lactation, it was $20 \pm 2.03$ and $28.6 \pm 3.05$ kg/d for primiparous and multiparous, respectively, in Holstein whereas it was $15.9 \pm 2.24$ and $19.3 \pm 2.22$ kg/d in Normande. Milk production was higher for high FS compared to low FS ($P < 0.01$ for primiparous and multiparous, $27.6 \pm 3.05$ vs $20.2 \pm 2.23$ kg/d for high and low FS, respectively, for multiparous and
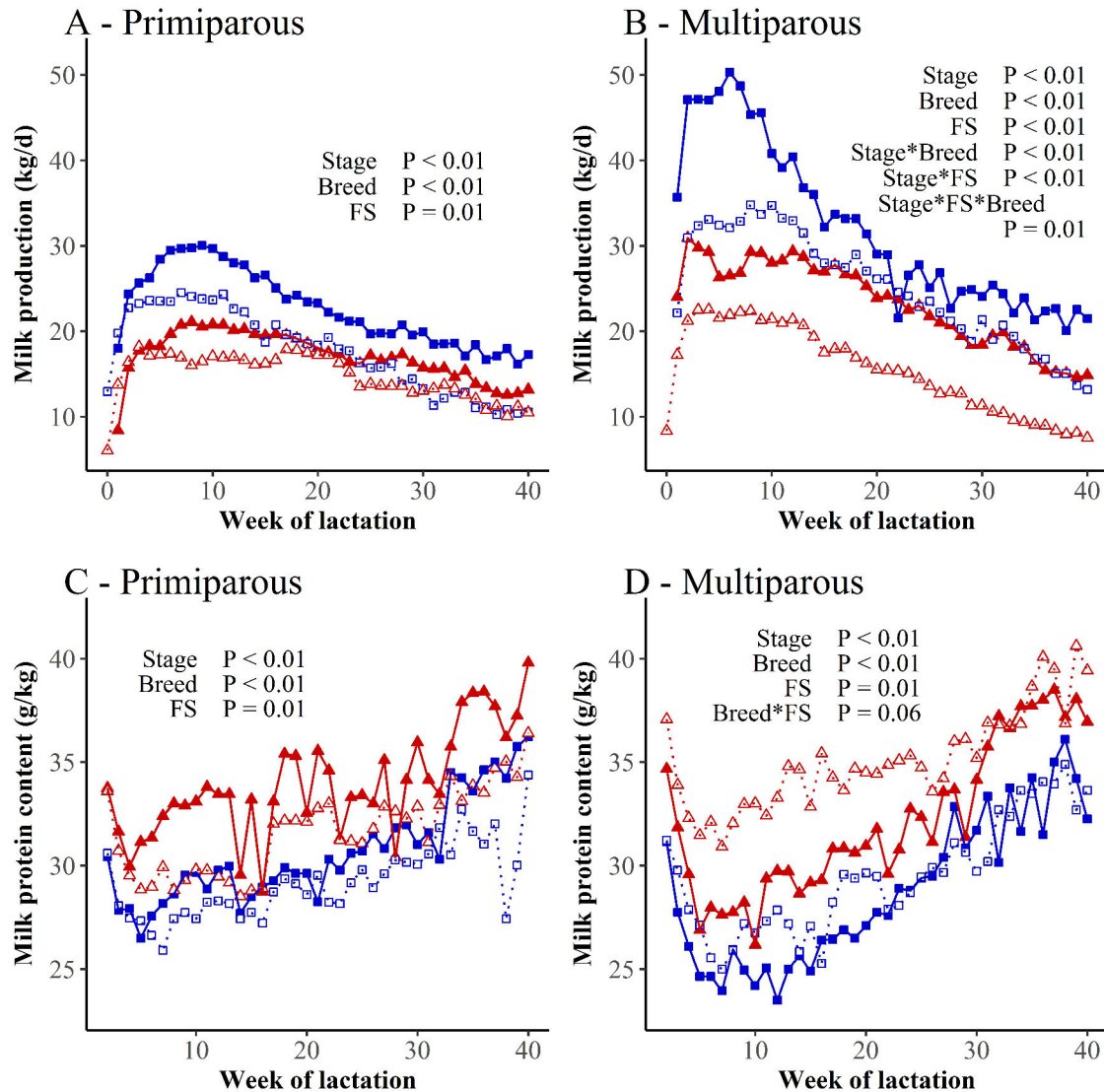
Figure IV.1: Effect of breed and feeding strategy on milk production (A and B) and milk protein content (C and D) according to parity. Dotted lines and white-filled shape are for the low feeding strategy and straight lines and color-filled shapes for the high feeding strategy. Holstein: ■/□; Normandes: ▲/△

19.9 ± 2.24 vs 16.2 ± 2.03 kg/d for high FS and low FS, respectively, for primiparous). Milk production at the peak of lactation was higher and peak of lactation was more pronounced with Holstein fed with high FS, leading to significant effects of the interactions stage of lactation x breed, stage of lactation x FS, and stage of lactation x breed x FS. Milk protein content was higher for Normande compared to Holstein, whatever the FS (P < 0.01, Figure IV.1C and D) over 40 weeks of lactation (33.6 ± 0.34 vs 29.1 ± 0.47 g/kg for multiparous Normande and Holstein, respectively). Milk protein content was also higher for the high FS compared with low FS for both primiparous and multiparous (P < 0.001, 32.3 ± 0.34 vs 30.4 ± 0.46 g/kg in multiparous fed with high vs low FS).

## 2  Plasma concentrations of Ca, Pi, OC, CTX and NEFA

For multiparous and primiparous cows, plasma OC, which is the blood biomarker of bone accretion, clearly decreased after calving to reach a minimum at 0.5 months of lactation, increased until 3 months of lactation, remained stable then before a decrease in late lactation after 8 months of lactation (Figure IV.2A and B, P < 0.01). OC concentrations were numerically more important for primiparous than multiparous (difference not tested). In primiparous cows, plasma OC was higher with high FS compared with low FS (P = 0.05, Figure IV.2A), especially at the end of lactation (P = 0.08). In multiparous cows, plasma OC was higher for Normande compared with Holstein (44.7 $\pm$ 3.87 vs. 26.7 $\pm$ 5.34 ng/ml on average over the 10 sampling times, P < 0.01, Figure IV.2B), particularly between the 3$^{rd}$ and the 8$^{th}$ month of lactation (P < 0.01 for interaction stage of lactation $\times$ breed)

For primiparous cows, plasma CTX, which is the blood biomarker of bone resorption, clearly increased after calving until 2.5 months of lactation in primiparous and decreased after 4 months of lactation (P < 0.001, Figure IV.2C). It was unaffected by the breed, the feeding strategy or their interactions. For multiparous cows, plasma CTX was relatively steady during the lactation with the low FS whereas it increased sharply after calving and decreased also sharply after 3 months of lactation with the high FS (P < 0.01 for stage and interaction stage x FS, Figure IV.2D). Plasma CTX was higher for Normande than Holstein (2.2 $\pm$ 0.23 vs. 1.5 $\pm$ 0.32 ng/mg on average over the 10 sampling times, P < 0.01), with higher amplitude of variation during the lactation (P < 0.01 for interaction stage of lactation $\times$ breed).

In primiparous cows, plasma NEFA increased after calving and decreased after the 2$^{nd}$ month of lactation (P < 0.01, Figure IV.2E), with maximal values being reached at 0.5 month of lactation. Plasma NEFA was unaffected by the breed, the feeding strategy or their interactions. In multiparous cows, with high FS, plasma NEFA hugely and sharply increased after calving, with values over 800 µmol/l during the 2$^{nd}$ month of lactation, whereas, with low FS the amplitude of variation were much lower (P < 0.01 for stage of lactation and its interaction with FS, Figure IV.2F). Plasma NEFA was also lower in Normande compared with Holstein (P < 0.01), mostly because Holstein showed a higher increase in NEFA concentrations after calving with the low FS.

Averaged plasma Ca per modalities of stage of lactation, parity, breed and feeding
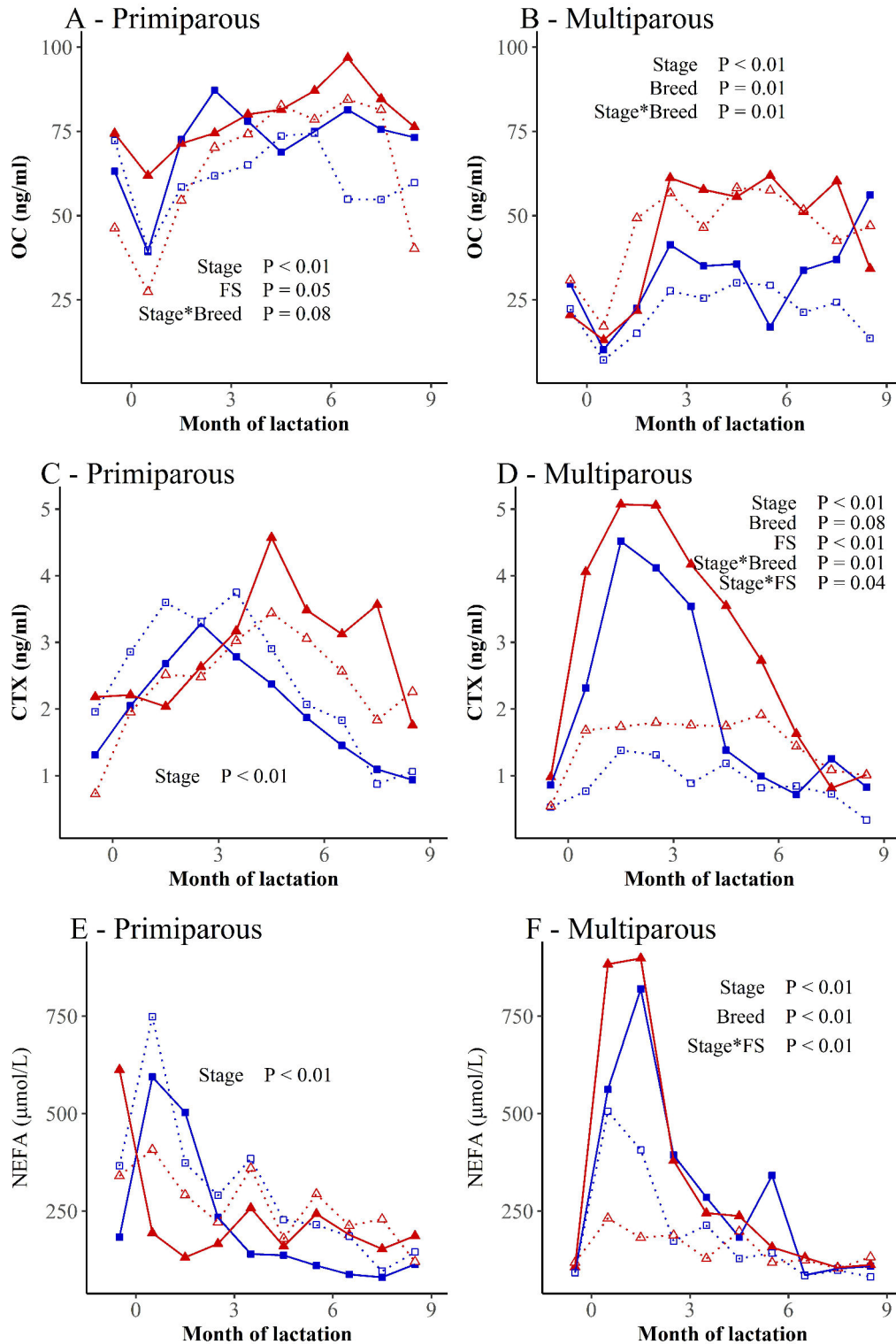
Figure IV.2: Effect of breed and feeding strategy on plasma concentrations of OC (A and B), CTX, (C and D) and NEFA (E and F) according to parity. Dotted lines and white-filled shape are for the low feeding strategy and straight lines and color-filled shapes for the high feeding strategy. Holstein: ■/□; Normandes: ▲/△

strategy was always comprised between 95 and 120 mg/L (Figure IV.3A and B), which is within the physiological range of variation for dairy cows. Only one cow was in
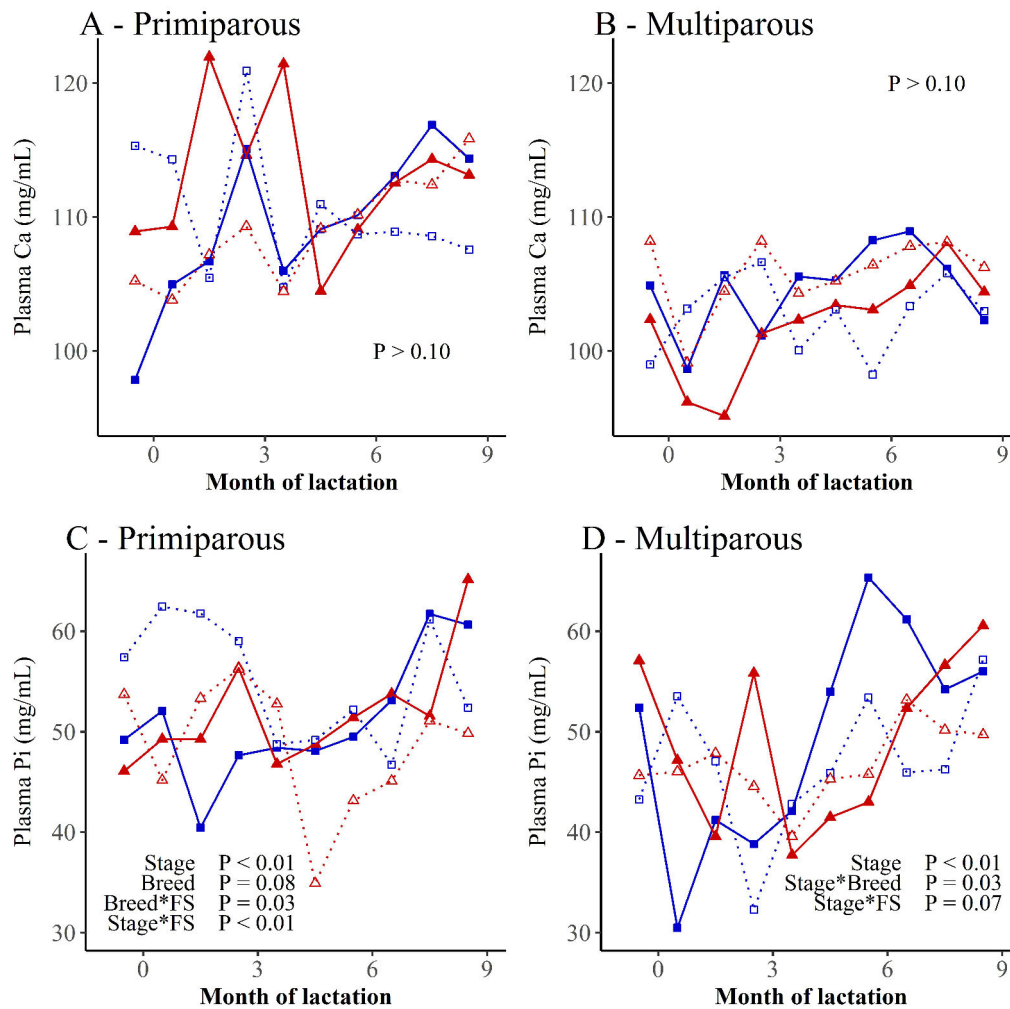
Figure IV.3: Effect of breed and feeding strategy on plasma concentrations of Ca (A and B) and Pi (C and D) according to parity. Dotted lines and white-filled shape are for the low feeding strategy and straight lines and color-filled shapes for the high feeding strategy. Holstein: ■/□; Normandes: ▲/△

hypocalcemia 15 days before calving, with a calcemia of 69.6 mg/L which is under 80 mg/L (Data not shown, Holstein cow within high FS). Plasma Ca remained relatively stable during the whole lactation (P > 0.10). Plasma Pi showed higher variations throughout lactation than plasma Ca, with average values over the 10 samplings times between 30 and 65 mg/L. For primiparous cows, plasma Pi was lower with high FS than with low FS until 3 months of lactation whereas it was lower after (P < 0.01 for interaction stage of lactation × FS). It was also lower with Normande than Holstein until 3 months of lactation whereas it was lower after and tended to be lower with Normande compared with Holstein over the lactation (P = 0.03 for interaction stage of lactation × breed, P = 0.08 for breed).

## 3   Milk Ca and P contents

In primiparous cows, both morning and evening, milk Ca content were higher with Normande compared with Holsetain (Figure IV.4A and C, P < 0.01 for morning and P = 0.07 for evening). In the morning, milk Ca tended to increase at the beginning of lactation and to decrease after for Normande whereas it tended to decrease at the beginning of lactation and increase after for Holstein (P = 0.07, interaction stage of lactation $\times$ breed, Figure IV.4C). In the morning at 3 and 4 months of lactation, milk Ca tended also to be higher with high FS compared with low FS for Normande whereas the contrary tended to be observed for Holstein (P = 0.08, interaction stage of lactation $\times$ breed $\times$ FS, Figure IV.4C). In multiparous cows, milk Ca clearly decreased at the beginning of the lactation to reach a minimum between 3 and 4 months of the lactation and increased after whatever the breed or the FS (P < 0.01 in both morning and evening, Figures IV.4B and IV.4D). Milk Ca content was also higher in Normande than Holstein in both morning and evening whatever the FS (P < 0.01, figure IV.4B and D, with for evening, average milk Ca over lactation of $1.3 \pm 0.02$ g/kg for Normande and $1.2 \pm 0.02$ g/kg for Holstein). Amplitude of variations of milk Ca during lactation was also higher in Holstein (P = 0.04 interaction stage of lactation $\times$ breed in the evening). Milk Ca was also higher with high than low FS (P = 0.01 in the evening, Figure IV.4B), especially after the $5^{th}$ month of lactation (Stage of lactation $\times$ FS, P = 0.05 in the morning, P < 0.01 in the evening),.

For primiparous cows, milk Ca to protein ratio did not significantly vary with the stage of lactation (P > 0.10, Figure IV.4E), whereas, in the evening, it increased during the $1^{st}$ month of lactation, reached a maximum between the $2^{nd}$ and the $4^{th}$ month of lactation and decreased after (P < 0.01, Figure IV.4G). It also tended to be lower with high FS compared to low FS in the evening (P = 0.10, Figure IV.4G). For multiparous cows, as for primiparous cows, the effect of the stage of lactation on milk Ca to protein ratio was limited (P = 0.10 for stage of lactation or P = 0.09 for the interaction stage of lactation $\times$ breed $\times$ FS, Figure IV.4F) but in the evening milk Ca to protein ratio followed a dynamic during lactation comparable to that of primiparous cows (P < 0.01). Milk Ca to protein ratio was high with high compared to low FS whatever the breed in the morning (P < 0.01, Figure IV.4F) or tended to be higher in the evening (P = 0.10). In the evening, amplitude of variation of the milk Ca to protein ratio during lactation was higher with the high FS (P < 0.01, interaction stage of lactation
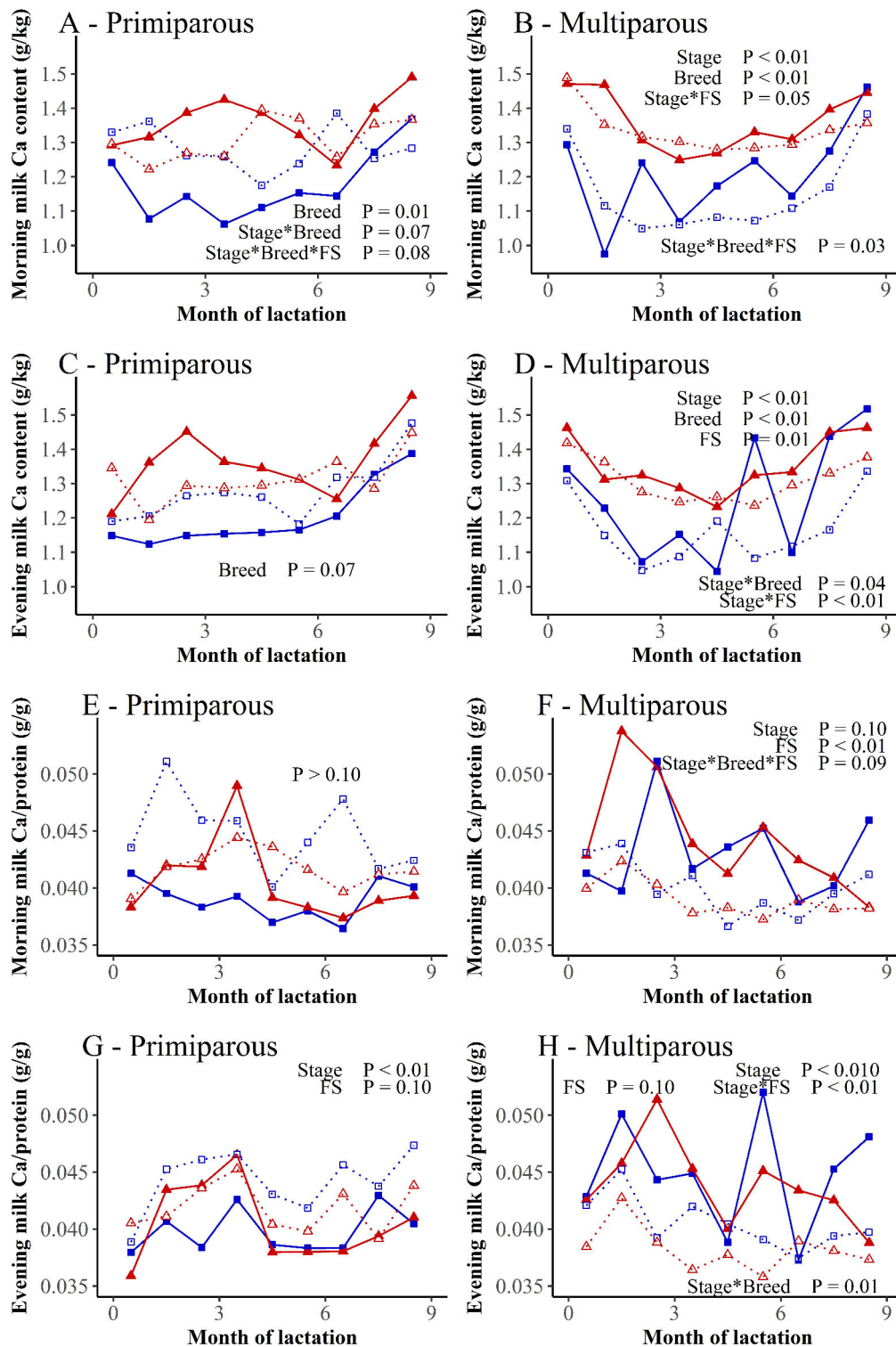
Figure IV.4: Effect of breed and feeding strategy on milk Ca content (A, B, C and D) and Ca to protein ratio (E, F, G an and H) according to parity and time of sampling. Dotted lines and white-filled shape are for the low feeding strategy and straight lines and color-filled shapes for the high feeding strategy. Holstein: ■/□; Normandes: ▲/△

× FS). In both evening and morning or for both primiparous or multiparous cows, the effect of the breed on the milk Ca to protein ratio was barely significant.

For primiparous cows, milk P did not significantly vary with the stage of lactation, except a transient increase at 5 months of lactation in the morning (P = 0.02 in the morning and P > 0.10 in the evening, Figure IV.5A and C). For multiparous cows, it decreased after the 1st month of lactation (P < 0.01 in the morning, P = 0.10 in the evening, Figure IV.5B and D). For both primiparous and multiparous cows, milk P was unaffected by either the breed or the FS.
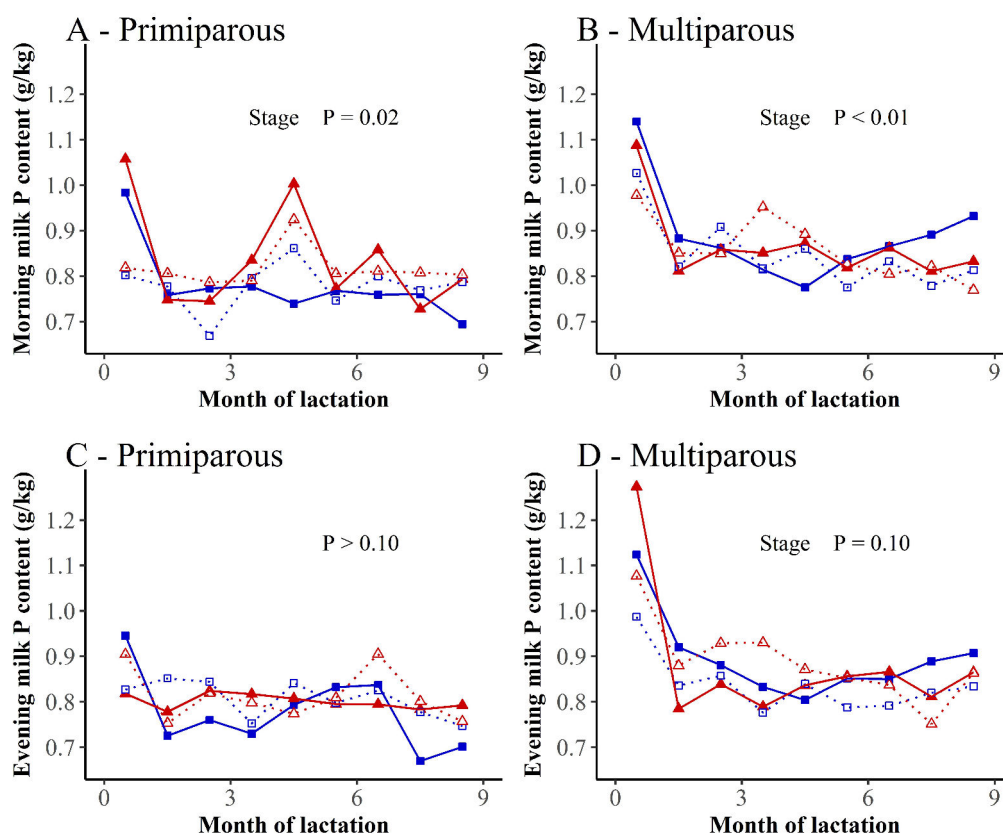


Figure IV.5: Effect of breed and feeding strategy on milk P content (A, B, C and D) according to parity and time of sampling. Dotted lines and white-filled shape are for the low feeding strategy and straight lines and color-filled shapes for the high feeding strategy. Holstein: ■/□; Normandes: ▲/△

## 4    Relationship between plasma OC to CTX ratio and milk Ca to P ratio

Plasma OC to CTX ratio only tended to be affected by milk Ca to P ratio when considering all the data of the experiment (P = 0.07), and was unaffected by either the cow or the interaction cow × milk Ca to P ratio (respectively P = 0.42 and P = 0.40).  Considering

only data obtained with Normande or with Holstein did not improve the model. When considering only data obtained within the high FS, plasma OC to CTX ratio was affected by milk Ca to P ratio (P = 0.05, Figure IV.6A) whereas it was unaffected by the same ratio when considering only data obtained with the low FS (P = 0.41, Figure IV.6B). However, the quality of the regressions remained low in both cases, respectively 0.34 and 0.42 respectively for the high and the low FS. Two of 12 cows in the high FS presented a negative relationship between plasma OC to CTX ratio and milk Ca to P ratio whereas 7 of 18 cows were in this case in the low FS. For both FS, plasma OC to CTX ratio was unaffected by either the cow of the interaction cow × milk Ca to P ratio.



Figure IV.6: Relationship between plasma OC to CTX ratio and milk Ca to P ratio in A) the high feeding strategy; B) The low feeding strategy. Bold line = inter-individual regression. Intra-individual regression = straight lines for high feeding strategy, dashed lines for low feeding strategy. Holstein: blue; Normandes: red

# D) Discussion

## 1 Differences on milk production and composition due to feeding strategies and breed along lactation and repartition of cows

Compared with previous results obtained from the same experiment (Bedere et al., 2017), the effects of feeding strategies and breed on milk production and composition were expected. High FS led to higher milk production than low FS in relation with the higher

energy density of the diets fed within the high FS. Normande produced less milk than Holstein but with higher protein and fat contents, which is a well described characteristic of the breed (Delaby et al., 2009, Cutullic et al., 2009, Dezetter et al., 2015). Amplitude of variation of milk production and fat and protein contents between feeding strategies and breeds were systematically lower in primiparous than multiparous.

## 2 Bone and adipose tissue mobilization during lactation according to feeding strategies, parity and breed

Whatever the parity, the dynamics of plasma biomarkers of both bone accretion, i.e. OC, and bone resorption, i.e. CTX, were very similar to those described in the literature with a sharp and transient decrease in bone accretion after calving and a more smooth and lasting effect on bone resorption (Liesegang et al., 2000, Taylor et al., 2009, Puggaard et al., 2014, Gaignon et al., 2018a). Both plasma biomarkers of accretion and resorption were mostly higher in primiparous than in multiparous cows as observed previously, indicating a higher bone remodeling (Iwama et al., 2004, Taylor et al., 2009, Gaignon et al., 2018a). Gaignon et al. (2018a) also observed a higher amplitude of variation of plasma CTX during lactation in primiparous than in multiparous cows. In the present experiment, this higher amplitude could only be observed when the dynamics of CTX of primiparous were compared to those of the multiparous cows fed the low FS.

In multiparous cows, amplitude of variation of plasma biomarker of bone resorption during lactation was clearly higher in high than in low FS. This was accompanied by a higher amplitude of variation of plasma NEFA. These results indicated that the multiparous cows with high FS mobilized more their bone and their adipose tissue than the multiparous cows fed the low FS (Cunningham and Klein, 2007). This should likely be explained, at least, partially, by the higher milk production of the cows fed with the high FS, leading to higher Ca, P and $NE_L$ requirements and maybe a more negative balance of those nutrients at the beginning of lactation. Liesegang et al. (2000) also described, in dairy cows, an increased bone mobilization with increasing milk production. Lean et al. (2014) highlighted a possible role of bone in energy regulation, which could be especially important in the case of dairy cows. However, the link they proposed was related to adiponectin, osteoblast activity and osteocalcin production but we did not observe any effect of the treatment of plasma OC in

the present experiment and thus there is no evidence that such mechanisms could have been involved. In primiparous cows, no effect of the feeding strategy on either plasma biomarkers of bone accretion or resorption or plasma NEFA could have been observed. This has to be related to the limited effect of the feeding strategy on the milk production of those cows. It is also likely that the high basal bone remodeling activity in those animals allow a maintenance of the higher calcemia, as numerically observed in figure IV.3, leading to a lower necessity of bone resorption for calcemia regulation. It is known also that younger cows present higher ability for digestive absorption of Ca, and it is possible that digestive absorption can also be involved more effectively for calcemia regulation in those animals (Horst et al., 1990). This shows the importance to consider the that diet effect on body reserve mobilization can be very different according to the parity of the cows.

An original result of the present study was that breed affected the dynamics of plasma biomarkers of both bone accretion and resorption, with higher amplitude of variation of both OC and CTX in Normande than in Holstein for multiparous cows. This showed a higher increase in bone remodeling and a relatively higher increase in bone resorption during lactation with Normande compared to Holstein. These differences could not be attributed to higher milk production given that milk production of Normande was lower than that of Holstein. These differences were not associated either to higher adipose tissue mobilization in Normande. It has been observed in human that ethnic origin can affect the basal level of blood biomarkers (Seibel, 2000). Such explanation should apply to breed for cows. However, whether or not these differences of dynamics of plasma biomarkers between cows could be related to actual differences of bone net mobilization remained to be determined. In primiparous cows, the effect of breed on the dynamics of plasma bone biomarkers was not significant, suggesting that the "basal level" would not be so different between breed but rather that the involvement of bone in calcemia regulation could be different between breeds. As evocated above, the fact that the effect of breed on plasma bone biomarker was not significant in young cows could be related to the fact that calcemia regulation would be easier in those animals due to higher basal bone remodeling and higher ability of digestive absorption of Ca.

The absence of effect of FS on plasma Ca is not surprising as it is very finely regulated (Horst et al., 1997), with very low variation all over the dataset. In contrast, plasma Pi

showed important variations along lactation, but they remained hard to explain. Plasma Pi is known to be more variable because it is less regulated (Horst et al., 2005). It can be noticed than phosphatemia decreased sometimes under 40 mg/mL, especially for Holstein of the high FS, meaning a possible P dietary deficiency for dairy cows (INRA, 2018).

## 3 Dynamics of milk Ca, P and Ca to protein ratio during lactation according to feeding strategies, parity and breed

The milk Ca content we observed in Normande has already been described (Gaucheron, 2005, Gaignon et al., 2018b). It was partly explained by the higher protein content in that breed, as suggested by the less significant effect of the breed on milk Ca to protein ratio compared to milk Ca. The dynamics of milk Ca content during lactation with multiparous cows was totally in accordance with that described by Gaignon et al. (2018b), with a clear decrease at the beginning of lactation and an increase at the end of lactation. The amplitude of dynamics was less pronounced in primiparous cows with limited effect of the stage of lactation. This could be due to low number of primiparous cows as the intersection between FS and breed leading to a high threshold of detection of significant effect. However, some of the dynamics of milk Ca measured for primiparous cows were similar to those reported by Gaignon et al. (2018a), with a constant increase along lactation. The results of the present study also illustrated a high variability of the milk Ca to protein ratio, suggested that milk Ca content is not determined by milk protein content even though Ca has an important place in casein micelle structure (Farrell et al., 2006). The ratio between Ca and casein in milk is as more determined by the equilibrium in the milk between Ca and micelles than by common pattern of secretion between casein and Ca (Farrell et al., 2006, VanHouten and Wysolmerski, 2007). Milk P content was mainly affected by the stage of lactation as described in the literature (Kaufmann and Hagemeister, 1987).

An hypothesis of this study was that the dynamics of milk Ca content would be related to that of plasma bone biomarkers. However, the clear positive effect of the high FS on the amplitude of variation on plasma bone biomarker of accretion was not accompanied by a higher decrease in milk Ca during lactation as we assumed from the study of VanHouten et al. (2004). This indicates that milk Ca alone may not be a good indicator of the amplitude of bone mobilization during lactation when comparing diets. A reason could be that several

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organs interact to regulate plasma Ca, and that the role of the mammary gland in plasma Ca regulation may be limited at the scale of the whole lactation.

## 4   Can milk ratio Ca to P be a predictor of plasma ratio OC to CTX within individual?

Gaignon et al.  (2018a) suggested that variations of milk Ca to P ratio could reflect those of plasma OC to CTX ratio during lactation within cows.  Thanks to the use of rapid methods of analyses of milk contents of Ca and P, such as MIR spectrometry analyses, this relationship could be an interesting tool to measure the shape of the dynamics of the equilibrium between bone accretion and resorption during lactation in important numbers of cows. The relevance of such a relationship partly relies on a negative relationship between milk Ca content and plasma CTX concentration that could be explained by the fact that the mammary gland is able to reduce Ca secretion in milk and to secrete PTHrP that increases bone mobilization, in case of low Ca diet, as observed in mice by VanHouten et al. (2004). The fact that Gaignon et al.  (2018a) observed that milk Ca to P ratio could be a better predictor of bone mobilization than Ca remained unexplained.  Anderson et al.  (2017) highlighted that plasma ratio Ca to Pi can be high in case of dietary P deficiency in some studies with ruminants.  This difference could be the fact that breeder cows observed by Anderson et al. (2017) were facing a P deficiency, when dairy cows may be more subject to face Ca deficiency.

In the present experiment, the effect of the milk Ca to P ratio on plasma OC to CTX ratio was not as clear as that observed by Gaignon et al. (2018a) given that it was even hardly significantly when considering all the data.  It became significant when considering only the data with the high FS, which corresponded better to the conditions in which Gaignon et al. (2018a) obtained their relationship.  The fact that milk ratio Ca to P did not affect plasma ratio OC to CTX with the low FS may be due low diet energy density limited bone mobilization and thus that maybe mammary gland did not have to adapt milk Ca content for regulation of plasma Ca concentrations (VanHouten et al., 2004). Our results could suggest that milk Ca to P ratio could only be used as a bone biomarker in a case of sufficient energy density in the diet.  However, this impossibility to use the milk Ca to P as bone biomarker ratio with low energy intake significantly decreases its field of application to follow bone

mobilization in commercial farms. In France, many dairy farms use pasture based diet in spring and summer with low or no energy supplementation (Gaignon et al., 2018b), making almost impossible to use this milk Ca to P ratio as a bone biomarker in commercial dairy farms.

# E) Conclusion

This experiment clearly illustrated that feeding strategy based on the $NE_L$ density of the diets can strongly affect the bone mobilization of cows. In the present experiment, the cows that mobilized the more their bones were those that mobilized the more their adipose tissue likely because of a high milk production with high feeding strategy. This experiment also illustrated that the bone mobilization may be different according to the breed and this effect would not be related to difference in milk production. In this experiment, the dynamics of milk Ca during lactation would not have allowed to predict that the cows within the high feeding strategy would have more mobilized their bones. This experiment also suggest that milk Ca to P ratio can be related to the equilibrium between bone accretion and resorption but only with high feeding strategy making difficult the general use of this ration as a predictor. From this study, it could be imagined that considering the breed and the parity in the calculation of absorbable requirement could be justified.

# Acknowledgements

# Effect of Calcium intake and dietary cation anion difference in early lactation on bone mobilization dynamics all over lactation in dairy cows

**Abstract**

This study aimed to evaluate the consequences of increased bone mobilization in early lactation on the dynamics of the milk Ca content during lactation and bone reconstitution in late lactation. Fifteen multiparous Holstein cows were distributed among 3 treatments 5 weeks before their expected calving date. Those treatments consisted of the provision of different diets beginning at 5 d after calving and continuing through 10 wks of lactation. During that period, the control treatment (NCa) consisted of a diet providing 100% of the Ca requirements, with a dietary cation-anion difference (DCAD) of 200 mEq/kg DM. The treatments LCa (Low Ca) and LCaLD (Low Ca, Low DCAD) consisted of diets providing 70% of the Ca requirements, with a DCAD of 200 and 0 mEq/kg DM for LCa and LCaLD, respectively. After 10 wks of lactation, all cows received the same total mixed ration which was formulated to meet 100% of the Ca requirements, with a DCAD of 200 mEq/kg DM. LCa and LCaLD tended to decrease the body retention of Ca at 3 wks of lactation compared with NCa (-0.95 vs 8.10 g/d, P< 0.09), but did not affect either the dynamics of the blood biomarkers of bone accretion (osteocalcin) and resorption (CTX) during the 32 wks of lactation or the body retention of Ca at 17 wks of lactation. Cows almost entirely compensated for the decrease in Ca supply in the LCa and LCaLD compared with the NCa by increasing their apparent digestive absorption of Ca at 3 wks of lactation (39.6 vs 30.1%, P = 0.03), whereas the apparent digestive absorption was unaffected by the treatments at 17 wks of lactation. The morning milk Ca content was higher with the LCa and LCaLD compared to that with the NCa, but as they only appeared after 10 wks of lactation, those differences may be attributed to genetic differences between cows. Milk production tended (P = 0.09) to be lower throughout lactation with the LCa and LCaLD compared with the NCa, with a mean difference of 2 kg/d, whereas milk production did not differ between the groups of cows affected by the treatments during the previous lactation. This study indicated that measuring the dynamics of the milk Ca content during lactation cannot be considered effective for indirectly estimating the dynamics of bone accretion and resorption of cows. The results also showed that at 3 wks of lactation, an increase in the apparent digestive absorption of Ca is a main way for cows to adapt to a low Ca supply and suggested that limited Ca intake at the beginning of lactation can have deleterious effect on milk production.

## Effect of calcium intake and dietary cation anion difference in early lactation on bone mobilization dynamics all over lactation in dairy cows

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**Interpretive Summary**

The dynamics of the milk Ca content was measured in dairy cows during lactation to determine whether an increased bone mobilization could be detected by milk Ca content dynamic at the beginning of lactation and to quantify the consequences of increased bone mobilization during early lactation on bone reconstitution in late lactation. Lowering the supply of dietary Ca to cows at the beginning of lactation only slightly increased bone mobilization, which was unexpected, but increased digestive absorption of Ca by cows. However, this experiment showed that such a practice could have a deleterious effect on milk production.

**Keywords:** dairy cows, calcium, lactation, bone, milk composition

# A)   Introduction

Dairy cows excrete an important amount of Ca during lactation due to the high milk Ca content (Horst et al., 1997), and this Ca flow suddenly and importantly increases later in lactation (Horst et al., 2005). During the first months of lactation, the dietary intake of Ca is generally lower than the amount of Ca secreted in the milk, feces or urine (Horst et al., 1997), and several responses occur to face this unbalance. The first response is an increase in bone resorption, mediated by the secretion of 2 hormones, PTH and PTHrP, which allow the use, by other organs, of the Ca contained in the mineralized matrix of bone (Mundy and Guise, 1999). The net mobilization of bone resulting from this increase in bone resorption at the beginning of lactation can reach up to 10 to 20% of the bone mass during lactation, with

the amount of mobilized Ca being replenished later in lactation (Benzie et al., 1955, Suttle, 2010, Dixon et al., 2017). The other responses, such as an increase in the intestinal Ca absorption or a decrease in urinary loss, occur later (Horst, 1986, Reinhardt et al., 1988), explaining the existence of cycles of bone mobilization and reconstitution during lactation in cows (Beighle et al., 1993, Ekelund et al., 2006, Taylor et al., 2009, Elizondo Salazar et al., 2013, Puggaard et al., 2014).

Questions remain about the consequences of the amplitude and the completeness of those cycles of bone mobilization and reconstitution on the health and productivity of dairy cows. Incomplete bone reconstitution at the end of the lactation can result in a higher susceptibility of cows to the restricted supply of P during the following lactation, with suboptimal production performances, as highlighted for sucker cows by Dixon et al. (2017), or maybe to higher susceptibility of milk fever at the beginning of the next lactation (McNeill et al., 2002). The effects of dietary Ca and P content and strategies for the supplementation of Ca and P on the amplitude and the completeness of the cycle of bone accretion and resorption have been quantified in several experiments in lactating ruminants (Braithwaite, 1983a, Ekelund et al., 2006, Moreira et al., 2009, Taylor et al., 2009, Elizondo Salazar et al., 2013, Puggaard et al., 2014).

Confirmation of an effect of the amplitude and completeness of the bone cycles on the health and productivity of dairy cows would consequently affect the estimations of the Ca and P requirements of those animals. Current recommendations are based on the principle that daily excretions of Ca and P allow a certain production level, with minimal fecal and urinary losses and with replacement by an equivalent amount of daily intake of those elements (AFRC, 1991, NRC, 2001, INRA, 2010). This principle does not consider that bone mobilization in early lactation and the reconstitution in late lactation that could constitute either an extra-supply or a specific requirement. Possibly, a strategy of supplementation would have to be determined at the scale of the whole cycle of lactation-gestation. However, the amount of published research available remains too limited to allow a definition of an optimal supplementation strategy of Ca and P at the scale of lactation (AFRC, 1991, NRC, 2001, INRA, 2010).

A major limit to addressing these issues is the lack of fast and cost-efficient methods for evaluating, in a significant number of cows, the amplitude and eventually the

completeness of the cycles of bone mobilization and reconstitution during lactation and gestation. Measurements of retained Ca and P at the animal scale (Taylor et al., 2009) allow an evaluation of the daily net flow of mobilized and reconstituted Ca and P reserves, but it is very time-consuming and requires keeping the animals individually stabled. Bone biopsies (Beighle, 1999) allow a good evaluation of bone reserve of Ca and P, but the number of repetitions of the measurement per animal cannot be multiplied on an important number of animals because of the time, cost and ethics. The concomitant use of blood biomarker analyses of bone accretion and resorption increased during the last 20 years, and this interesting method allows monitoring of the relative dynamics of bone accretion and resorption during lactation (Liesegang et al., 2000, Puggaard et al., 2014, Anderson et al., 2017). This method can be applied to a relatively high number of cows but is limited by the necessity for conducting several blood samplings during lactation, with relatively expensive analyses.

VanHouten et al. (2004) showed that a decrease in Ca intake in mice induced a lower Ca secretion in milk and a higher bone resorption mediated by PTHrP secretion, with both mediated by the Ca-sensing receptor (**CaSR**) in the mammary gland; those results suggest that the monitoring of milk Ca content during lactation could be an inexpensive way to indirectly estimate the dynamics of bone resorption. Mid-infrared spectra technology allows a rapid and inexpensive way to determine the milk Ca content (Soyeurt et al., 2009, Gaignon et al., 2018b). Data collected during several stages of lactation in dairy cows with different parities (Gaignon et al., 2018a) suggested that milk Ca and P contents could be related to the plasma concentrations of biomarker of bone accretion and resorption. However, whether those variations are specifically related to the cycle of bone accretion and mobilization or other interfering effects is unknown. Thus, the objective of this experiment was to induce bone mobilization in lactating cows through dietary treatments and to determine the consequences on (1) the dynamics of milk Ca and P contents, of the plasma concentrations of biomarkers of bone accretion and resorption, and the retention of Ca and P by the body, and (2) on bone reconstitution dynamics in late lactation. The dietary treatments either supplied Ca and P according to the French recommendation (INRA, 2010), restricted the Ca supply, or restricted the Ca supply and decreased the dietary anion cation difference (**DCAD**) known to increase bone resorption.

# B)   Materials and Methods

## 1   Animals and experimental design

The 3 compared treatments consisted of 3 differentiated mineral supplementations delivered between 5 d after calving and 10 wks from the beginning of lactation. The Ca content of the supplement was either calculated to allow a fully meet the Ca requirements of the cows according to the INRA feeding system (INRA, 2018), with a DCAD of 200 mEq/kg DM (Normal Ca, **NCa**), or it was calculated to provide only 70% of the Ca requirements of the cows, with a DCAD of either 200 (Low Ca, **LCa**) or 0 (Low Ca and Low DCAD, **LCaLD**) mEq/kg DM. These 3 treatments were planned to allow comparisons among the 15 lactating cows according to a complete randomized block design, with lactation stage as the blocking factor. Five wks before the calving date of the cow that was expected to calve first, 18 multiparous Holstein cows were blocked into three groups of 6 cows according to their expected dates of calving.  Cows were assigned to the three treatments to allow a homogenous representation of groups and parity within each treatment, and as similar as possible, similar averages of mature equivalent milk production and milk protein contents as observed in the first 32 wks of the previous lactation between treatments. The mature equivalent milk production was estimated as equivalent to milk production for a third lactation cow, i.e., 120% of the milk production for primiparous cows and 104% for cows lactating for a second time, as established from the data used by Gaignon et al. (2018b). Measurement started 3 wks before the average expected date of calving for each group, on 5 cows of the initial 6, with the extra cow being kept only for blood analyses to replace a cow whose actual calving date might occur too far from the expected date.

The experiment was conducted at the INRA experimental farm of Méjusseaume (longitude -1.71°, latitude +48.11°, Brittany, France) from September 1$^{st}$ 2016 to June 30$^{th}$ 2017. During the experiment, the cows were housed in a free-stall barn cubicle, covered with rubber carpet, except during 3 periods of 3 wks, for measurements of Ca retention, during which they were transferred to individual tie stalls (1.4 × 2.0 m). The individual stalls were also covered with a rubber mat with individual troughs and individual water bowls. During lactation, a total mixed ration (**TMR**) was distributed twice a day, in 2

equal-sized meals, at 0830 h and 1630 h.  The TMR was distributed by an automatic dispenser into the individual troughs, specific to each animal, identified by RFID, in the free-stall barn and directly by animal technicians in the individual tie stalls. Cows always had free access to the trough and to water during the day.  Lactating cows were fed *ad libitum* and offered quantities calculated to allow 10% orts.  Orts were weighed daily before the morning feeding.  Cows were milked twice a day, at 0630 h and 1630 h.  Milk production and DMI were recorded daily for each individual.  Milk composition (fat and protein contents) and somatic cell count were measured twice a week, at the evening and morning milkings.  Before calving, each cow was fed a fixed amount of diet, with one distribution per day.  Procedures related to care and use of animals for the experiment were approved by an animal care committee of the French Ministry of Agriculture, in accordance with French regulations (project number, 7096-20 16082515505689v2).

## 2  Diets

During the experiment, cows were fed 4 or 5 successive diets according to their physiological stage and the treatment to which they were assigned (Table V.1). During the dry period, the offered diets were formulated to cover the requirements for $NE_L$, protein digestible in the intestine (**PDI**) and minerals and vitamins of cows according to the INRA recommendations (INRA, 2010), with restricted quantities offered.  The diets offered during the far-off period, i.e., more than 3 wks before the expected calving date, and the close-up period, i.e., less than 3 wks before the expected calving date, differed with the specific objective to lower the dietary Ca and the DCAD. For the first 5 d of lactation, all cows received the TMR corresponding to the NCa treatment, whose composition was formulated to cover requirements for $NE_L$, PDI, macrominerals, trace minerals, and vitamins of cows according the INRA recommendations (INRA, 2010), with a target DCAD of 200 mEq/kg DM. DCAD is defined as the sum of the diet content of $Na^+$ and $K^+$ minus the sum of the dietary content $Cl^-$ and $S^{2-}$ content, which are expressed in mEq/kg DM. Five d after calving and until the end of the $10^{th}$ wk of lactation, cows were assigned to one of 3 TMRs corresponding to the compared treatments.  The LCa and LCaLD TMRs were formulated to meet the requirements for $NE_L$, PDI, and all macrominerals and trace minerals, except for Ca, of cows, according to the INRA recommendations (INRA, 2010). The treatment diets were isocaloric and isonitrogenous with a similar content of P.

| | From -5 to -3 weeks | -3 weeks to calving | 0 to 70 days of lactation | | | After 70 days of lactation |
|---|---|---|---|---|---|---|
| | | | NCa | LCa | LCaLD | |
| **Offered amount (kg DM/d)** | 12 | 15 | TMR *ad lib* | TMR *ad lib* | TMR *ad lib* | TMR *ad lib* |
| **Ingredients, % of DM** | | | | | | |
| Corn Silage | 53.7 | 80.5 | 70.0 | 70.2 | 70.1 | 72.1 |
| Hay | 14.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Hay (wrapping) | 24.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Energy concentrate | 0.0 | 4.1 | 15.6 | 15.7 | 15.1 | 11.1 |
| Tanned meal | 0.0 | 0.0 | 10.3 | 10.3 | 10.2 | 0.0 |
| Soybean meal | 0.0 | 10.1 | 0.0 | 0.0 | 0.0 | 13.1 |
| Colza meal | 5.9 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Straw | 0.0 | 3.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| Urea | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 0.7 |
| Mineral Feed | 0 | 0.7 | 2.9 | 2.3 | 3.3 | 2.5 |
| Commercial Mineral Feed | 1.31[1] | 1.32[2] | 0.0 | 0.0 | 0.0 | 0.0 |
| **Mineral Feed g/kg of DM** | | | | | | |
| Calcium Carbonate | 0.0 | 0.0 | 10.7 | 4.7 | 4.4 | 9.9 |
| Dicalcium phosphate | 0.0 | 0.0 | 5.3 | 4.6 | 4.4 | 4.7 |
| Sodium Sulfate | 0.0 | 0.0 | 2.7 | 2.3 | 2.3 | 2.4 |
| Sodium bicarbonate | 0.0 | 0.0 | 2.2 | 2.8 | 0.0 | 2.4 |
| Sodium carbonate | 0.0 | 0.0 | 3.1 | 3.7 | 0.0 | 0.0 |
| Magnesium oxide | 0.0 | 0.0 | 0.9 | 0.9 | 0.0 | 1.4 |
| Hexahydrate Magnesium chloride | 0.0 | 6.7 | 3.6 | 3.7 | 21.5 | 3.8 |
| Premix[3] | 0.0 | 0.0 | 0.2 | 0.2 | 0.2 | 0.2 |
| **Nutrient Content** | | | | | | |
| CP (g/100 g DM) | 11.1 | 10.5 | 15.5 | 15.5 | 15.5 | 12.7 |
| NEL (MJ/kg) | 3.9 | 6.4 | 6.7 | 6.7 | 6.7 | 6.6 |
| PDI/NE$_L$ (g/MJ) | 9.5 | 11.0 | 15.8 | 15.8 | 15.8 | 11.9 |
| Ca (g/ kg DM) | 0.74 | 0.42 | 0.83 | 0.60 | 0.58 | 0.78 |
| P (g/ kg DM) | 0.37 | 0.30 | 0.41 | 0.39 | 0.39 | 0.40 |
| DCAD (mEq/kg DM) | 156.8 | 34.8 | 218.0 | 279.5 | 0.0 | 219.9 |

Table V.1: Diet centesimal composition and nutritional value
[1]: Kéomine Repro Semoulette; [2]: Kéomine Prépa Taries: [3]: 3 for trace elements and vitamins. CP: Crude protien; PDI: Protein digestible in the instestine; NE$_L$: NEt energy for lactation

Differences in the Ca content and DCAD between the 3 TMRs were achieved only by formulating different mineral supplements, with all cows receiving the same base ration.

## 3  Blood and milk sampling

For each group, blood was sampled 3 wks before the average expected date of calving in the group and at 1, 3, 8, 12, 17, 22, 27 and 31 wks of lactation (average stage of lactation of the group).  After being milked, before being fed, and while restrained in self-locking head gates at the feedline, the cows were sampled for blood by venipuncture of the tail vessels. The samples were collected in vacutainer tubes (Monovette, Sarstedt, Nümbrecht, Germany) coated with lithium heparin for Ca and inorganic P (**Pi**) analyses, and in tubes coated with EDTA for osteocalcin (**OC**) and carboxy-terminal telopeptide of type I collagen (**CTX**). OC and CTX are, respectively, biomarkers of bone accretion and resorption (Seibel, 2000).  Plasma was recovered after centrifugation at 3,000 x g for 12 min within 30 min of sampling and stored at -80°C for OC analysis and at -20°C for other analyses. Milk samples were collected during the morning milking preceding blood sampling.  They were stored at 4°C for analyses of fat and protein contents and for separation of the N, crude protein, Ca and P fractions (i.e., non-protein nitrogen (**NPN**), non-casein nitrogen (**NCN**), urea, soluble Ca and P) and frozen at $-20$°C for analysis of the total Ca and P contents.  Additional samples of milk were also collected at the morning and evening milkings, twice per wk for determination of milk fat and protein contents (stored at 4°C before analyses), and on wks 1, 3, 6, 8, 10, 12, 14, 17, 19, 22, 24, 27, 29 and 31 of lactation, samples were collected for analyses of the milk total Ca and P content (frozen at -20°C before analyses).

## 4  Measurement of Ca and P retention in cows

For each group, all input (feed and water intake) and output (excretion in milk, urine and feces) flows of Ca and P were measured 3 times during the experiment, i.e., 3 wks before the average expected calving date of the group and 3 and 17 wks after the average calving date. For each measurement, cows were moved from the free-stall barn 2 wks before beginning the measurements and sent to individual tie stalls for habituation. The individual tie stalls were located in the same building as the free-stall barn.  All cows were kept in a same room and were able to smell and hear each other.  The feeding modalities remained similar to those applied in the free stall barn.  To determine the fecal excretion of Ca and P, large

trays were positioned behind the cows on d 15 after the entry of the cows into the stall at 0900 h. Gross fecal output was weighed and sampled from d 16 at 0900 h to d 19 at 0900 h. Two representative samples (500 g fresh each) were dried in a forced air oven (80°C, 72 h) to determine the daily amount of fecal DM excreted. These dried samples were pooled by cow and period for the Ca and P determination. The daily volume of excreted urine was measured from d 15 at 0900 h to d 19 at 0900 h, by equipping cows with urinary catheters connected by a Tygon tube to a 25-L container, which was closed with a rubber plug. To prevent urine deterioration, 250 mL of sulfuric acid (20% vol/vol) was added to the container. The urine was weighed and emptied daily at 0900 h. Each day and for each cow, a sample of 1% of the daily excreted volume was stored at $-20$°C. At the end of the experiment, these samples were pooled by animal and by period for further Ca and P content analyses.

## 5   Chemical analysis

Samples of the offered diets, orts, and feces were ground with a 3-blade knife mill through a 0.8-mm screen. Ash was determined by calcination at 550°C for 5 h in a muffle furnace. Nitrogen concentration was determined by the Dumas method, according to the Association Française de Normalisation (AFNOR, 1997), on a LECO apparatus (LECO, St. Joseph, MI). The dietary, fecal, urine and milk Ca contents were measured by atomic absorption spectrophotometry (Spectra-AA20 Varian, Les Ulis, France) after mineralization of the solid samples (500°C for 12 h). Phosphorus contents were determined using a KONE PRO multi-parameter analyzer (Thermo Fisher Scientific, Illkirch, France) by the Allen method for P (Pien, 1969). Milk fat and protein contents were determined by a commercial laboratory using mid-infrared analysis (Mylab, Châteaugiron, France). Milk content of total N (Kjeldahl), nonprotein N (precipitation at pH 4.6 with trichloroacetic acid and filtration), NCN (precipitation at pH 4.6 with 10% acetic acid and 1 M sodium acetate) content, and urea (colorimetric analysis) were determined according to the methods described in Hurtaud et al. (2000). Plasma OC and CTX concentrations were determined by ELISA with a CrossLaps kit from IDS (Paris, France) for CTX and a kit from Quidel (San Diego, CA) for OC.

## 6 Statistical analysis

Data were analyzed using PROC GLIMMIX in SAS (SAS Institute, 2013), with a generalized linear mixed model with repeated values given here:

$$Y_{ijk} = \mu + Treatment_i + Stage\,of\,lactation_j +$$
$$Treatment : Stage\,of\,lactation_{ij} + Cow(Treatment)_k + \epsilon_{ijk}$$

where $Y_{ijk}$ was a dependent variable of a cow k, within treatment i at the stage of lactation j. Treatment, stage of lactation and their interaction were fixed factors, and the cow was a random factor. Measurements repeated over the stage of lactation were considered by using a covariance matrix. The choice of the structure of the matrix was determined according to the structure of the data and then was performed using an AIC for analyses of all variables. Average flows of Ca and P during the 4 d of measurements were also analyzed independently during each stage of lactation with a generalized linear model using proc GLM in SAS and included only the fixed effect of the treatment. These analyses supplemented the previous analyses, performed with the complete model because it has been shown that with a lower number of data, the inclusion of repeated measurements in a generalized linear mixed model can deteriorate the quality of detection of significant effect by increasing the second species risk (Liu et al., 2012).

# C) Results

The distribution of the cows among the 3 treatments had to be modified before the differentiation of the TMR 5 d after calving. Two cows calved more than 2 wks before the expected calving: one was assigned to the NCa treatment; the other, to the LCaLD treatment. They were removed from the experiment and replaced by the extra cows, kept in each of the considered group, and only one blood sampling before calving was performed for each replacement cow. Another cow, initially assigned to the LCaLD treatment and diagnosed with a milk fever, was replaced by a cow initially assigned to the NCa treatment and belonging to the same group of calving date. At wk 7 of lactation, one cow of the NCa treatment died due to bowel obstruction, and its data were removed from the data set. Despite these events, the pre-experimental characteristics of the 3 experimental lots
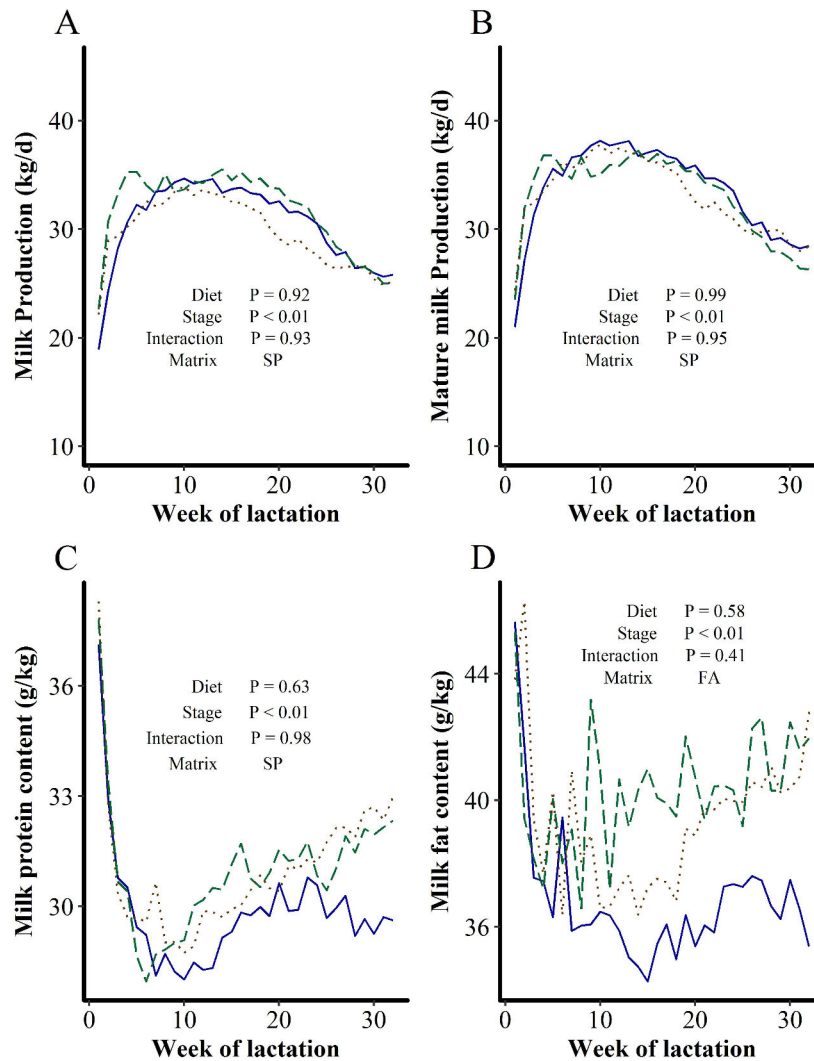
Figure V.1: Differences between cows according to their treatments on A) milk production, B) mature equivalent milk production, C) milk protein content and D) milk fat content during the first 32 wks of the lactation preceding that described in this chapter. NCa: normal line, LCa: dashed line, and LCaLD: dotted line

remained similar. Average parities were 2.4 for the LCaLD and 3.0 for the TEM and LCa. Milk production during the first 32 wks of the previous lactation was not affected by the treatments (Figure V.1A, P = 0.92 for the treatment effect, and P = 0.93 for the effect of the interaction treatment × stage of lactation). Mature milk yield was not different between the treatment either (Figure V.1B, P = 0.99 for the treatment effect, and P = 0.95 for the effect of the interaction treatment × stage of lactation). The average mature equivalent milk productions over the first 32 wks of lactation were 33.6 (± 3.94), 33.2 (± 3.52) and 33.1 (± 3.52) kg/d for the treatments NCa, LCa and LCaLD, respectively. Neither milk protein content (Figure V.1C, P = 0.62) nor milk fat content (Figure V.1D, P = 0.58) were affected by

the treatments during the first 32 wks of the previous lactation, but individual variability of those last parameters was high. The average milk protein contents over the 32 first wks of lactation were 29.8 ($\pm$ 0.97), 31.0 ($\pm$ 0.87) and 31.0 ($\pm$ 0.87) g/kg for the treatments NCa, LCa and LCaLD, respectively. The average milk fat contents over the first 32 wks of lactation were 36.8 ($\pm$ 2.48), 40.3 ($\pm$ 2.22) and 39.3 ($\pm$ 2.22) g/kg for the treatments NCa, LCa and LCaLD, respectively.

## 1   Ca, P and DM intake

During the period of differentiation of the TMR between treatments, i.e., between d 5 and d 70 of lactation, the Ca intake was significantly lower with the LCa and LCaLD treatments compared with NCa (Figure V.2A, P < 0.01). During this period, the average daily intake of Ca was 136.0 ($\pm$ 4.98) g/d and 126.5 ($\pm$ 4.98) g/d for LCa and LCaLD, respectively, and 184.1 ($\pm$ 5.06) g/d for NCa, leading to Ca intake that was 31% lower for the LCa and LCaLD treatment compared with the NCa, as expected. During the same period, the fulfillment of Ca requirements, i.e., the difference between the Ca intake and Ca requirements calculated according the INRA feeding system (INRA, 2010), was negative for the LCa and LCaLD treatments (-13.4 $\pm$ 1.36 and -15.5 $\pm$ 1.36 g/d, respectively), whereas it was positive for the NCa treatment (5.4 $\pm$ 1.42 g/d, P < 0.01, data not shown). After d 70 of lactation, when cows received the same ration, the Ca intake (Figure V.2B) was not affected by the treatments, and the fulfillment of the Ca requirements always remained positive, with an average value of 10.9 $\pm$ 1.12 g/d. The DMI tended (P = 0.08, Figure V.2B) to be higher for LCa compared with the LCaLD and NCa treatments, with DMIs of 23.0 $\pm$ 0.4, 21.6 $\pm$ 0.4 and 22.0 $\pm$ 0.5 kg/d for the treatments LCa, LCaLD and NCa, respectively, throughout the lactation. Consequently, the P intake also tended (P = 0.08, data not shown) to be higher with LCa compared with the LCaLD and NCa treatments across lactation, with P intake of 91.8 $\pm$ 1.70, 86.1 $\pm$ 1.7 and 89.4 $\pm$ 1.90 g/d for the treatments LCa, LCaLD and NCa, respectively. Required P coverage was, on average, 4.09 $\pm$ 0.90 g/d during the first 70 d of lactation.

## 2   Plasma concentrations of biomarkers of bone accretion and resorption, Ca and Pi

The plasma concentrations of OC and CTX were affected neither by the treatments nor by the interaction treatment $\times$ stage of lactation (Figure V.3). For all treatments,
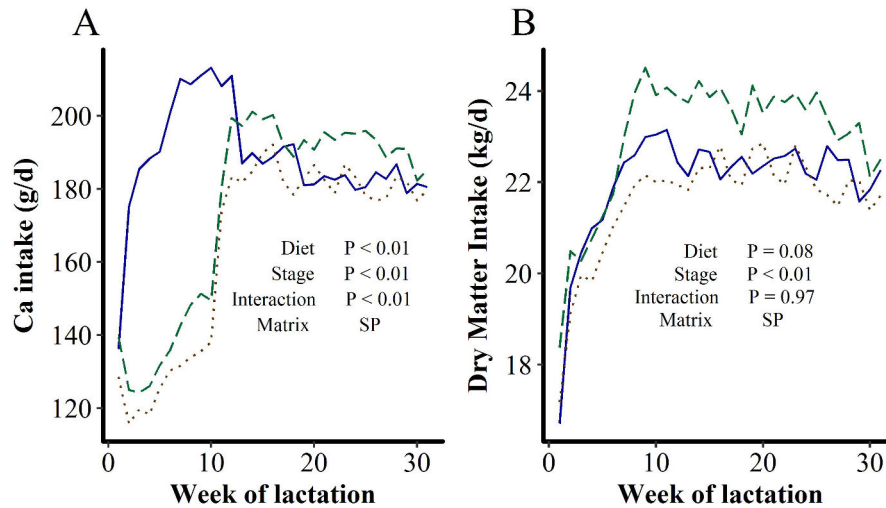
Figure V.2: Effect of dietary content of Ca and DCAD between d 5 and d 70 of lactation on A) Ca intake and B) DM intake. NCa: normal line, LCa: dashed line, and LCaLD: dotted line

plasma OC concentrations decreased after calving to reach a minimal value one wk after calving, whereupon it increased sharply for 10 wks and then more slowly until the end of the lactation (Stage of lactation, effect P < 0.01). For all treatments, the plasma CTX concentrations increased sharply after calving to reach a maximal value between wk 3 and 8 of lactation and then decreased until the end of lactation (Stage of lactation effect P < 0.01). The plasma Ca concentration was, on average, 100.3 ($\pm$ 1.84) mg/L, and individual values always remained between 80 to 120 mg/L, with only one cow in hypocalcemia at one wk of lactation (76 mg/L). Plasma Ca concentration tended to be lower at 1 and 3 wks of lactation compared with the other sampling times (Stage of lactation effect P = 0.06, data not shown) but was affected neither by the treatments (P = 0.63) nor by the interaction treatment $\times$ stage of lactation (P = 0.31). Plasma Pi concentration was 50.7 ($\pm$ 1.78) mg/L on average. Some individual values could be lower than 40 mg/L at 3 weeks of lactation, but no individual data were higher than 80 mg/L. Plasma Pi sharply decreased after calving, at 1 wk and 3 wks of lactation, increasing afterward and leveling off at 17 wks of lactation (stage of lactation effect, P < 0.01, data not shown). It was affected neither by the treatment (P = 0.89) nor by the interaction treatment $\times$ stage of lactation (P = 0.63).

## 3 Ca and P partitioning and retention

During the 4 d of measurement of Ca and P retention, the Ca intake was lower for the LCa and LCaLD treatments compared with the NCa at 3 wks of lactation, i.e., during the
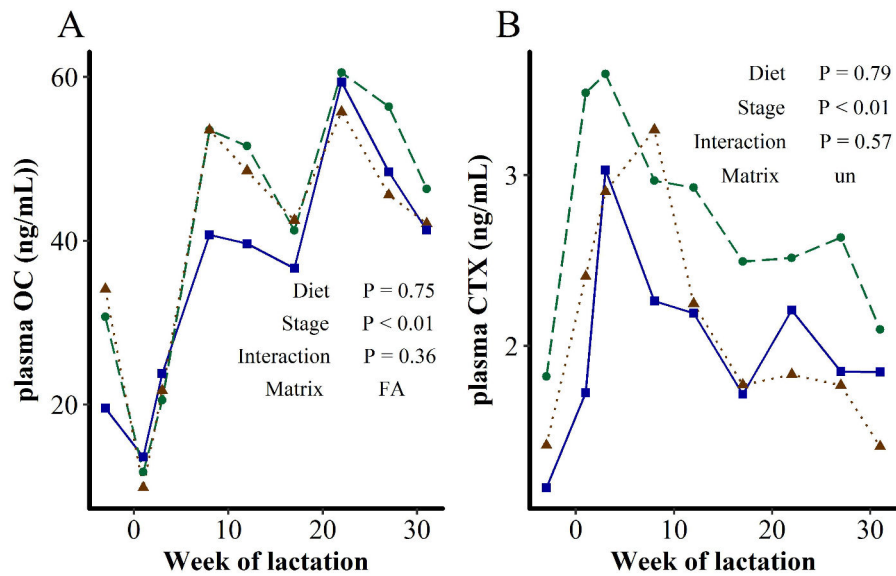
Figure V.3: Effect of dietary content of Ca and DCAD between d 5 and d 70 of lactation on A) plasma OC concentration and B) plasma CTX concentration. NCa: normal line, LCa: dashed line, and LCaLD: dotted line

period of TMR differentiation between treatments (P < 0.05, Figure V.4A), whereas the Ca intake was not affected by the treatment 3 wks before calving and at 17 wks of lactation (P > 0.10 for both stages, interaction treatment × stage of lactation P < 0.001). The daily amount of Ca excreted in the feces was also lower for the LCa and LCaLD treatments compared with the NCa at 3 wks of lactation (P < 0.001, Figure V.4B) and was not affected by the treatment 3 wks before calving and at 17 wks of lactation (P > 0.10 for both stages, interaction treatment × stage of lactation P < 0.001). Apparent digestibility of the Ca increased after calving (Figure V.4C, P < 0.001) from 21.0% (± 2.33) 3 wks before calving to 36.4% (± 2.10) and 33.4% (± 2.10) at 3 and 17 wks of lactation, respectively. Apparent digestibility of Ca was not affected by the treatments 3 wks before calving and at 17 wks of lactation but it was higher for the LCa and LCaLD treatments compared with NCa at 3 wks of lactation (P = 0.03, average of 37.3 ± 3.5% for LCa, 41.7 ± 3.5% for LCaLD and 30.1 ± 3.9% for NCa). Daily amounts of Ca excreted in urine were low, 2.0 g/d on average, compared to the other flows of the input-output retention, as expected, and this flow tended to be affected only by the stage of lactation (P = 0.09, Figure V.4D). However, the daily amount of Ca excreted in the urine was higher with the LCaLD treatment compared with the NCa treatment and the LCa treatment at 3 wks of lactation (P < 0.05, interaction treatment × stage of lactation P = 0.004, 4.4 ± 0.5 g/d for LCaLD vs. 0.5 ± 0.6 g/d for NCa and 0.7 ± 0.5 g/d for LCa). The
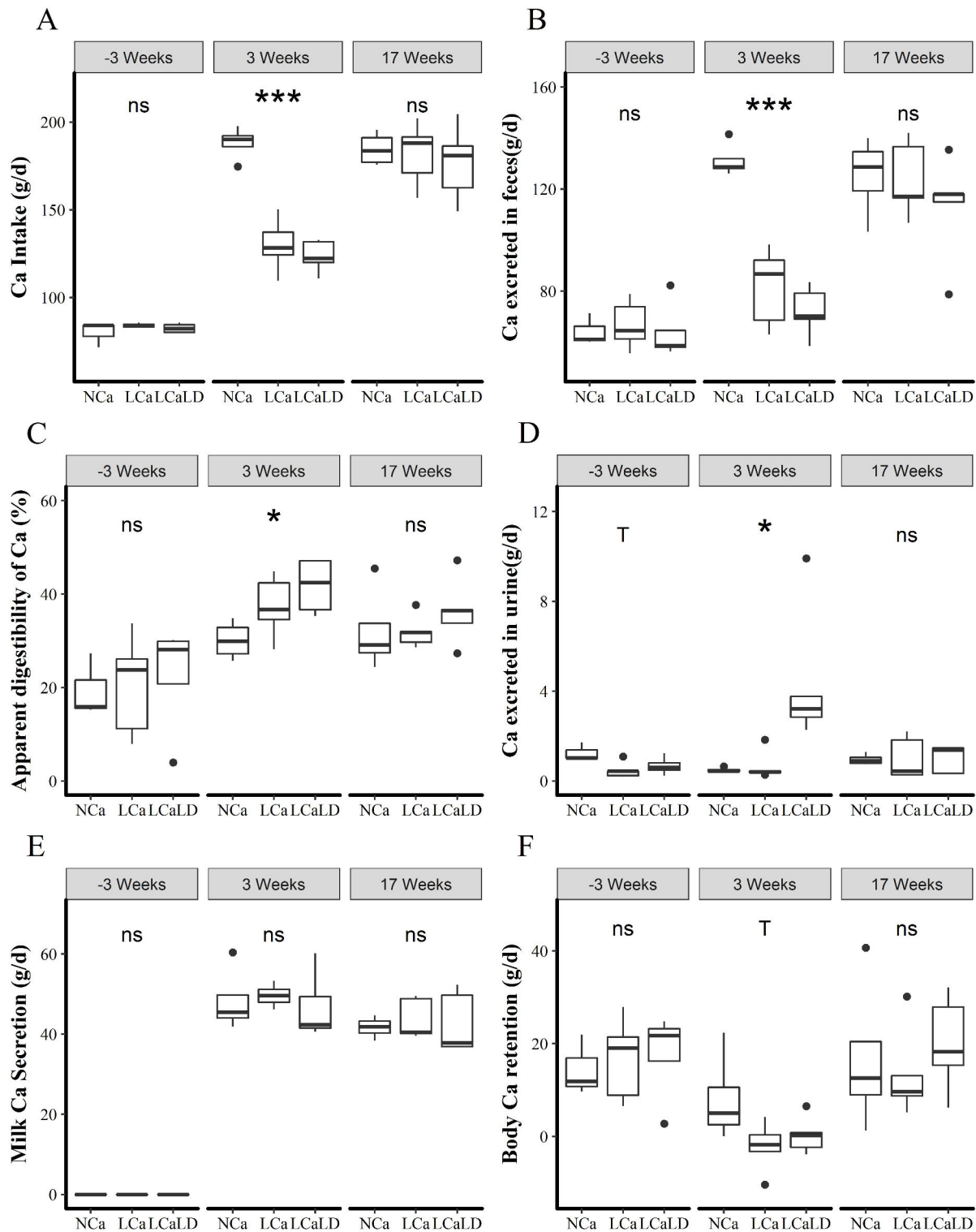
Figure V.4: Effect of dietary content of Ca and DCAD between d 5 and d 70 of lactation A) daily Ca intake, B) fecal losses of Ca, C) Ca secretion in milk, D) urinary losses of Ca, E) apparent digestibility of Ca and F) Ca balance. Boxplots are obtained from mean data per animal. Signs give p-values of the dietary effect for intra-period analyses: ns = not-significant (P > 0.10); T = Tendency (P<0.10), * = significant (P<0.05), ** = significant (P < 0.01); *** = significant (P < 0.001)

daily amount of Ca secreted in milk was on average 48.2 ±1.42 g/d at 3 wks of lactation and 42.7 ±1.42 g/d at 17 wks (Figure V.4E). The amount of Ca secreted in milk daily decreased slightly between 3 and 17 wks of lactation (P < 0.001) and was not affected by the treatments at any stage. Daily Ca retention, i.e., the difference between input and output of Ca, was very positive before calving (+17.3 ± 2.84 g/d, Figure V.4F), decreased at 3 wks of lactation to an average value close to 0 (2.1 ± 2.62 g/d) and increased at 17 wks of lactation to a positive value (+16.7 ± 2.62 g/d, stage of lactation P < 0.001). At 3 wks of lactation, the daily Ca retention tended to be lower for the LCa and LCaLD treatments compared with the NCa treatments (P = 0.09), with values around equilibrium for the LCa and LCaLD (-2.1 ± 4.3 g/d for LCa, + 0.3 ± 4.3 g/d for LCaLD and + 8.1 ± 4.8 g/d for NCa). The daily Ca retention was unaffected by the treatment 3 wks before calving and at 17 wks of lactation (P > 0.10).

Neither the DMI, intake of P, fecal or the excretion of DM and P were affected by the treatment or the interaction treatment × stage of lactation (Data not shown). Both the apparent digestibility of the DM and P increased at calving and leveled off after 3 wks of lactation. Apparent DM digestibility was 65.1 ± 0.86 at 3 wks before calving, 72.7 ± 0.79% at 3 wks of lactation and 71.6 ± 0.79% at 17 wks of lactation (P < 0.001), and apparent P digestibility was 10.9 ± 2.17% 3 wks before calving, 54.7 ± 1.98% at 3 wks of lactation and 47.5 ± 1.98% at 17 wks of lactation (P < 0.001). Daily P retention tended to increase at 17 wks of lactation compared with both other stages of maturity, with values of 4.3 ± 1.66 g/d at 3 wks before calving, 7.9 ± 1.48 g/d at 3 wks of lactation and 10.0 ± 1.48 g/d at 17 wks of lactation (P = 0.08).

## 4 Milk production and composition

Milk production tended to be lower throughout the 32 weeks of lactation for treatments LCa and LCaLD compared with treatment NCa (P = 0.09, Figure V.5A), with average values of 36.8 ± 0.9 kg/d for LCa, 35.9 ± 0.9 kg/d for LCaLD and 39.2 ± 1.1 kg/d for NCa. This production led to a difference in cumulative milk production at 200 d of lactation between the low Ca treatments (LCa and LCaLD) and the control treatment NCa of more than 400 kg. The difference in milk production between the low Ca treatments and NCa was maximal at the fourth week of lactation, with a milk production difference of more than 4.5 kg/d. Milk Ca content sharply decreased to reach a minimal value at 3 wks of lactation (Stage of lactation effect, P < 0.01, Figure V.5B). Then, after 8 wks of lactation, the milk Ca content increased
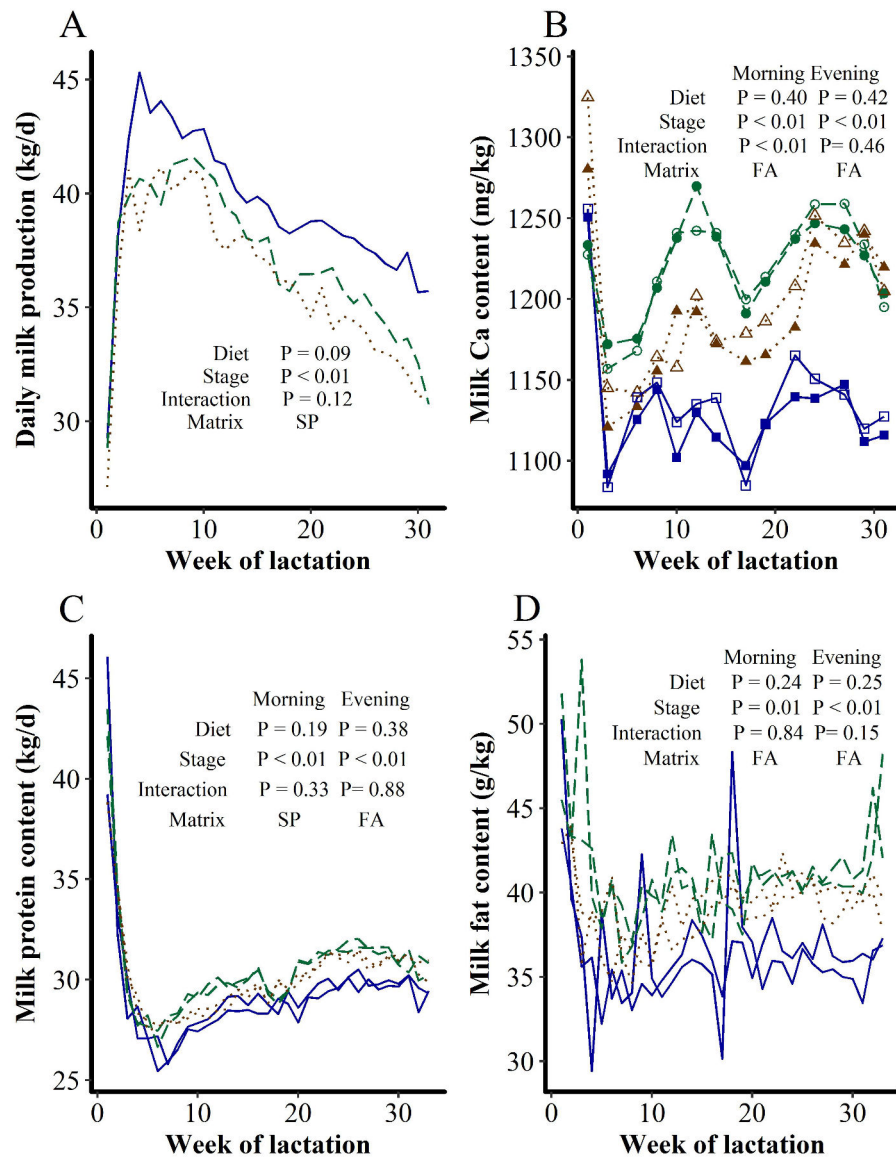
Figure V.5: Effect of diet content of Ca and DCAD between d 5 and d 70 of lactation on A) daily milk production, B) milk Ca content, C) milk protein content and D) milk fat content. NCa: normal line, LCa: dashed line, and LCaLD: dotted line. For milk Ca content, color-filled shapes are for morning milk Ca content and the white-filled shapes for evening milk Ca content.

for the LCa and LCaLD treatments, whereas it remained stable for NCa (interaction stage of lactation × treatment P < 0.01 in the morning, non-significant in the evening). After the 24th wk of lactation, the morning milk Ca contents were 1230 (± 48.4) and 1228 (± 48.4) mg/kg for the LCa and LCaLD treatments, respectively, and 1128 (± 54.2) mg/kg for NCa (P < 0.01). Milk protein content was numerically higher for cows affected by the treatments LCa and LCaLD compared with those affected by the NCa between 5 and 9 wks of lactation and after 20 wks of lactation, but the treatment effect was not significant (Figure V.5C).

Milk fat content was also unaffected by the interaction of stage of lactation × treatment (Figure V.5D, P > 0.10).

## 5  Milk Ca and protein partitioning between soluble and colloidal phases

Milk casein content was higher with the LCa and LCaLD treatments compared with the NCa (Figure 6A, P < 0.001), with this difference being more distinct after 17 wks of lactation (interaction stage of lactation × treatment, P < 0.01). At the end of the period of diet differentiation between treatments, the milk casein content of the NCa treatment increased transitorily. The ratio between the milk contents of colloidal Ca and casein increased at the beginning of the lactation and remained relatively stable after 8 wks of lactation at a value approaching 36 mg/g (Figure 6B). This ratio was not affected by either the treatments or the interaction stage of lactation × treatment (P > 0.10). The proportion of soluble Ca among total Ca was lower for the LCa and LCaLD treatments compared with the NCa (Figure 6C, P < 0.01). This proportion decreased transitorily at the end of the period of diet differentiation between treatments for the NCa treatment (Interaction stage of lactation × treatment, P = 0.03). On average, 27% of the milk Ca was in the soluble form. The ratio between the milk Ca and protein contents increased at the beginning of the lactation to peak at approximately 6-8 wks of lactation and then decreased to level off after 17 wks of lactation to a value close to 39 mg/g on average (Figure 6D, P < 0.001). It was affected neither by the treatment nor by the interaction stage of lactation × treatment.

# D)  Discussion

## 1  A limited effect of dietary Ca supply and DCAD on the dynamics of bone mobilization and reconstitution during lactation

The objective of the treatments LCa and LCaLD was to induce an increased bone mobilization during the first 10 wks of lactation. For this purpose, the dietary Ca supply was limited to 70% of the recommended supply to cover the cows' requirements according
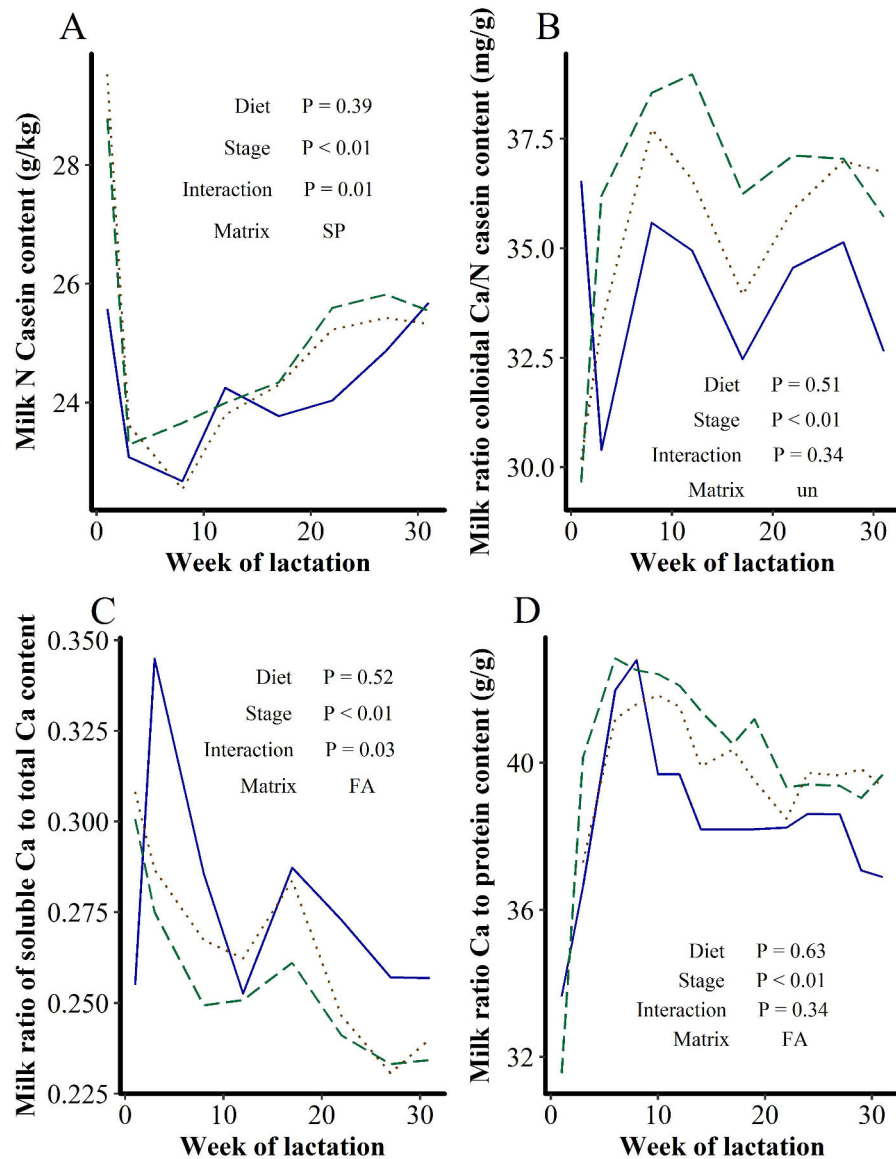
Figure V.6: Effect of dietary content of Ca and DCAD between d 5 and d 70 of lactation on morning milk A) casein content, B) ratio of colloidal Ca to N casein content, C) ratio of soluble Ca to total Ca content and D) ratio Ca to protein content. NCa: normal line, LCa: dashed line, and LCaLD: dotted line.

to the expected milk production and intake according to the INRA feeding system (INRA, 2010). With such restriction of Ca supply, some studies highlighted a decrease in the body Ca retention at the beginning of lactation in dairy cows, with this decrease in body Ca retention later reaching negative values (Wohlt et al., 1986, Taylor et al., 2009), whereas some studies highlighted an increase in the serum concentration of pyridinoline, which is a biomarker of bone resorption (Moreira et al., 2009). Both results suggested an increase in mobilization of Ca from bones. To increase the chance to induce a bone mobilization in our experiment, the DCAD was also decreased for treatment LCaLD. The positive effect of

low DCAD on bone mobilization is used to prepare the bones of cows to be mobilized before lactation for milk fever prevention (Goff et al., 2014). An expected effect of lowering the DCAD is to decrease the pH of the blood and to avoid changes in the PTH receptor conformation, which is caused by alkalinity, thereby decreasing the receptor affinity for PTH (Goff et al., 2014). Another expected effect is that bone can release Ca from bicarbonates when the local pH is low as a buffering system (Bushinsky, 2010). The DCAD of the LCaLD treatment in our experiment approached 0, which is a maximal limit under which a positive effect on the prevention of milk fever, and perhaps even on bone mobilization, could be expected (Charbonneau et al., 2006). In our experiment, the diet offered to the cows during the first 10 wks of lactation was also intentionally formulated with a high dietary PDI to $NE_L$ ratio, with the objective being to maximize the milk production capacity by the mammary gland and perhaps the necessity of bone mobilization (Liesegang et al., 2000). Primiparous cows were excluded from the experiment, and special care was applied to balance the average parity between treatments because parity is, in dairy cows, a strong determinant of bone accretion and resorption throughout lactation (Iwama et al., 2004, Taylor et al., 2009, Gaignon et al., 2018a) and digestive absorption of Ca (Horst et al., 1990).

However, treatments LCa and LCaLD, compared with NCa, only tended to induce a small decrease in Ca retention 3 wks after calving, with no effect on the dynamics of the blood biomarkers of accretion and resorption throughout the lactation. Even at 3 wks of lactation, the Ca retention remained close to 0 with the treatments LCa and LCaLD and was not clearly negative as it might have been expected from other studies (Wohlt et al., 1986, Taylor et al., 2009). A debate exists about the effect of a low supply of Ca and P on the amplitude of bone mobilization at the beginning of lactation. The results of Braithwaite (1983) illustrated that a strong restriction of the Ca and P supply did not affect the amount of Ca mobilized from bone at the beginning of lactation of ewes but strongly reduced the amount of Ca retained in bone for bone reconstitution at the end of lactation. Benzie et al. (1955) also showed that a restriction of the Ca supply lowered the mineral contents of the bones of ewes slaughtered at 100 d of lactation. These results suggest that bone mobilization at the beginning of lactation was programmed in response to homeorhesis regulation, which was linked to the stage of lactation, and the level of the Ca supply did

not affect it. Because we observed only a limited effect of restriction of Ca dietary supply on bone mobilization at the very beginning of lactation, i.e., at 3 wks of lactation, the possibility exists of such regulation. However, the data of Taylor et al. (2009) illustrated, in Holstein dairy cows, a clear effect of dietary Ca supply on the body Ca retention even at 2 to 5 wks of lactation. With Ca supplies approaching those of the low Ca treatments in our experiment, these authors measured Ca retention of -14.5 g/d, whereas with Ca supplies approaching those of the NCa treatment, the measured Ca retention was 7.4 g/d. With high Ca supply, i.e., 1.03 g/kg MS, the Ca retention they measured was even as high as 31.7 g/d. These observations contradicted the hypothesis that bone mobilization at the beginning of lactation would be programmed in response to homeorhesis regulation linked to the stage of lactation independently from the level of Ca supply. After 20 wks of lactation, as observed by Taylor et al. (2009) and Braithwaite (1983), at the end of lactation in ewes, the Ca supply also affected the bone reconstitution, with a proportionally higher Ca retention with diets providing more Ca. In our experiment, we did not observe any effect of the treatment on the Ca retention at 17 wks of lactation. This result may be explained by the fact that the dietary supply was not differentiated among the treatments after 10 wks of lactation, that is during a stage preceding the period of end of bone mobilization, i.e., 3 months in dairy cows (Horst et al., 2005). This might suggest that the immediate Ca supply may be more important than the bone status to drive the amplitude of the reconstitution. However, the difference in the Ca mobilization among the treatments in our experiment may have been too low to significantly affect the status of bone reserves at 17 wks of lactation.

The dynamics of the blood bone biomarkers throughout lactation observed in our experiment agreed with those previously observed (Liesegang et al., 2000, Taylor et al., 2009, Puggaard et al., 2014), with a sharp decrease in OC being observed at calving and an increase in CTX being observed at the beginning of lactation. These results agreed with the measurements showing lower Ca retention at the beginning of lactation compared to that measure before calving or at 17 wks of lactation, illustrating a net bone reconstitution at those times.

## 2 Dairy cows have adapted to low dietary Ca supplies by increasing digestive absorption of Ca in early lactation

In our experiment, as with that of Taylor et al. (2009) and Braithwaite (1983), the evolution of calcemia during the lactation-gestation cycle was not affected by the dietary Ca supply, which confirms that calcemia is very tightly regulated. This suggests that, if bone mobilization was not the main effector mobilized for the regulation of calcemia when the Ca supply was lowered in our experiment, other Ca flows must have allowed this regulation. Our results clearly illustrate that the decrease in Ca intake with the treatments LCa and LCaLD was almost entirely compensated, at the scale of the organism, by an equivalent decrease in the daily amount of Ca excreted in feces, i.e., by an increased apparent digestive absorption of Ca. The apparent digestibility of Ca was even quite high, with an average higher than 40% for the LCaLD. These results contrasted with those of Taylor et al. (2009) and Moreira et al. (2009), who observed lower apparent digestibility of Ca at a similar stage of lactation, with highest values at approximately 35%. A reason for that may be that a significant proportion of the dietary Ca was provided by alfalfa silage or hay in those studies, whereas it was mainly provided by a mineral source of Ca in ours. Ca from alfalfa is known to be less available for absorption in ruminants (Suttle, 2010). Possibly, in contrast to our findings, these authors could observe an increase in bone mobilization under conditions of low calcium supply at approximately 3 wks of lactation because the cows could not increase their apparent absorption of Ca because of the low availability of dietary Ca in their feed. In our experiment, the cows may have experienced an increase in digestive absorption rather than a mobilization of Ca from bone to regulate calcemia because dietary Ca was more available for absorption. This hypothesis would require confirmation. The effect of the dietary Ca content on the apparent or real absorption of Ca had been clearly illustrated by Ramberg et al. (1976) on nonpregnant, nonlactating cows on diets that did not contain alfalfa. A clear increase in bone mobilization at the beginning of lactation with low dietary supply of Ca has also been observed by Braithwaite (1983) in ewes. However, these authors also observed a concomitant increase in the digestive absorption of Ca. The reason for the adaptive mechanisms to co-exist in this study in contrast to our study may be explained by the very important restriction of Ca supplied compared to that in the experiment of Taylor et al. (2009), Moreira et al. (2009) or ours. A high restriction of Ca likely necessitates the

implementation of more adaptive mechanisms.

We clearly observed a strong effect of the physiological stage of the cows on the apparent digestibility of Ca, with higher digestibility at 3 or 17 wks of lactation compared with 3 wks before calving. The increase in the absorption capacity of Ca by the digestive tract between gestation and lactation agrees with the increase in PTH release and 1,25-(OH)2D3 synthesis at the onset of lactation (Horst et al., 2005) but has not been so clearly illustrated. We also observed a clear positive effect of the LCaLD treatment on the daily amount of Ca excreted in urine, but the size of the flow, i.e., less than 4 g/d at 3 wks of lactation, is limited compared with that of Ca secreted in milk, i.e., approximately 50 g/d or in feces, approximately 100 g/d. The increase in this flow with the treatment providing a DCAD close to 0 agrees with the idea that a low DCAD enhances renal affinity for PTH and thus promotes the reabsorption of Ca from proximal renal tubular fluids (Goff et al., 2014). This possibility suggests that the low DCAD of this treatment may also have enhanced the affinity of osteoblasts for PTH and thus induced the osteoclast activity (Goff et al., 2014), but this was not observable with either the blood biomarkers of bone accretion or resorption or the Ca retention.

## 3   The relation between the dynamics of milk Ca content and bone accretion and resorption during lactation

Experiments with lactating mice have demonstrated that a decrease in Ca intake can induce an increase in bone resorption and a concomitant decrease in the milk Ca to protein ratio (VanHouten et al., 2004). Those effects have been shown to be mediated by the CaSR of the epithelial cells of the mammary gland, with a lack of Ca on the CaSR decreasing Ca transport into the milk and increasing PTHrP secretion by the mammary gland, and thus bone resorption. Our hypothesis was that low Ca intake would induce both an increase in bone resorption and a decrease in the secretion of Ca in milk. However, the effects of the low Ca supply in our experiment had only a limited effect of bone mobilization at the beginning of lactation; thus, this experiment did not fully allow testing our hypothesis.

The cows affected by the LCa and LCaLD treatments tended to have higher milk Ca content after 10 wks, which did not agree with our hypothesis for 2 reasons. First, this difference appeared when the diet no longer differed by treatment. Second, according to our hypothesis, a lower milk Ca content was expected for those treatments. Because the genetics

of the cow is known to be a major determinant of milk Ca content in cows (Van Hulzen et al., 2009) and because the milk Ca content was not measured prior to the previous lactation when attributing cows to the treatments, possibly the cows of the NCa treatments had lower milk Ca contents because of their genetics. The milk casein content of these cows was also lower, and most milk Ca is bound to casein (Malacarne et al., 2014), with a stable ratio between colloidal Ca and casein, as observed in our experiment. However, we could also observe a very transient change in the proportion of the milk soluble Ca and even of the total milk Ca contents when the TMR changed at 10 wks of lactation milk. This result suggests that milk Ca content may be related to the Ca homeostasis of cows in a very transient way on the first day of perturbation. Detecting and fully explaining such quick change with a sampling interval of 2 wks is impossible. This would indicate that the milk Ca content may not be an indicator of the whole shape of bone resorption at the scale of the lactation.

## 4 A possible effect of restricted Ca intake on milk production and cow longevity?

The difference of 2 kg/d in milk production between the NCa treatment on one side and the LCa and LCaLD treatments on the other side was an unexpected result that could not be attributed to the measured pre-experimental characteristics of the cows. The discrepancy between the treatments appeared approximately 2 wks after the differentiation of the diets according to the treatment, i.e., at 3 wks of lactation, and lasted until the end of the experiment, i.e., largely after 10 wks of lactation, when all cows began to receive the same diet. Thus, the low Ca intake possibly impaired the potential milk secretion by the mammary gland at peak lactation by altering either the proliferation of the mammary epithelial cells or their exfoliation. Ca has been shown to be involved in cell proliferation as an important messenger, notably for the breakdown of the nuclear envelope (Pinto et al., 2015). Possibly, the milk production potential of cows in the LCa and LCaLD was not totally expressed because Ca may have limited the capacity for cell proliferation during early lactation. Wohlt et al. (1986) also observed a decrease in the milk production of cows with lower Ca supply, with dietary Ca contents between 0.9 and 0.6% DM, but the response depended on the Ca source. Taylor et al. (2009) did not observe any effect of the dietary Ca contents on milk production but two-thirds of the cows were

primiparous. Moreira et al. (2009) did not observe such an effect either, with multiparous cows, but their experiment stopped after one month of lactation. Older studies (Becker et al., 1934) highlighted an effect of Ca supplementation on milk production of dairy cows, but bone meal was used as Ca supplementation, and bone meal also contained P, which is known to affect the DM intake and milk production. Possibly, because we used a high level of protein supplementation, with tanned soybean meal partially protected from protein ruminal degradation, and multiparous cows, the milk production potential may have been maximized and the Ca could have been a limiting factor, but this remains to be confirmed on more animals.

Another unexpected observation in our experiment was that the culling rate of cows before the next calving was numerically clearly higher in the LCa and LCaLD treatments compared with the NCa treatments. With the LCa treatment, 3 of the 5 cows were culled before the next lactation, 1 because of the absence of estrus detection, and 2 because of claw disorders. With the LCaLD treatment, 1 of the 5 cows was culled because of failures to be artificially inseminated. All cows from the NCa treatment were kept for subsequent lactation without health or reproductive problems before the calving. Due to the low number of cows involved in this experiment, affirmation of an effect of the dietary Ca content on cow's reproduction and health from our results was not possible. The subclinical hypocalcemia during the first three days of lactation has a negative effect on the reproductive performances of cows. Our results suggest that Ca supply under the requirements during the first weeks of lactation could also have a detrimental effect (Caixeta et al., 2017), but this remains to be demonstrated with more animals.

# E)   Conclusion

Lowering the dietary Ca content to between 0.8 and 0.6 g/kg DM, clearly increased the apparent digestive absorption of Ca of the cows at 3 weeks of lactation but marginally affected the body retention that remained nearly zero. This result suggests that bone mobilization of cow at the beginning of lactation can be unaffected by the supply of Ca, as long as the source of Ca is available or absorption. The low supply of Ca did not clearly affect the Ca milk content but may have lowered the milk production and may have

affected the reproductive performances of the cows and their probability to continue for a subsequent lactation. These results need to be confirmed using a higher number of animals, while also suggesting that Ca supplementation must be carefully checked at the beginning of lactation.

# Acknowledgments

General Discussion

The issue of this PhD thesis is that dairy cows are submitted during lactation and gestation to cycles of bone mobilization and reconstitution with a likely net bone mobilization at the beginning of lactation and a likely net bone reconstitution between 3 months of lactation and next calving. The effects of the amplitude and the completeness of these cycles on cow's health and especially on the susceptibility to milk fever are not known. Recent publications showed that even subclinical hypocalcemia at the beginning of lactation can have consequences on reproduction or occurrence of infectious diseases (Caixeta et al., 2017; Neves et al., 2017). A limit to quantify the effect of the amplitude and the completeness of the cycles of bone mobilization and reconstitution on cow's health and reproductive performance is that these cycles are difficult to measure. The question of this PhD thesis was to determine if the dynamics of milk Ca content could reflect that of instantaneous bone resorption and/or accretion. The hypothesis was that, given that the mammary gland is involved in the regulation of calcemia by modifying concomitantly Ca secretion in milk and bone mobilization via PTHrP secretion, variations of milk Ca content and that of bone biomarkers of bone accretion and resorption could be concomitant.

The first step of this PhD was to characterize the variability of milk Ca content in dairy cows that was not related to genetic factors. It has been observed, thanks to the PhénoFinLait database that milk Ca content varies according to the stage of lactation, the diet of the cows or their parity. The second step was to quantify concomitantly dynamics of milk Ca content and of blood biomarkers of bone accretion and resorption during lactations in dairy cows. In a first experiment with an unique diet, it has been observed that primiparous cows had higher amplitude of variation of plasma CTX (biomarker of bone resorption) and milk Ca during lactation compared to multiparous cows. Even though the dynamics of milk Ca variation during lactation did not allow predicting that of blood biomarkers of bone accretion and resorption when considering individuals. The milk Ca to P ratio appeared to be correlated to the plasma OC to CTX ratio within individuals. In a second experiment, the link between both ratios was much weaker but this experiment showed a clear effect of the breed and of the energy level of the diet on the dynamics of blood biomarkers of bone resorption and accretion. The last step was to quantify the consequences on milk Ca and P contents of an increased bone mobilization at the beginning of lactation due to dietary treatments. Unfortunately, the treatments

designed to increase bone mobilization mainly increased intestinal Ca absorption and consequences of increased bone mobilization on milk Ca content could not be tested. However, this experiment suggests consequences of low Ca supply at the beginning of lactation on milk production and maybe on reproductive performances and health of cows.

In this last part of my PhD, three questions will be addressed. The first one will be to determine what are the non-genetic factors of variation of milk P content and to understand why the milk Ca to P ratio could be related to plasma OC to CTX ratio within individuals. The second one will be to determine across the realized experiments, if it can be concluded or not that variations of milk Ca content or Ca to P ratio allow a prediction of the shape of bone accretion and/or resorption dynamics at the scale of the lactation. The last one will be to determine what can be drawn from the measured dynamics of bone accretion and resorption considering the role of bone in the regulation of calcemia in various breeding conditions in dairy cows.

# A) Non-genetic factors of variation of milk P content

The non-genetic factors of variation of milk P content and milk Ca to P ratio were quantified thanks to the PhénoFinLait database described in the Chapter II.

## 1 Complements of Material and Methods

Descriptions of the PhénoFinLait bank of milk samples and database are given in Chapter II. A prediction equation of milk P content from MIR spectra was established. To achieve this, the milk P content of 495 frozen milk samples taken from the bank of samples of the PhénoFinLait program was analyzed by the Allen method using a KONE PRO multi-parameter analyzer (Thermo Fisher Scientific, Illkirch, France, Pien, 1969). Those samples were chosen to maximize the diversity of the potential factors affecting variations in P content (i.e., parity, lactation stage, breed, localization, cow diet, milk yield, and protein yield). The samples were split into 2 groups: the first group contained 348 samples for calibrating the prediction equation, whereas the second group contained 147 samples used as external data to validate the equation. The prediction equation was then applied to the 200,000 MIR spectra stored in the PhénoFinLait database. Milk Ca to P ratio estimations were obtained by the ratio of predictions for milk Ca and P contents, and not by a specific prediction equation.

The database used for analysis of variations of milk P content and milk Ca to P ratio was slightly different from that used for analysis of variations of milk Ca content in Chapter II because milk P content was only predicted for milk samples kept in database used in Chapter II, in which statistical individual with extreme values of predicted milk P content were removed. From 223,309 milk spectra used for Ca analysis, 222,274 spectra were kept before the restrictions due to date, stage of lactation, feeding strategy or number of points per herd that are detailed in Chapter II and were repeated in the present analysis.

Statistical models and definition of feeding strategies used to analyze variations of milk P content and milk Ca to P ratio were the same as those described in Chapter II . Moreover, considerations that were given for the Ca, like independence between stage of lactation and seasonality will be considered as still accurate for P, unless specified. As in Chapter II, because of the large amount of data gathered in the data basis, P-values could easily be low (< 0.001). Thus, the effect size (ES) of each simple fixed factor included in the ANOVA model

for all explicated variables was given. Statistical analyses were performed within breed.

## 2 Results and Discussion

### a Prediction equation of milk P content from MIR spectra

Results of milk P content prediction was good in our case, but a little less precise than those obtained by Soyeurt et al. (2009) and Toffanin et al. (2015). However, slope of the regression between predicted and measured values stayed close to 1. The mean milk P content we observed was similar to Toffanin et al. (2015), with a mean of 933, but lower than Soyeurt et al. (2009) that had a mean above 1070 mg/kg. The $R^2_{cv}$ we obtained, i.e. 0.76, was lower than those obtained by Soyeurt et al. (2009) and Toffanin et al. (2015), respectively 0.88 and 0.85, even if we used more milk samples for the calibration of the prediction equation and we had lower variation within selected samples (80 mg/kg in our case vs above 100 mg/kg for the two others studies). Our quality of prediction seemed less good than those obtained by Soyeurt et al. (2009) and Toffanin et al. (2015), but may be more reliable as more factors of variations may have affected milk P content in our case.

### b Description of the database and restrictions

The numbers of data points involved in the mixed models used for the analyses of milk P content or milk Ca to P ratio were 74,547, 59,130, and 76,819 for the Holstein, Montbéliarde, and Normande breeds, respectively. Repartition of calving dates and mean stages of lactation according to breed were similar to those obtained for Ca in Chapter II (Figures II.1 and II.2). Under-represented feeding strategies were removed (fewer than 2,000 milk samples, data not shown). It gave the same restrictions as those described for Ca analyses in Chapter II, i.e., strategies "grazing and FC hay" and "grazing and BD hay" were removed for Holstein and Normande cows, and the "grazed temporary pasture" strategy was removed for Montbéliarde cow. A high variability in the estimations of adjusted means was observed for data in August, September and November, as already observed in Chapter II.

### c Non-genetic factors of variation of milk P content

As for Ca, higher milk P contents were found for Normande than for Holstein and Montbéliarde cows, with average milk P contents over lactation of $987 \pm 1.5$, $951 \pm 1.6$ and $958 \pm 2.0$ mg/kg respectively. The effect of genetics, including breed, on milk P content has

been described several times in dairy cows (Cerbulis and Farrell, 1976, Hermansen et al., 2005, Van Hulzen et al., 2009, Nantapo and Muchenje, 2013, Chassaing et al., 2016). Jersey cows have been described to have a higher milk P content than Holstein or Friesian cows (Hermansen et al., 2005, Nantapo and Muchenje, 2013). Van Hulzen et al. (2009) found a high heritability for milk P content (0.60) but this was contradicted by Toffanin et al. (2015) who found a heritability of 0.12.

Milk P content decreased with parity, whatever the breed (TableVI.1). Differences between 1$^{st}$ and 5$^{th}$ or higher rank of lactation were 67.5 $\pm$ 1.14, 63.5 $\pm$ 0.84 and 91.2 $\pm$ 0.98 mg/kg respectively for Holstein, Montbéliarde and Normande cows. Parity was the factor of the model explaining the higher proportion of milk P content variability for the 3 breeds (ES for parity = 0.36 for Holstein, 0.35 for Montbéliarde and 0.48 for Normande; Tables VI.1 and VI.2) whereas the stage of lactation was the factor of the model explaining the higher proportion of variability for milk Ca content (Chapter II). This decrease in milk P content with the parity has been described in the literature over three lactations without possible explanation (Forar et al., 1982, Kume et al., 1998, Toffanin et al., 2015b).

| Parity | Holstein | Montbéliarde | Normande |
|---|---|---|---|
| 1 | 995.3 $\pm$ 1.34 [a] | 1000.0 $\pm$ 1.76 [a] | 1036.4 $\pm$ 1.24 [a] |
| 2 | 956.0 $\pm$ 1.34 [b] | 963.6 $\pm$ 1.78 [b] | 1009.5 $\pm$ 1.25 [b] |
| 3 | 940.9 $\pm$ 1.40 [c] | 948.7 $\pm$ 1.80 [c] | 983.9 $\pm$ 1.29 [c] |
| 4 | 936.7 $\pm$ 1.50 [d] | 943.6 $\pm$ 1.85 [d] | 961.2 $\pm$ 1.38 [d] |
| 5+ | 927.7 $\pm$ 1.55 [e] | 936.5 $\pm$ 1.80 [e] | 945.2 $\pm$ 1.37 [e] |

Table VI.1: Effect of parity ($P < 0.001$) on milk P content (mg/kg) for each breed. [a-e]Letters indicate the results of comparison between parities within a breed. Different letters indicate signifiant differences in P content ($P < 0.05$)

Milk P content sharply decreased between the first and second month of lactation by 53.8 $\pm$ 1.32 mg/kg for Montbéliarde, 42.6 $\pm$ 1.43 mg/kg for Holstein and 29.7 $\pm$ 1.30 mg/kg for Normande cows (Figure VI.1). Then it slightly increased between the 2$^{nd}$ and the 5$^{th}$ month of lactation for Holstein and Montbéliarde cows but the variation after the 2$^{nd}$ month of lactation remained low for the 3 breeds. The amplitude of the variations after the 2$^{nd}$ month of lactation was lower than 20 mg/kg for Montbéliarde and Normande cows, and around 30 mg/kg for Holstein cows. The proportion of variability explained by the stage of lactation was clearly lower than that explained by the parity for the 3 breeds (ES = 0.13 for Holstein, 0.19 for Montbéliarde and 0.09 for Normande; $P < 0.001$; Table VI.2). Similar effect of stage

# A). NON-GENETIC FACTORS OF VARIATION OF MILK P CONTENT

| | Breed | $P^1$ | $Ca/P^1$ | $Ca^2$ |
|---|---|---|---|---|
| Parity | Holstein | 0.36 | 0.28 | 0.10 |
| | Montbéliarde | 0.48 | 0.32 | 0.10 |
| | Normande | 0.35 | 0.26 | 0.16 |
| Stage of lactation | Holstein | 0.13 | 0.26 | 0.33 |
| | Montbéliarde | 0.09 | 0.23 | 0.35 |
| | Normande | 0.19 | 0.28 | 0.21 |
| Calendar Month | Holstein | 0.15 | 0.16 | 0.24 |
| | Montbéliarde | 0.16 | 0.16 | 0.24 |
| | Normande | 0.15 | 0.16 | 0.16 |
| Feeding Strategy | Holstein | 0.05 | 0.08 | 0.05 |
| | Montbéliarde | 0.09 | 0.12 | 0.16 |
| | Normande | 0.05 | 0.07 | 0.06 |
| Feeding Strategy x Calendar Month | Holstein | 0.08 | 0.08 | 0.05 |
| | Montbéliarde | 0.07 | 0.05 | 0.16 |
| | Normande | 0.10 | 0.08 | 0.06 |
| Calendar Month, | Holstein | 0.16 | 0.17 | 0.25 |
| Feeding strategy and | Montbéliarde | 0.19 | 0.15 | 0.31 |
| Feeding Strategy x Calendar Month | Normande | 0.16 | 0.15 | 0.18 |

Table VI.2: Effect size of explanatory variables of milk P and Ca contents and milk Ca to P ratio. [1]: ES estimated from database used for P. [2]: ES estimated from the database used for Ca analysis
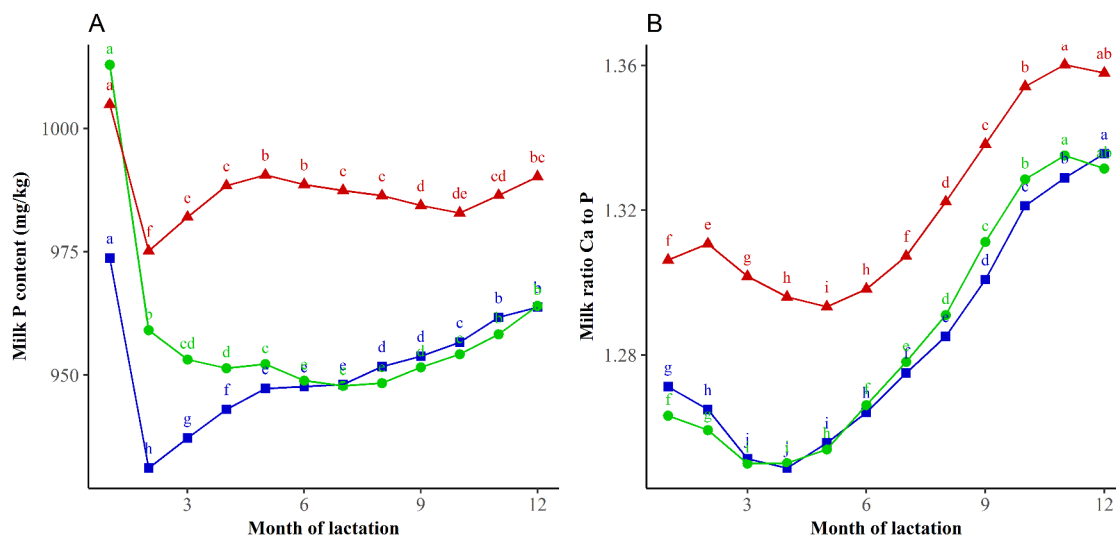


Figure VI.1: Effet of stage, within each breed on lactation on : A) milk P content, B) milk ratio Ca to P content for Holstein (blue), Montbéliarde (green) and Normande (red). [a-e]Letters indicate the results of comparison between parities within a breed. Different letters indicate signifiant differences in P content ($P < 0.05$)

of lactation on milk P content has been described by Hidiroglou et al. (1982) and Kaufmann and Hagemeister (1987), but not by Neville et al. (1995) who could not describe a constant effect of the stage of lactation when comparing milk P content obtained from several studies.

Milk P content decreased between March and July for the three breeds, whatever the
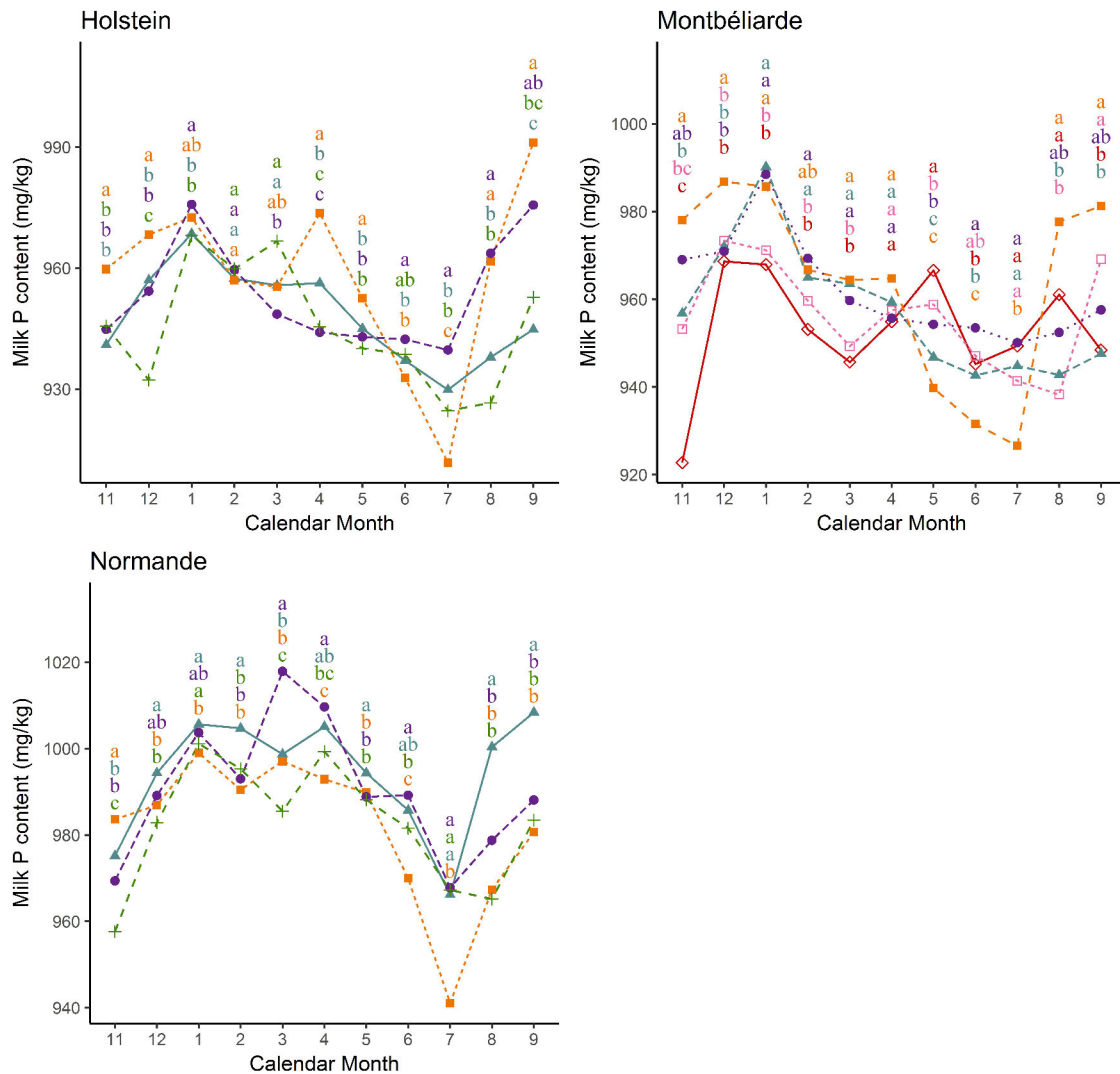
Figure VI.2: Effet of calendar month and feeding strategy on milk P content according to breeds. [a-d]Letters represent feeding strategy over a side-by-side comparison within a month ($P < 0.05$): grazing and field-cured hay (◇), grazing and barn-dried hay (□), maximum grazing (■), grazing and corn silage (▲), corn silage (●), grazed temporary pasture (+)

feeding strategy. It reached its lowest values in July for most strategies (Figure VI.2), except for the strategies "grazing and FC hay" and "grazing and BD hay". This period of July corresponded to a decrease in the proportion of grazed pasture in the diet for most feeding strategies, except "grazing and FC hay" and "grazing and BD hay" (Figure II.4). The proportion of variability explained by the calendar month was in the same order of magnitude than that explained by the stage of lactation (ES = 0.15 for Holstein and Montbéliarde, 0.16 for Normande, Table VI.2). Such variations in milk P content due to seasonality, with lower values during summer, have been already described (Lenstrup, 1926, Forar et al., 1982, Poulsen et al., 2015, Chassaing et al., 2016) but no explanation

has been proposed. In the PhénoFinLait database, the decrease in milk P content in July was concomitant to a decrease in milk protein and lactose contents (data not shown). This is coherent with the idea that P secretion in milk is related with protein and lactose secretion via exocytosis (Shennan and Peaker, 2000). This relationship has been shown in milk with the possibility to predict milk P content using lactose and protein contents (Klop et al., 2014). At the contrary of milk Ca content, milk P content does not seem to be affected by the day length (Boudon et al., 2016).

Even though the effect of the feeding strategy or the interaction between the feeding strategy and the calendar month on the milk P content were significant, the proportion of variability explained by those variables remained low (ES for feeding strategy = 0.05 for Holstein and Normande, 0.09 for Montbéliarde and ES for the interaction = 0.08 for Holstein, 0.07 for Montbéliarde and 0.10 for Normande). Differences in milk P content between feeding strategies were quite low, barely exceeding 20 mg/kg at a given calendar month. The ES of the effect of both the feeding strategy and the interaction between the feeding strategy and the calendar month were higher for Montbéliarde compared with Holstein and Normande because in Montbéliarde, milk P content was more steady during the year with the strategies "grazing and FC hay" and "grazing and BD hay" compared with others. At the contrary of milk Ca content, milk P content did not seem to be affected by the gradient of the proportion of grazed pasture in the diet in summer. Publications describing an effect of the diet on milk P content in dairy cows are rare. Ferris et al. (2010) did not found any effect of dietary P content on milk P content, but they compared levels of dietary P content that were sufficient to cover P requirements of dairy cows. Alvarez-Fuentes et al. (2016) showed a decrease in milk P content with lower dietary Ca content. As it has been shown that Ca content of pasture is the lowest in June and July (Metson and Saunders, 2012), this could be an explanation of the lower milk P content observed in summer in the PhénoFinLait database.

### d   Non-genetic factors of variation of milk Ca to P ratio

The milk Ca to P ratio was higher in Normande compared to both Holstein and Montbéliarde, with averages over the lactation of 1.32± 0.002, 1.28 ± 0.004 and 1.28 ±0.003 g/g for Normande, Holstein and Montbéliarde respectively (Figure VI.1B). These values were high compared to those published by Cerbulis and Farrel (1976) which were

comprised between 1.01 and 1.20 g/g. These authors also reported a clear effect of the breed with higher values for Holstein when compared to Jersey or Guernsey. The milk Ca to P ratios measured in the PhénoFinLait database were also in the highest values reported by Kaufmann and Hagemeister (1987) from studies realized in the 60s. The higher ratios observed in the PhénoFinLait database than in the experiments realized in the 60s and 70s could suggest an effect of the evolution of the genetics of the cows. Bijl et al. (2013) observed that both milk Ca and P contents increased in the same range since the 50s but these contents were expressed in mmol/kg in this study. When considering that Ca has a higher molecular weight than P, this would confirm the idea that milk Ca to P ratio expressed in g/g increased since the 50s.

Milk Ca to P ratio decreased with parity, whatever the breed (table VI.3) and parity explained an important part of variability (ES = 0.28 for Holstein, 0.26 for Montbéliarde and 0.32 for Normande, Table VI.2). This decrease was coherent with the fact that the decrease in milk P content with parity was more pronounced with higher ES than that of milk Ca content. Milk Ca to P ratio also slightly decreased after calving but increased sharply and continuously between 5 and 11 months of lactation (Figure VI.1). Stage of lactation explained as much variability as parity with similar range of ES (ES = 0.26 for Holstein, 0.28 for Montbéliarde and 0.23 for Normande, Table VI.2). This effect of the stage of lactation was mostly driven by its strong effect on milk Ca content (ES = 0.33 for Ca and 0.13 for P in Holstein).

| Parity | Holstein | Montbéliarde | Normande |
|--------|----------|--------------|----------|
| 1 | 1.24 ± 0.002 [d] | 1.25 ± 0.002 [e] | 1.27 ± 0.002 [e] |
| 2 | 1.28 ± 0.002 [c] | 1.28 ± 0.002 [d] | 1.31 ± 0.002 [d] |
| 3 | 1.30 ± 0.002 [b] | 1.30 ± 0.002 [c] | 1.32 ± 0.002 [c] |
| 4 | 1.30 ± 0.002 [b] | 1.30 ± 0.002 [b] | 1.34 ± 0.002 [b] |
| 5+ | 1.30 ± 0.002 [a] | 1.31 ± 0.002 [a] | 1.36 ± 0.002 [a] |

Table VI.3: Effect of parity ($P < 0.001$) on milk Ca to P ratio (g/g) for each breed. [a-e]Letters indicate the results of comparison between parities within a breed. Different letters indicate signifiant differences in P content ($P < 0.05$)

Whatever, the feeding strategies, lowest milk Ca to P ratios were obtained in April, if not considering November, August and September, during which unexplained variability of milk composition was high (Figure VI.3). Those lower values in April were concomitant with the decrease in milk Ca content. The variability of milk Ca to P ratio explained by the
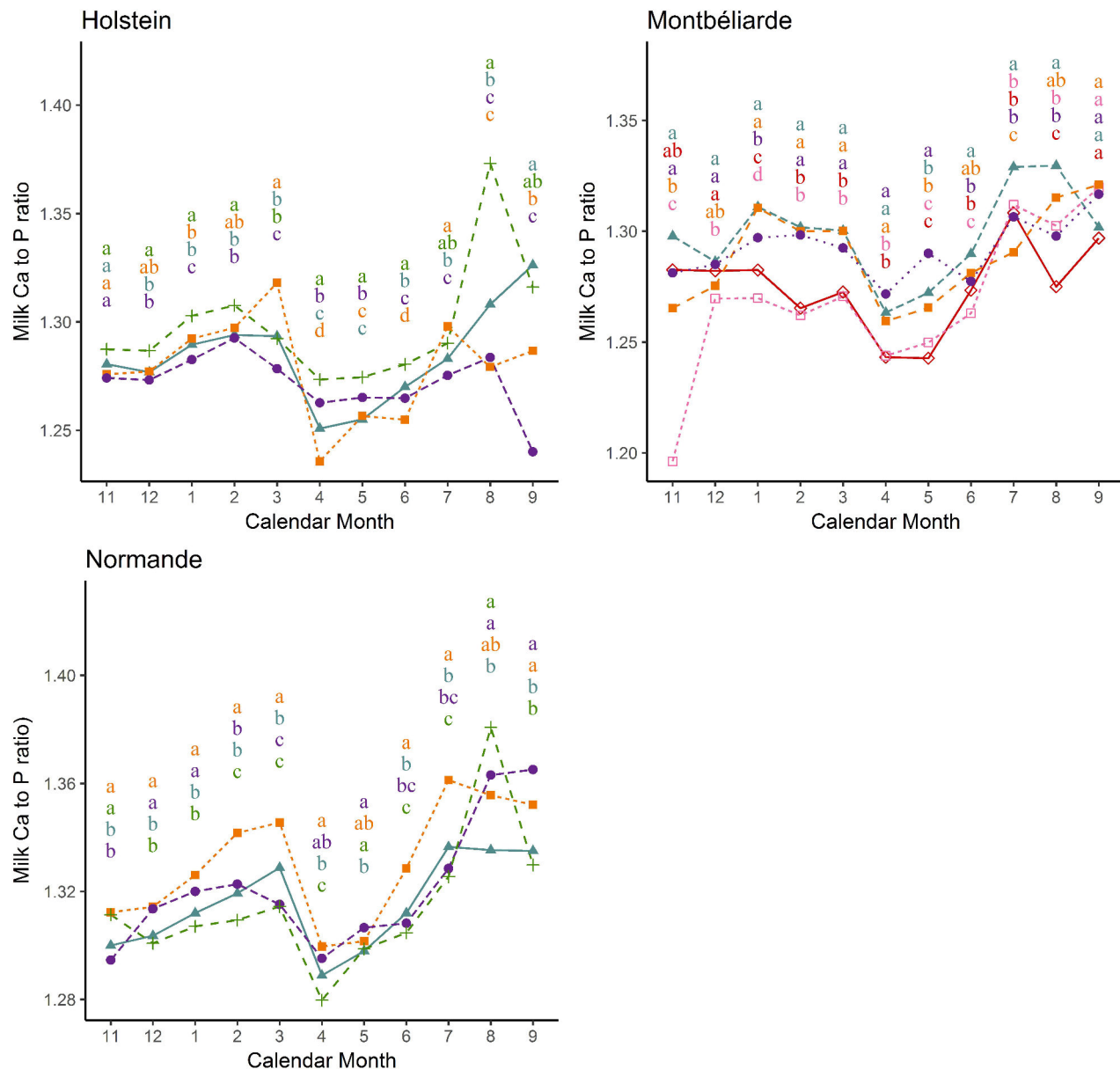
Figure VI.3: Effet of calendar month and feeding strategy on milk Ca to P ratio (g/g) acconding to breeds. [a-d]Letters represent feeding strategy over a side-by-side comparison within a month($P < 0.05$): grazing and field-cured hay (◇), grazing and barn-dried hay (□), maximum grazing (■), grazing and corn silage (▲), corn silage (•), grazed temporary pasture (+)

calendar month was lower than that explained by the parity or the stage of lactation (ES for calendar month = 0.16 for the three breeds, ES for feeding strategy = 0.08 for Holstein, 0.12 for Montbéliarde and 0.07 for Normande, TableVI.2). The higher ES for the feeding strategy for Montbéliarde was due to the fact that, the strategies "grazing and FC hay" and "grazing and BD hay", specific to Montbéliarde, drove to lower milk Ca to P ratio compared with other strategies, especially in winter (Figure VI.3).

## 3 Conclusion about non-genetic factors of variation of milk contents of Ca and P

This supplementary analysis of the PhénoFinLait database showed that the factors of variation of milk Ca and P contents are quite different. The milk content of both elements similarly depends on breed with higher contents in Normande compared to Holstein and Montbéliarde and slightly higher contents in Montbéliarde than in Holstein. The differences in milk P content between breeds were clearly lower than those of milk Ca content. Within breed, parity is the factor explaining the higher part of the variability of milk P content whereas stage of lactation and calendar month explained higher parts of the variability of milk Ca content. The proportion of grass in the diet, as hay or grazed herbage, that decreases milk Ca content does not seem to affect milk P content. The effect of the stage of lactation on milk P content is mainly explained by a drop at the beginning of lactation whereas this drop was accompanied by an increase at the end of lactation for milk Ca content.

A consequence of those differences in the variations of milk Ca and P contents is that milk Ca to P ratio is affected by the stage of lactation with relatively steady value until 4 months of lactation and an increase after. On gross averages, these variations were quite concomitant to those of the plasma OC to CTX ratio we could observe during my experiments (see figure VI.6 later in this chapter) with steady values until 3-4 months of lactation, due to high values of plasma both OC and CTX, and an increase after 4 months of lactation with more bone accretion than resorption. The fact that milk Ca to P ratio could be correlated, within cows, to plasma OC to CTX ratio can also make sense from a physiological point of view. In a literature review, Anderson et al. (2017) highlighted a relationship between plasma Ca and Pi concentrations, particularly in situation of dietary deficiency of P or eventually Ca in breeder cows. A general underlying mechanism could be that when the supply of one of those two elements is too low, a bone mobilization occurs and releases concomitantly Ca and P. The proportional rate of uptake of Ca and Pi released from bones by others organs would be higher for the element that is not sufficiently provided, which would increase, at least transitory, the plasma concentrations of the other one, at the origin of this negative relationship between plasma Ca and Pi concentrations. In parallel, in adequate dietary situations compared to dietary deficiency, it could be considered that the plasma

concentration of the element that could be deficient would comparatively slightly increase. The inverse relationship between plasma Ca to Pi ratio and plasma CTX in situation of low dietary supply of P in breeder cows obtained by Anderson et al. (2017) illustrated this principle. Our assumption was that, during lactation, dairy cows adequately supplemented are more susceptible to be submitted to situations with difficulties of regulation of plasma Ca concentration rather than plasma Pi concentration. The principles outlined before would draw to a negative relationship between plasma Ca to Pi ratio and CTX, as the increase in bone resorption by Ca challenge would lead to a higher plasma Pi concentration. This would lead to a positive relationship between plasma Ca to Pi ratio and OC to CTX ratio, when Ca is driving the bone mobilization, when it would become negative when P drives the bone mobilization, like it is the case for Anderson et al. (2017). However, plasma Ca is more regulated by the organism than plasma Pi and it would be consistent that this relationship would not be as accurate as that shown by Anderson et al. (2017). If we consider the role of the mammary gland in the regulation of plasma Ca concentration demonstrated by Van Houten et al. (2004), the fact that the relationship between milk Ca to P ratio and plasma OC to CTX ratio we observed could be more accurate than that between plasma Ca to Pi ratio and plasma OC to CTX ratio makes sense, as both milk Ca and P contents would be affected, due to low plasma Ca concentration. Van Houten et al. (2004) showed that the amount of Ca secreted in milk could decrease to allow the regulation of plasma Ca concomitantly with higher bone resorption in case of very low Ca dietary supply. This would mean that variation of milk Ca could be more pronounced than variation of plasma Ca in case of Ca deficiency. At the contrary the link between plasma Pi and milk P is less known, but it cannot be excluded that high plasma Pi can increase milk P. This is a only a suspicion given that the main parameters known to affect milk content of P are milk protein and lactose contents, and that Pi is suspected to be excreted via Golgi secretory membrane and Pi content in Golgi vesicles is either generated by UDP uptake for lactose synthesis or provided by casein phosphorylation (Shennan and Peaker, 2000). Finally, the results of Anderson et al. (2017) and ours, suggest that the relationship between plasma Ca to P or milk Ca to P ratios and plasma OC to CTX ratio exists but could be very dependent on the nutritional situations or homeostatic challenges considered in the comparison and more specifically on the Ca and P dietary supplies.

# B) Can the dynamics of milk Ca content during lactation be used to predict that of bone mobilization and reconstitution?

## 1 Methods for cross analysis of the 3 experiments carried out in this thesis

To answer this question, a cross analysis of the results of plasma concentrations of biomarkers of bone accretion and resorption and of milk Ca contents in the 3 experiments described in this thesis was carried out. To facilitate the reading of this part of the text, the 3 experiments were named as follow: 'Parity-Unique TMR' for the experiment described in Chapter III, 'Breed and E density' for the experiment described in Chapter IV, 'Mineral supplementation' for the experiment described in Chapter V.

An objective of this section was to analyze the variability of the measured parameters between the three experiments. To achieve this, cows used in the 3 experiments were split between 13 groups according to the experiment, their parity (primiparous or multiparous), their breed (Holstein or Normande) and the dietary treatments that were specific to each experiment. The definition of the 13 groups, the numbers of cows constituting each group and the names of the dietary treatments are resumed in table VI.4. For each group, only data from the day when milk and blood were both sampled on a same cow were kept. Thus, plasma concentrations of OC and CTX obtained before calving were discarded, as well as milk contents of Ca, P or protein that were obtained with no concomitant analyses of plasma OC and CTX concentrations in the experiment 'Mineral supplementation'. For the statistical effect of the dynamics of plasma OC and CTX and milk Ca, protein and P during lactation, times of sampling were approximated to the month of lactation during which they occurred. As no sample was collected during the 4$^{th}$ month of lactation for the 'Mineral supplementation' experiment, data from this month of lactation were removed from statistical analysis, but not from figures, to obtain a complete block design. If several samples of blood or milk were collected on the same month of lactation, values were averaged.

Several statistical models were used for the cross analysis of the 3 experiments. To

| Experiment (Chapter) | Breed | Treatment [1] | Primiparous | Multiparous |
|---|---|---|---|---|
| Parity-Unique TMR (III) | Holstein | UTMR | 17 | 16 |
| Breed and E density (IV) | Holstein | HFS | 4 | 2 |
| | | LFS | 3 | 4 |
| | Normande | HFS | 2 | 4 |
| | | LFS | 4 | 7 |
| Mineral supplementation (V) | Holstein | NCA | | 4 |
| | | LCa | | 5 |
| | | LCaLD | | 5 |

Table VI.4: Number of cows constituting the 13 groups used for cross analysis of the 3 experiments described in this PhD thesis. [1]: UTMR = Unique TMR fed in experiment 'Parity-Unique TMR' (Chapter III), HFS and LFS = High and Low feeding strategies used in experiment 'Breed and E density' (Chapter IV), NCa, LCa, and LCaLD for the 'Normal Ca', 'Low Ca', 'Low Ca and Low DCAD', respectively in experiment 'Mineral supplementation' (Chapter V).

analyze the variability between groups of average values and of the dynamics over lactation of plasma concentrations of bone biomarkers and milk contents of Ca and P, the following model was used:

$$Y_{ijk} = \mu + Stage\,of\,lactation_i + Group_j + Stage\,of\,lactation : Group_j + $$
$$Cow(Group)_{k|j} + \epsilon_{ijk}$$

(VI.1)

where $Y_{ijk}$ is the explained variable, and stage of lactation and group were considered as qualitative fixed effects; cow was a random effect within a group. Analyses were realized using PROC GLIMMIX (SAS Institute, 2013). The matrix covariance selected was that presenting the lower AIC.

For the analysis of the relationship between milk contents of Ca and protein or between plasma OC to CTX ratio and milk Ca to P ratio, an intra-individual regression was realized, using R (R Development Core Team, 2008), and the following model was used:

$$Y_{ij} = \mu + \beta X + Cow_i + \beta : Cow_i \times X + \epsilon_{ij}$$

(VI.2)

where $Y_{ij}$ is the explained variable by the continuous variable ($X$). The effects of the cow and the interaction between the cow effect and the $X$ predictor were considered as fixed,

according to the question.

## 2  An important variability of the dynamics of plasma CTX across experiments

We observed, previously in this thesis, that plasma OC concentrations were higher in primiparous than in multiparous cows in experiments 'Parity-Unique TMR' and 'Breed and E density', as well as in experiment 'Breed and E density' with multiparous Normande compared to multiparous Holstein cows.  The cross-analysis of the plasma OC concentrations shows a high variability of this parameter across experiments (Group, P < 0.001, Figure VI.4) but also a rather consistent pattern of variation with the stage of lactation across groups.  The parity explains a high proportion of the variability of the average plasma OC concentrations between groups.  The lowest concentrations were observed for the multiparous cows of the experiment 'Breed and E density' whatever the feeding strategy.  The effect of the stage of lactation was quite consistent among experiments (Stage P < 0.001) with lowest values during the 1st month of lactation, a sharp increase after, mostly before the 2nd month of lactation, and a plateau more or less steady according to the groups after.  The increase at the beginning of lactation could be smoother for some groups of multiparous cows from the experiments 'Breed and E density' and 'Mineral supplementation' and some irregularities could be observed after 2 months of lactation according to the groups (Interaction group × stage of lactation, P = 0.01).  The clear effect of the parity on plasma OC concentrations is consistent with the idea that primiparous cows are still growing with more bone accretion and remodeling as already observed by Iwama et al. (2004) or Taylor et al. (2009) during the first months of lactation.  The shape of the dynamics over lactation we observed was also quite consistent with consensual observations from the literature, with a sharp increase at the beginning of the lactation and quite constant concentrations after two months of lactation (Liesegang et al., 2000, Taylor et al., 2008, Taylor et al., 2009, Puggaard et al., 2014).  The studies of Holtenius et Ekelund (2005) and Ekelund et al. (2006) were the only studies, as far as we know, to illustrate a regular increase until 25 weeks of lactation and, nothing, excepting maybe strong particularity of the used breed, Swedish Red and White Breed, explained this specific result.  The clearly effect of the breed we observed, with higher OC

concentrations for Normande compared with Holstein, was not as strong as that of the parity.
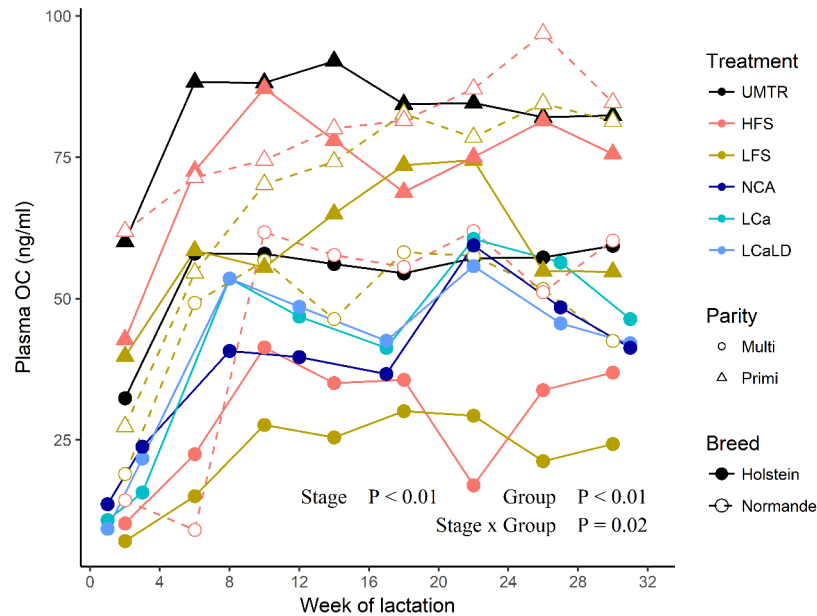


Figure VI.4: Effect of the experimental group on the dynamics of the plasma osteocalcin (OC) concentration over lactation

We also observed in the previous parts of this thesis that the average plasma CTX concentration was higher in primiparous than in multiparous cows (experiments 'Parity-Unique TMR' and 'Breed and E density') and tended to be higher in Normande than in Holstein cows ('Breed and E density'). It also appeared that the amplitude of variation of CTX during lactation was higher with the high feeding strategy compared with the low feeding strategy ('Breed and E density'), with multiparous Normande compared to multiparous Holstein ('Breed and E density'). It could be higher in primiparous that in multiparous cows ('Parity-Unique TMR', 'Breed and E density'-LFS) but this latter result depended on the feeding strategy given that the amplitude of variation of CTX during lactation was lower in primiparous cows compared with multiparous cows for the high feeding strategy in the experiment 'Breed and E density'. The cross-analysis of the plasma CTX concentrations confirms a high variability of the dynamics of plasma CTX concentrations across groups (Figure VI.5), higher than that of plasma OC concentrations. Plasma CTX concentrations increased during the first month of lactation in all groups and decreased after 4 months of lactation in most groups (Stage, $P < 0.001$), but highest values were reached at different stages of lactation according to the

group. The variability between 2 and 4 months of lactation could be high and the amplitude of variation within groups between stage was also very variable (Interaction group × stage of lactation, $P < 0.001$). Higher variability of the dynamics of plasma CTX concentration was observed for the experiment 'Breed and E density' compared to both others. Even though part of this higher variability in this experiment could be attributed to erratic variation due to the low number of individuals in certain groups, the amplitude of variation remained higher in this experiment. The fact that the dynamics of bone resorption over lactation, and more specifically that of plasma CTX concentration, are more variable than that of accretion (OC) is consistent with the literature. Most specifically, several authors showed that dynamics of plasma concentration of bone biomarker of bone resorption over lactation can be affected by the diets of the cows or the balance between cows' requirements and supply, which is less the case for bone accretion. Liesegang et al. (2000) observed an increased bone resorption with increased milk production. Moreira et al. (2009) observed an increase in serum PYD with lower dietary Ca content. Puggaard et al (2014) observed an increase in serum CTX with lower dietary P content. All those authors did not observe any effect of the compared dietary Ca or P contents on the dynamics of bone accretion. Only Boudon et al. (2016) observed effect of DCAD and day length on bone accretion and not on bone resorption. From these results, it seems that bone resorption responds to insufficient mineral supply, due to higher milk production (Chapter IV, Liesegang et al., 2000) or inadequate mineral supplementation (Moreira et al., 2009, Puggaard et al., 2014, Anderson et al., 2017). This is in accordance with the action of PTH and PTHrP in case of hypocalcemia, which effect is to increase bone resorption. Effect of DCAD on bone accretion may be linked with the fact that a part of Ca in bone is transferred into blood to maintain blood pH. It is possible that this Ca comes from bone liquid, and thus less Ca is available for mineralization of organic matrix in bone.

When considering the analysis of the plasma OC to CTX ratio across experiments, it appears that it was only affected by the stage of lactation ($P < 0.001$, figure VI.6) and the interaction between stage of lactation and group ($P < 0.001$) and not by the group ($P = 0.50$). This suggests than even with high differences between groups on mean values of OC and CTX concentrations due to parity, breed or treatments, the ratio smoothed those differences. The plasma OC to CTX ratio increased continuously during the 4 first months
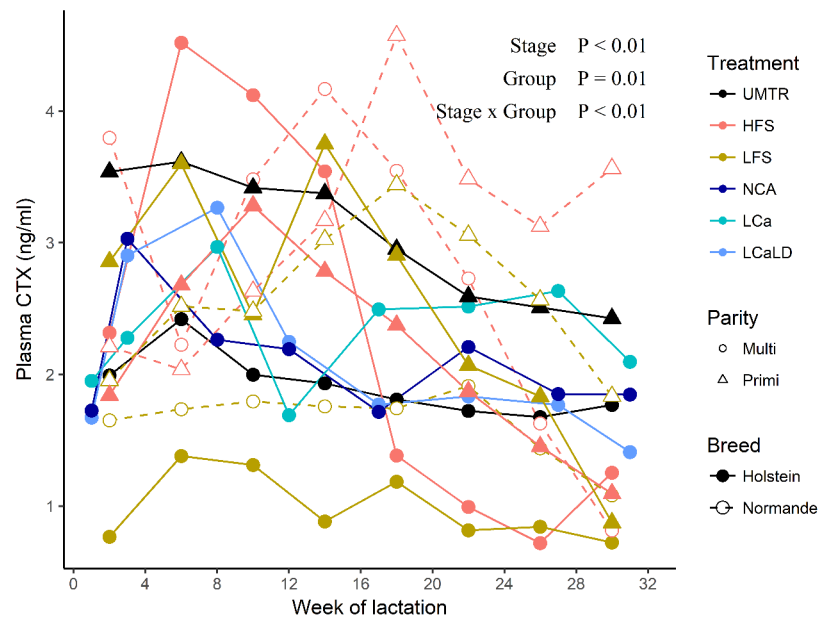
Figure VI.5: Effect of the experimental group on the dynamics of the plasma carboxy- terminal cross-linking telopeptides of collagen type I (CTX) concentration over lactation

of lactation but the increase could be sharper after, especially for the experiment 'Breed and E density'. The increase in the plasma OC to CTX ratio during the lactation, is coherent with the literature suggesting that the bone mobilization is important in early lactation and is replaced gradually by bone reconstitution (Ramberg et al., 1970, Braithwaite, 1983a, Ekelund et al., 2006, Puggaard et al., 2014).
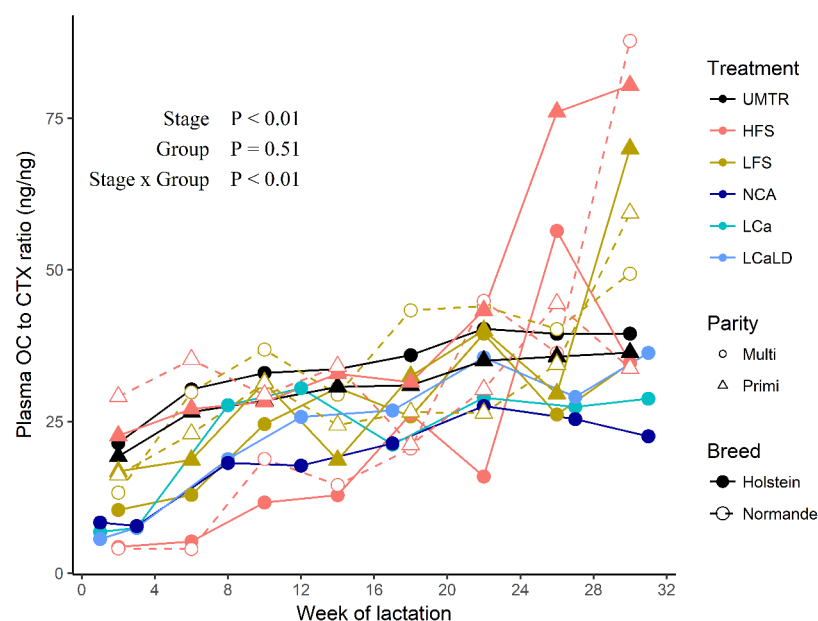


Figure VI.6: Effect of the experimental group on the dynamics of the plasma OC to CTX ratio over lactation

Finally, the cross analysis of the experimental results of this thesis illustrates that we obtained a clear variability of the dynamics of plasma CTX concentrations over lactation. Part of this variability could be attributed to the breed and the parity of the cows, but also to the feeding strategy. Thus it can be verified that we obtained enough variability to test our hypothesis that milk Ca content could be related to bone resorption when considering within cows variations during the lactation.

## 3 A weak link between the variability of milk Ca content and that bone resorption at the scale of the whole lactation

### a Cross-analysis of milk Ca content

When considering experiments independently we observed several links between dynamics of bone accretion during lactation and of milk Ca contents. In the experiment 'Parity-Unique TMR', the younger cows showed higher amplitude of variation during the lactation for both plasma CTX and milk Ca. In the experiment 'Breed and E density', the Normande cows showed both higher plasma CTX concentration and amplitude of variation during lactation that were also accompanied by higher milk Ca content and higher variation of milk Ca content during lactation. However, these links were weak and not always consistent. When looking at the variability of the dynamics of milk Ca content between individuals, it appears, in experience 'Parity-Unique TMR', that it was not related to that of plasma CTX. The high effect of the feeding strategy on the dynamics of CTX in multiparous cows in the experiment 'Breed and E density' did only affect that of milk Ca content at the very end of lactation. The amplitude of the dynamics of milk Ca content during lactation was more pronounced in multiparous cows than in primiparous cow in the experiment 'Breed and E density', at the contrary of what we observed in the experiment 'Parity-Unique TMR'. Milk Ca content also differed at the end of lactation between 3 tested groups in experiment 'Mineral supplementation' whereas plasma CTX was not affected.

The cross-analysis shows that the average of milk Ca contents is strongly affected by the group (P < 0.001, Figure VI.7). The differences between the groups are partly explained by higher values for Normande compared with Holstein which was consistent with the observations issued from the analyses of the PhénoFinLait database. However, strong variations within breed could also be observed in our set of data. Given the high effect

of the genetic of the cow on the milk Ca content (Van Hulzen et al., 2009) and the low number of cows involved in most groups, it is likely that most of this variability is explained by genetic factors that remained to be elucidated. In most groups, the milk Ca content decreased during the first 2 months of lactation and slowly increased until the end of the lactation (stage of lactation, P < 0.001), which was consistent with the observations issued from the analyses of the PhénoFinLait database. However the dynamics of milk Ca content could be very different from one group to another (Interaction group × stage of lactation, P < 0.001). Part of this variability could be due to the erratic variations of the milk Ca content that could be observed in the 'Breed and E density' experiment due to the low numbers of animals in some of those groups but also to instability of the diet between pasture during the turnout in Spring due to difficult meteorological conditions on the year of the experiment. It can be noticed that the overall range of variation of milk Ca content, over 0.5 g/kg in our dataset, is important in comparison to the variability described in the literature (Van Hulzen et al., 2009, Poulsen et al., 2015, Toffanin et al., 2015b, Chassaing et al., 2016). However, dynamics in the 'Breed and E density' experiment were far from the one observed from the PhénoFinLait program, with important variations in comparison with the groups from the 'Parity-Unique TMR' and 'Mineral supplementation' experiments.
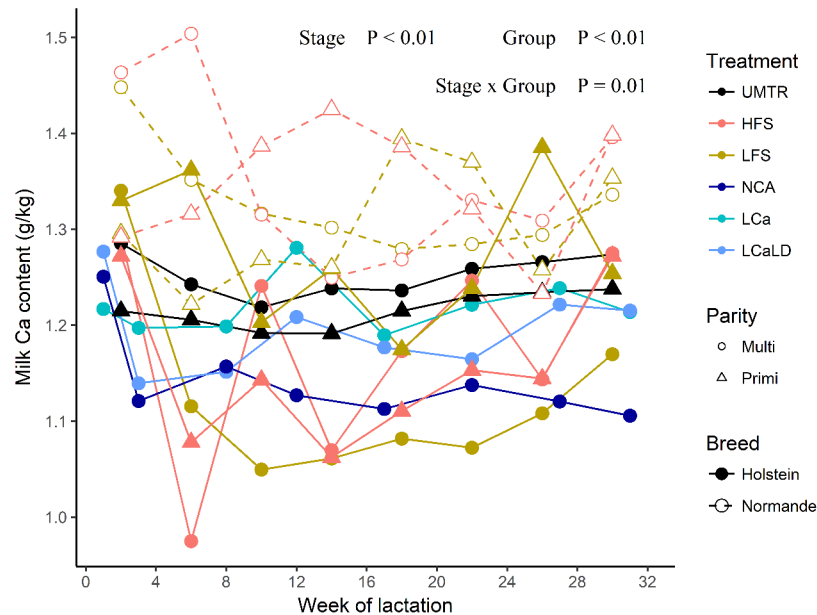


Figure VI.7: Effect of the experimental group on the dynamics of the milk Ca content over lactation

All those results illustrate that the variability of average milk Ca contents over lactation between animals is very determined by the genetics and thus cannot be an

indicator of environment or feeding conditions. If some links could be observed between the dynamics of milk Ca content and plasma CTX concentration during lactation, those links lack of consistency and it is not possible to imagine that the dynamics of milk Ca content over lactation within an individual could be an indicator of that of bone resorption. This conclusion can be drawn even more if we consider that the variability of the plasma CTX was relatively high in the dataset obtained from our experiments. The variability of the dynamics of milk Ca is more difficult to explain than that of CTX and several publications suggested that milk Ca content can be explained by milk protein content (Alais, 1984, Gaucheron, 2005). A next question is to explain if a part of variations of milk Ca content during the lactation could be explained by variation of milk protein content in our dataset.

### b   Cross-analysis of milk Ca to protein ratio

It has been considered that milk Ca content was mostly determined by milk protein content (Alais, 1984, Gaucheron, 2005), due to 1) the role of Ca in the stability of the structure of micelles of caseins in milk (Malacarne et al., 2014), 2) the fact that secretion of Ca by the mammary epithelial cells into milk was firstly described as mediated by the incorporation of Ca in caseins (Neville and Peaker, 1979), 3) the high correlation between milk contents of Ca and protein when comparing species (Jenness, 1979). The analysis of the PhénoFinLait database (Chapter II) suggested that, even though a correlation exists between milk Ca and protein contents, milk protein content only explains a low part of the variability of milk Ca content. Indeed, the correlation between milk contents of Ca and protein was relatively weak with a $R^2$ that did not exceed 0.3 within a breed. A certain variability of the dynamics of the milk Ca to protein ratio during lactation could be observed (Figure II.7). When looking at the relationship between milk Ca and protein contents that could be observed on the dataset gathered from the experimental part of this thesis, it appears that both milk contents of Ca and protein were weakly correlated as observed in the PhénoFinLait database ($R^2$ = 0.24, Figure VI.8A). Part of the correlation was explained by the breeds given that Normande cows had higher contents of both Ca and protein than Holstein and that, regressions performed on data from either Normande or Holstein explained less variability with lower slopes (Figure VI.8B). Our question is to determine if a part of the variability of milk Ca content during lactation, i.e. within cow,

could be explained by the milk protein content. Within-cow regressions with model VI.2 show that average Ca content for a given milk protein content was strongly explained by the cow (Cow effect $P < 0.001$, Figure VI.8B). However, in most cows, a positive relationship between milk Ca and protein contents could be explained with, nevertheless, a high variability of the slope between individual (Effect of the interaction cow $\times$ milk protein, $P < 0.001$). The model VI.2 explained 72% ($R^2$) of the variability of milk Ca content but the contributiosn of the effects of the cow and the interaction cow $\times$ milk protein were similar. Finally, this means that only a small part of the variability of milk Ca content during lactation, i.e. within cow, could be explained by the milk protein content.
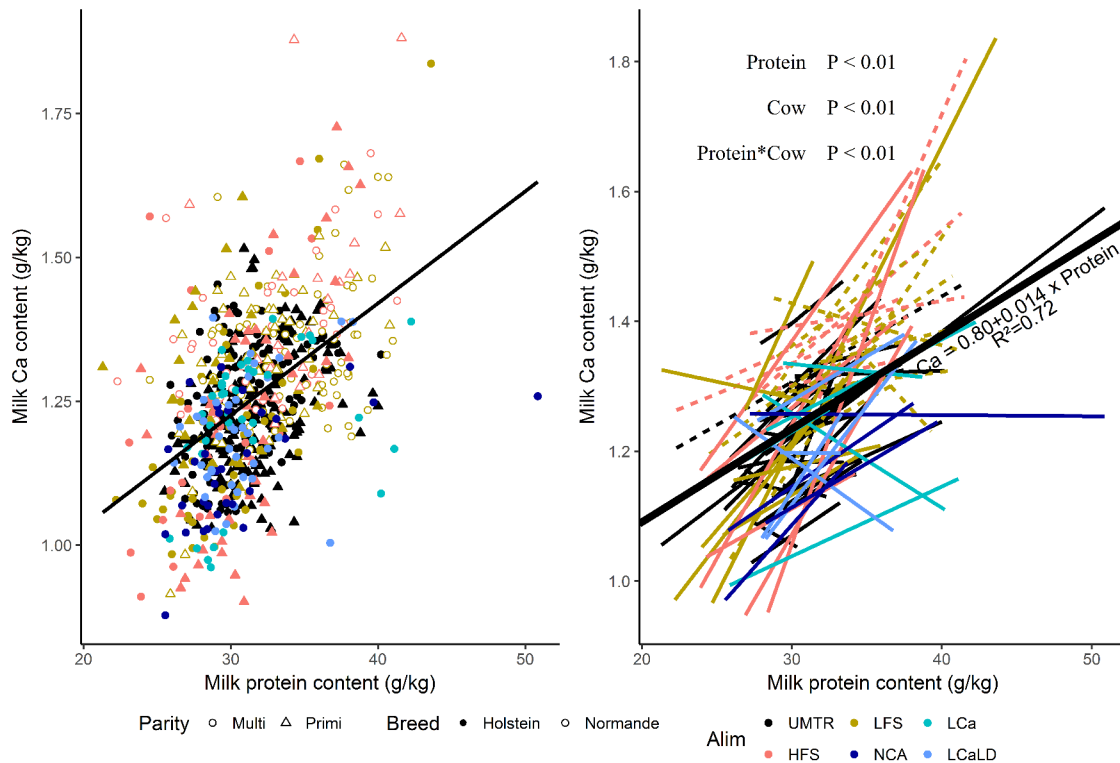


Figure VI.8: Regression between milk Ca content and protein content. Black bold line is A) regression between Ca and protein contents B) regression between Ca and proteins contents corrected from the effect of cow

When looking at the effect of the group of cows on both milk protein content and milk Ca to protein ratio (Figure VI.9), dynamics of milk protein content during lactation had similar shapes according to the groups, even if some dynamics could show some breaks, like that of primiparous Normande with HFS between 10 and 14 weeks of lactation in the experiment 'Breed and E density' (Figure VI.9A, interaction stage of lactation $\times$ group, $P < 0.001$). The milk protein content decreased during the second and third month of lactation, as expected,

and increased after until the end of lactation (Stage of lactation P < 0.001). Normande showed higher milk protein content and some groups showed very low milk protein contents (Group, P < 0.001). Dynamics of milk Ca to protein ratio exists during lactation with a clear effect of the stage of lactation (P < 0.001, Figure VI.9B). For the groups from the experiments 'Parity-Unique TMR' and 'Mineral supplementation', milk Ca to protein ratio sharply increased during the first month of lactation and then gradually decreased until the end of the lactation. Important and erratic variations of milk Ca to protein ratio could be observed for the groups from the experiment 'Breed and E density' and may explain the significant effect of the interaction stage of lactation × group (P < 0.001). The group effect significantly affected the milk Ca to protein ratio (P < 0.001).

The inconsistent links we could have observed between dynamics of milk Ca content and that of plasma CTX concentration in our experiments was not observed when considering milk Ca to protein ratio rather than milk Ca content. In the experiment 'Parity-Unique TMR', the youngest cows showed higher amplitude of variation of plasma CTX during lactation for both plasma CTX and milk Ca but not for milk Ca to protein ratio. In the experiment 'Breed and E density', the Normande cows showed both higher plasma CTX and higher amplitude of variation during lactation that were also accompanied by higher milk Ca content and higher variation of milk Ca content during lactation but not by similar variation of milk Ca to protein ratio.

From a physiological point of view, the higher relevance of milk Ca to protein compared with milk Ca content, as an indicator of bone resorption, is not clear. In the publication of Van Houten et al. (2004) showing an implication of the mammary gland in the regulation of Ca in mice, the low dietary Ca content induced a decrease in the milk Ca to protein ratio and not in the milk Ca content because milk protein content increased. The authors concluded that the mammary gland was able to secrete less Ca to avoid a decrease in plasma Ca concentration. However, their conclusion was based on the hypothesis that Ca secretion in milk would be firstly determined by the amount of casein secreted and that the mammary gland would be able to marginally affect Ca secretion by modulating the amount of Ca linked to casein. However, it can be argued, from results published after 2004, that this view has to be modulated. VanHouten et al. (2007) illustrated that the equilibrium established between casein and Ca in the Golgi apparatus of MEC is different from that established in the milk
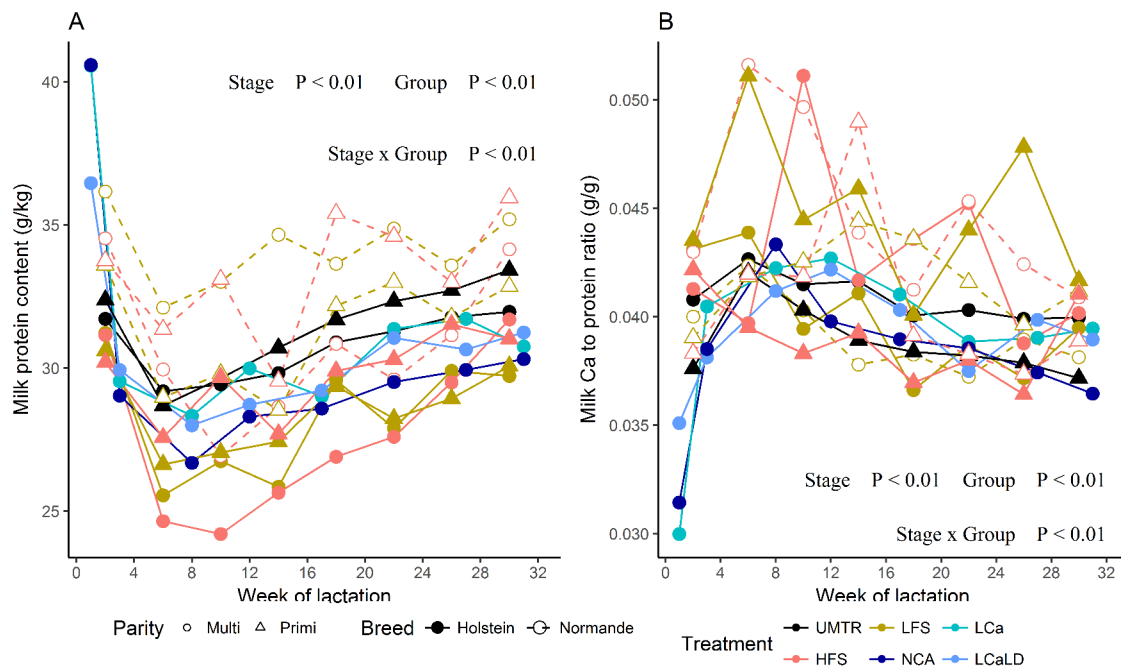
Figure VI.9: Effect of the experimental group on the dynamics of A) the milk protein content and B) Ca to protein ratio over lactation

after secretion. Indeed, only 40% of Ca secreted in milk by the MEC is secreted with casein, the reminder being secreted as free Ca via the transporter PMCa2. At the contrary, the proportion of colloidal Ca in milk, i.e. associated to casein, is close to 66% as observed in 'Mineral supplementation' experiment and in the literature (Kaufmann and Hagemeister, 1987, Flynn and Cashman, 1997), the reminder being free Ca. It cannot be argued either that secretions of Ca and casein in milk from the MEC are completely independent. It may even be likely that, for a given casein secretion, the Ca secretion is limited to a maximum value because a presence of high quantity of soluble Ca in milk, that could not be included in casein micelle, has to be limited to avoid formation of solid precipitates (Neville et al., 1995, Gaucheron, 2005). This limitation may occurs because at high concentrations of free Ca secreted in milk by the MEC, concentration gradient between MEC and milk would be high and secretion of Ca via PMCA2 would require an important amount of ATP, which could become too important for the CEM maintenance. However, under this maximum limit, the results synthetized by Farrel et al. (2006) and Malacarne et al. (2014) suggest that the amount of Ca secreted by the MEC can be modulated without affecting the stability of the micelle of casein because of a decrease in the size of the micelle when the quantity of Ca secreted in proportion of casein decreases. This would mean that the mammary gland has

latitude to modulate the amount of Ca secreted for a given amount of casein secreted. Our results suggest that a maximum of Ca to protein ratio is barely reached for both Holstein and Normande in our experiments, as it only happens during the second month of lactation in 2 groups of cows (Figure VI.8). This could mean that in the range of variation of milk Ca to protein ratio obtained in our experiment, the mammary gland would have had important latitude to modulate independently Ca and casein secretion.

This point will be discussed in the last section of this general discussion. However, our data illustrated a clear variation of milk Ca content that could be linked to bone resorption with the underlying idea that lowering milk Ca content in some cases could be concomitant with a decrease in milk Ca and increase in bone resorption. However, this general rules could not be generalized.

### c Cross-analysis of milk Ca to P ratio

In experiment 'Parity-Unique TMR' (Chapter III), the variations of milk Ca to P ratio during lactation, i.e. within cows, seemed to be correlated to that of plasma OC to CTX ratio. The correlation was still present but less good in the experiment 'Breed and E density' (Chapter IV), especially for LFS. The relationship between both ratios was not tested in the experiment 'Mineral supplementation' because our objective to increase plasma CTX with low Ca treatments was not achieved. The aim of the section is to illustrate the variability of milk P content and milk Ca to P ratio, to compare the relationship between plasma OC to CTX and milk Ca to P ratios across experiments and to try to conclude about the relevance of this ratio.

Milk P content clearly decreased during the first month of lactation and could be quite steady after for some groups (experiments 'Parity-Unique TMR', 'Mineral supplementation') with erratic variation in the 'Breed and E density' (Figure VI.10A , stage, $P < 0.001$, interaction stage $\times$ group P=0.01). Milk P content was clearly affected by the group with lower values for the experiment 'Breed and E density' (P<0.001). The higher milk P contents with Normande compared with Holstein, expected from the PhenoFinlait database (cf. Section A of this general discussion), was not observed in figure VI.10A due to the very low value observed for both breeds of cows in the experiment 'Breed and E density' compared to others. Similarly, effect of parity within a treatment tended to be opposite to that expected from the PhénoFinLait database, with higher P content for

multiparous in 'Breed and E density' experiment. As for Ca, the observed range of variation of milk P content we observed, almost 0.5 g/kg, was far higher than those reported in the literature, i.e. less than 0.2 g/kg for Toffanin et al. (2015). The coefficient of variation was 37% in our case, when Chassaing et al. (2016) reported a coefficient of 5%. In experiments 'Parity-Unique TMR' and 'Mineral supplementation', the milk Ca to P ratio increased after the 1st month of lactation and remained relatively steady after whereas it was much higher in the experiment 'Breed and E density' with huge variation between months after the 1st month of lactation (P < 0.001 for stage of lactation, group and their interaction, Figure VI.10B). The milk Ca to P ratios observed in the experiment 'Breed and E density' are very high compared with those observed with the PhénoFinLait database (Figures VI.1 and VI.3).
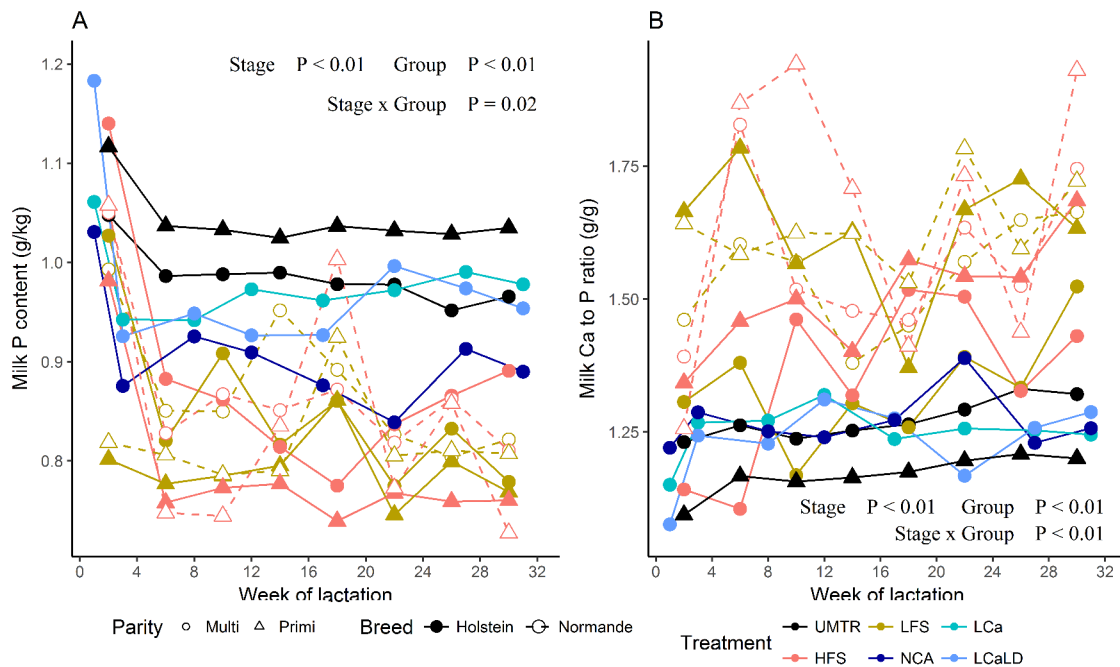


Figure VI.10: Effect of the experimental group on the dynamics of A) the milk P content and B) milk Ca to P ratio over lactation

To compare the relationship between plasma OC to CTX and milk Ca to P ratios across experiments, the groups were redefined by avoiding the differentiation of breeds and parities. Six groups were compared resulting from the 6 feeding strategies that were tested across the experiments (Figures VI.11 and VI.12 ). The reason of that choice is that 1) we observed in experiments 'Parity-Unique TMR' and 'Breed and E density' that breed and parities were not factors of differentiation of the relationship between plasma OC to

CTX and milk Ca to P ratios, 2) we wanted to dispose of groups with a significant number of individuals. A positive and significant relationship between both ratios could be observed from the cross-analysis, when all data of the dataset gathered in the experimental part of this thesis were included in the analysis, with a significant effect of the milk Ca to P ratio on plasma OC to CTX (P < 0.0009, Figure VI.11) and a $R^2$ of 0.48. Neither the effect of the cow or the interaction between the cow and milk Ca to P ratio were significant, which would suggest that, theoretically, this ratio could even allow an estimation of the plasma OC to CTX ratio even though measurements were not repeated within individuals. This is possible because milk Ca and P on one sides and plasma OC and CTX on the other side vary in the same direction when considering various parities or breeds (experiments 'Parity-Unique TMR' and 'Breed and E density'). However, given the high variability of the results and the relatively low $R^2$, an isolated interpretation of milk Ca to P ratio remains difficult to consider.
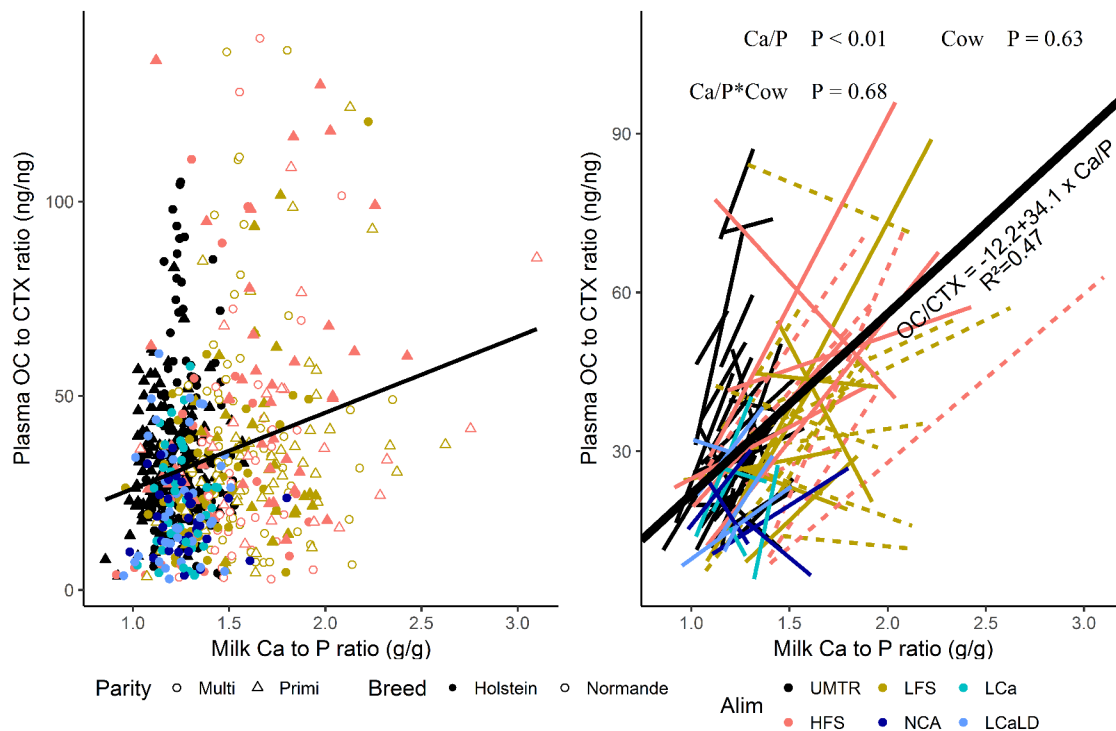


Figure VI.11: Regression between milk Ca to P ratio and plasma OC to CTX ratio within each treatment. Black bold line are mean regression A) between OC/CTX and Ca/P B) between OC/CTX and Ca/P corrected grom individuals differences

When considering regressions within groups of feeding strategies (Figure VI.12), it appears that within cow, correlation was relatively high in the experiment 'Parity-Unique

TMR' ($R^2$=0.69, with a significant effect of the milk Ca to P ratio, P < 0.001), and lower for the treatments HFS of the experiment 'Breed and E density' ($R^2$ = 0.34 with a significant effect of the milk Ca to P ratio, P < 0.008). The effect of the milk Ca to P ratio on plasma OC to CTX ratio was not significant for LFS of the experiment 'Breed and E density' with a $R^2$ of 0.42. It tended to be significant for the treatment LCa of the experiment 'Mineral supplementation' with a $R^2$ of 0.48 and was not significant for the treatments NCa and LCaLD of the same experiment.
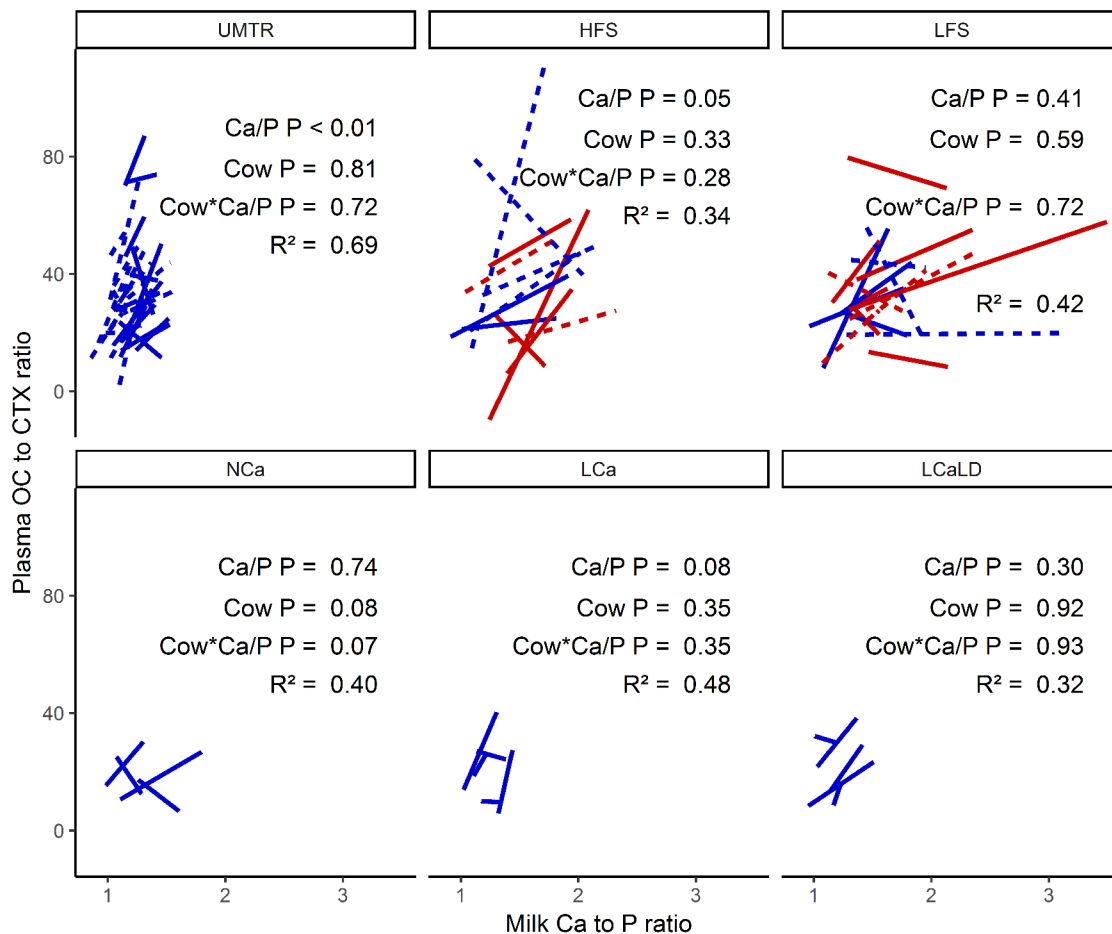


Figure VI.12: Regression between milk Ca to P ratio and plasma OC to CTX ratio within each treatment. Blue = Holstein; Red = Normande; straigth line = Multiparous; dashed line = Primiparous

As explained in the first section of the general discussion, the relationship between milk Ca to P ratio and plasma OC to CTX ratio could be explained by the ideas that plasma Ca and Pi vary inversely due to the relative deficiency of P or Ca in the organism compared to the requirements and that those variation in plasma would induce variation in milk Ca and P contents. From these principles, it would be expected that the positive relationships that we

could observe in our experiment were due to situations where the relative deficiency in Ca was higher than that in P. Anderson et al. (2017) observed a negative relationship between plasma Ca to Pi ratio and CTX in breeder cows which would lead to a negative relationship between milk Ca to P ratio and plasma OC to CTX ratio in cows if the principles given above were respected. With those assumptions, it could be considered that differences in the regression we could observe between groups of feeding treatments could be due to the relative deficiency of P or Ca in the organism compared to the requirements. Indeed, the regression was better in the experiment 'Parity-Unique TMR' than in 'Breed and E density' which may be due to higher relative deficiency of P compared to Ca in the experiment 'Breed and E density'. In this latter experiment, the dietary P content was similar to that of the 'Parity-Unique TMR' but the milk production was also clearly higher. The regression was also better in the treatment HFS compared with LFS of the 'Breed and E density'. Dietary P content was similar for both treatments but milk production was higher on the HFS treatment which could induce a higher deficiency of P. The number of cows in each treatment in the experiment 'Mineral supplementation' may have been too low for possible interpretation.

To validate the hypothesis that differences in the slopes of the regression between milk Ca to P ratio and plasma OC to CTX ratio could be due to the relative deficiency of P or Ca in the organism compared to the requirements, it would be necessary to measure concomitantly both ratios during lactation in cows submitted to various situations of relative deficiency of P compared with Ca. If this hypothesis was verified, it could be imagined that the dynamic recording of milk Ca to P ratio, thanks to MIR, either during lactation of individual cows or during the year in milk tank, could allow the detection of breaking point meaning that the relative ratio between relative deficiencies of Ca and P have changed. It cannot be excluded that the drop in milk Ca to P ratio observed in the PhénoFinLait database during the turnout (Figure VI.3) could not be explained by such phenomenon.

Another question that arises is how milk P variations can reflect those of plasma Pi. The measurement of milk Pi rather than plasma Pi may provide a better relationship between milk Ca to Pi ratio with plasma OC to CTX ratio. From the actual knowledge, milk Pi content is the result of equilibrium of a gradient of concentrations between the vesicles of secretion and cytosol in MEC. Pi enters vesicles of secretion with ATP, that releases Pi after

being hydrolyzed for the formation of lactose and proteins (Neville et al., 1995) and then a part of Pi returns into cytosol. In case of higher bone resorption, plasma Pi concentration should increase, and thus cytosol Pi concentrations also. In such case, the entry of Pi in vesicle of secretion may be more difficult due to higher Pi concentration in cytosol, leading to lower Pi exchange, higher Pi concentration in vesicle of secretion and thus in milk. The distinction between soluble and micellar Ca and P seemed less relevant because of the permanent equilibrium between colloidal Ca and P linked to micelles of casein and soluble Ca and P.

However, as long as mechanisms of secretion of Ca and P into milk are not totally described and understood, it remains difficult to fully determine if milk content of those minerals can help to constitute indicators of bone mobilization in cows and in which conditions. Even though important progress has been made during the last years to understand the actual knowledge of Ca secretion, the lack of knowledge around its entrance in MEC, that may be determinant for the Ca secretion in milk, compromises the understanding of how milk Ca content is determined in case of Ca insufficient supply. Mechanisms of P secretion in milk has been less studied. The fact that Pi is only excreted in milk by vesicle of secretion is now established but the molecular mechanisms determining the equilibrium of Pi between cytosol and vesicle of secretion remain to be described. Another limit to the use of those fractions of milk Ca and P in milk is their method of analysis that are still long and thus expensive as long as MIR equation of prediction will not be tested and maybe established.

# C) The role of the mammary gland in the regulation of calcemia

The part of the discussion aimed to show how the results described in this thesis bring information about the link between regulation of plasma Ca concentration and milk Ca content in lactating cows, and more generally about the role of the mammary gland in the regulation of plasma Ca concentration. More specifically two questions arise in relation with this general context: 1) Can milk Ca content may be a more efficient indicator of short term variations of regulation of plasma Ca concentration than of the shape of bone mobilization at the scale of the lactation? 2) Is the effect of the mammary gland on the regulation of plasma Ca concentration mainly mediated by the modulation of the milk Ca content? To answer those questions, an interpretation of the temporal integration of organ responses for the regulation of the plasma Ca concentration is necessary.

## 1 Temporal integration of organ responses for the regulation of plasma Ca concentration

In non-lactating animals, the responses allowing the regulation of calcemia in response to a stimulus of hypocalcemia are multiple with different temporalities, the first one being a very fast increase in PTH secretion into the blood. The detection of a decrease in calcemia by CaSR in the parathyroid glands is the triggering event, resulting into an almost instantly release of PTH into blood (Brown, 1991). This is possible because PTH is permanently synthetized in the chief cells of the parathyroid glands, which constitute a stock, only secreted when required. The increase in PTH in the blood is then rapid but transient because notably of the short half-life of PTH (Bilzekian et al., 2014). The PTH increase in blood lasts no more than one hour before blood PTH decreases. At the level of the cells able to respond to PTH, PTH bounding to receptor has a decuple effect because PTH receptors are coupled with G protein (Durand and Beaudeux, 2011). This blood PTH increase results into more bone resorption by higher osteoclasts activity, higher kidney Ca reabsorption and higher 25-OH-vitamin D activation into $1,25\text{-}(OH)_2$-vitamin D by the second phosphorylation in the kidney. This activation of 25-OH-vitamin D and its effect on the Ca absorption at the intestinal level arrives in a second time after the PTH secretion

but the consequences of the action of 1,25-(OH)$_2$-vitamin D last longer than that of PTH (Zull et al., 1966), due to action of 1,25-(OH)$_2$-vitamin D on gene expression and cell proliferation. This leads to an increase in the capacity of Ca absorption in intestine and in osteoclasts proliferation in bones, and thus in bone resorption (Bouillon et al., 1995, Horst et al., 1997). In case of long hypocalcemic challenge, an increase proliferation of chief cells allows increasing the capacity of PTH secretion (Brown et al., 1995).

In lactating animals, and more specially in lactating cows, the actions of the mammary gland, and among them the secretion of PTHrP, are also responses allowing the regulation of calcemia during hypocalcemic challenge. The kinetics of PHTrP secretion and the duration of its action are far less known than those of PTH, particularly because the effects of CaSR activation on MEC are not totally described yet. However, if considering that milk PTHrP concentration may reflect the amount of synthetized PTHrP (Ardeshirpour et al., 2006), several days would be necessary for a complete response of the mammary gland to an hypocalcemic challenge, in term of PTHrP synthesis (Uemura et al., 1997). The time during which PTHrP remains active in blood is not known either, given that concentration of circulating PTHrP is very low and remains hard to detect, even during lactation. In the specific case of the hypocalcemic challenge that occurs at the onset of lactation in dairy cows, the first physiological response that allows the regulation of calcemia is considered to be an increased bone mobilization (Braithwaite, 1983a, Horst, 1986, Kovacs and Kronenberg, 1997, Beighle, 1999, Horst et al., 2005, Holtenius and Ekelund, 2005, Ekelund et al., 2006, Taylor et al., 2009), confirming that bone mobilization is a faster response to regulate plasma Ca concentration than increased Ca absorption in the intestine.

The results we obtained in Chapter V (experiment 'Mineral supplementation'), i.e. an increase in intestinal absorption with low dietary Ca content at 3 weeks of lactation and a very weak increase of bone mobilization, suggested that, with long term hypocalcemic challenge, an increased intestinal absorption is favored over an increase bone mobilization. As discussed in Chapter V, this may have been because of the high absorbability of the Ca provided to the cows in our experiment. This would mean that the relative involvement responses allowing the regulation, among increased intestinal Ca absorption or increased bone resorption, would depend both of the time after the start of the hypocalcemic challenge

but also on the amplitude of the challenge. Increased bone mobilization may be the first response involved after the start of an hypocalcemic challenge, but it seems that when the cows can adapt to hypocalcemic challenge by increasing Ca intestinal absorption, this latter adaptive response is favored over increasing bone resorption that would more affect the animal, by decreasing body Ca reserved and bone strength.

## 2 Can milk Ca content be a more efficient indicator of short term variation of regulation of plasma Ca concentration than of the shape of bone mobilization at the scale of the lactation?

The hypothesis of this thesis was that an increased variation of milk Ca content during lactation could be related to an increased amplitude of dynamics of bone mobilization. However, our results suggest that this is not systematic. Indeed, we observed in the experiment 'Parity-Unique TMR' that an increased amplitude of variation of milk Ca content during lactation in primiparous cows was accompanied by an increase variation of the plasma CTX concentration. However, in the experiment 'Breed and E density', a huge increase in the amplitude of variation of the plasma CTX concentration during lactation was not accompanied by any increase in the range of variation of milk Ca content. Considering the milk Ca to P ratio, instead of milk Ca content, allows a prediction of the plasma OC to CTX ratio, i.e. of the equilibrium between bone accretion and resorption during the lactation. However, the prediction is not precise enough to allow a prediction of the amplitude of bone mobilization at the scale of the lactation.

A reason of the fact that the amplitude of variation of milk Ca content during lactation does not reflect that of bone mobilization may be that the decrease in milk Ca content, as an adaptive mechanism for regulation of calcemia in case of hypocalcemia challenge, is only transient, as could be bone mobilization when intestinal absorption has the ability to increase. In this thesis, we observed several situations with transient variation of milk Ca content that could have been explained by variation of Ca requirement or supply. For instance, data of the PhénoFinLait database (Chapter II) illustrated that a transient decrease in milk Ca content was concomitant with an increase in milk production at the turnout to grazing, meaning more Ca requirement for lactation. In the experiment 'Mineral supplementation' (Chapter V), at the end of the period of differentiation of

treatment at 10 weeks of lactation, milk Ca content of cows of the NCa treatment decreased when their dietary Ca content decreased, whereas the milk Ca content of cows of the LCa and LCaLD treatments increased when their dietary Ca content increased. In the experiment 'Breed and E density' (Chapter IV), important variation of milk Ca content could also be observed at the beginning or ending of grazing periods (Figure IV.4).

To conclude whether a decrease in milk Ca content could be a response of an hypocalcemia challenge, it would be necessary to measure daily the succession of adaptive mechanism regulating calcemia, with cows submitted to hypocalcemia challenges with several levels of severity. The measurement of the succession of adaptive mechanism among bone mobilization, increased intestinal absorption or decreased Ca secretion in milk would allow determining the temporality of the response of the mammary gland. Considering several hypocalcemia challenges with various levels of severity would allow determining the priority between responses in case of moderate challenges. Hypocalcemia challenge could be low dietary Ca content as tested in the experiment 'Breed and E density' (Chapter IV).

If milk Ca content was only transiently affect by the regulation of calcemia, a daily following of milk Ca content, via MIR measurement for instance, could allow detecting rupture in the Ca homeostasis of cows due to diet change for instance, which could constitutes a tool to check if the mineral supplementation is adapted. It would also be worth to determine if following variation of milk Ca content between milkings at the onset of the lactation could allow quantifying the difficulties of the cows to regulate their calcemia and then their susceptibility to milk fever. This would be very useful to apply targeted preventive action after the first or second milking or for epidemiological monitoring of subclinical hypocalcemia that can only be detected with blood sampling currently, allowing then a better understanding of milk fever and its consequences (Caixeta et al., 2017, Neves et al., 2017, Rodríguez et al., 2017, Wilhelm et al., 2017).

# 3 Is the effect of the mammary gland on the regulation of plasma Ca concentration mainly mediated by the modulation of the milk Ca content?

The implication of the mammary gland in the regulation of calcemia is well demonstrated (VanHouten et al., 2004, Mamillapalli et al., 2013, Kovacs, 2016), as non-expression of CaSR in mice induced dysfunctions in Ca regulation during lactation (Mamillapalli et al., 2013). A question arising when reading the literature is to determine whether this effect is mediated by a specific decrease in the Ca secretion by the MEC or by a less specific decrease in milk production. VanHouten et al. (2004, 2007) suggested that it would be mediated by a decrease in milk Ca content, but in their study, only the milk Ca to protein ratio decreased in situation of decreased dietary Ca content, which was only due to an increase in the milk protein content given that the milk Ca content, expressed by kg of milk did not vary. Kovacs et al. (2016) suggested, from these data, that the decrease in dietary Ca content could also have reduced water transport into milk and thus milk production, which could have explained the increase in milk protein content. This conclusion could have been contradicted by studies showing no effect of CaSR depletion on milk production (Ardeshirpour et al., 2006, Mamillapalli et al., 2013). However, a major limit in those latter studies is that they were realized with mice, with indirect measurement of milk production thanks to pups' growth. In such conditions, it is likely that the measured milk production is more representative of milk solids production than actual milk volume including water.

The short-term adaption of milk Ca content we could observed in the analysis of the PhénoFinLait database at turnout and in the experiment 'Mineral supplementation' at the end of the differentiation of the diets between treatments suggests that the decrease in milk Ca content could be an adaptive mechanism to hypocalcemia challenge but it may be limited. Indeed, the results issued from the PhénoFinLait database illustrated that Ca milk content decreased during the turnout to pasture, in relation with an increased milk production, without avoiding the increase in the daily amount of Ca secreted in milk. The results obtained from the experiment 'Mineral supplementation' (Chapter V) suggested that a lower milk production could occur with diet with low dietary content. The fact that the effect of the treatments in this experiment on milk production remained after the differentiation of

the treatments suggests that potential of milk production was altered maybe because low Ca in cells that limit cell proliferation or maybe because CasR can be involved in apoptosis.

# Conclusion

This thesis showed that the variations of milk Ca content during lactation cannot be a good indirect indicator of the amplitude of bone mobilization throughout lactation. Thus, following dynamics of milk Ca to better understand effect of bone mobilization on cow's health and performances seems difficult. It is possible that pressure of measurement, with one sampling time in a month in most cases, was too low to allow detecting variations in milk Ca content related to calcemia regulation in dairy cows. A higher sampling pressure, with stronger challenge of calcemia regulation may have been necessary to study how animals regulate Ca secretion in milk to maintain calcemia. However, our results suggested that, with a monthly sampling frequency, and in certain conditions, the dynamics of milk Ca to P ratio could give a general idea of the evolution of the equilibrium between bone accretion and resorption throughout lactation.

This thesis resulted in the identification of factors that could affect bone remodeling or the amplitude of bone mobilization during lactation in dairy cows. The diet fed to the cows and notably the diet energy density strongly affected the amplitude of bone mobilization during lactation, in relationship with the induced effects induced milk production. On the contrary, no effect of dietary Ca content was observed. Dairy breeds also showed differences in the amplitude of bone mobilization during lactation, with higher bone mobilization in Normande than in Holstein cows. Bone remodeling was also higher in primiparous than in multiparous cows and primiparous cows may also have a higher amplitude of bone mobilization during lactation than multiparous cows. This latter result

remain, nevertheless, to be confirmed and seems to be highly dependent on environmental conditions.

This thesis also showed that bone mobilization is not the only response to hypocalcemic challenge during lactation, with the observation that an increase in Ca absorption at 3 weeks of lactation may be sufficient to maintain calcemia if the digestive availability of the dietary sources of Ca is high. This would mean that bone mobilization is not a preferential response to a long term Ca challenge for the organism, at least if digestive absorption can be enhanced. The results obtained during this thesis also raised the question of a possible effect of a low dietary Ca supply in early lactation on milk production all over lactation. This surprising result needs to be confirmed on a larger scale. If verified, this result would be a demonstration of the necessity to supplement dairy cows to cover their Ca requirements at the beginning of their lactation. Consequences of low Ca at the beginning of lactation on cows' health and reproductive performance would also have to be investigated.

Finally, it would also be interesting to determine if daily milk Ca content variation can reflect important hypocalcemic challenge in dairy cows. This possibility would be very useful for the study of subclinical hypocalcemia in early lactation, to better understand how it can be avoided and its consequences on cows' health and performances.

[Abdi et al., 2013] Abdi, H., Williams, L. J., and Valentin, D. (2013). Multiple factor analysis: principal component analysis for multitable and multiblock data sets. *Wiley Interdisciplinary Reviews: Computational Statistics*, 5(2):149–179.

[AFRC, 1991] AFRC (1991). A reappraisal of the calcium and phosphorus requirements of sheep and cattle. *Nutrion Abstract and Reviews*, 61:573–612.

[Alais, 1984] Alais, C. (1984). *Science du lait: principes des techniques laitières*. Edition SEPAIC.

[Allen, 2003] Allen, M. J. (2003). Biochemical markers of bone metabolism in animals: uses and limitations. *Veterinary Clinical Pathology*, 32(3):101–113.

[Alvarez-Fuentes et al., 2016] Alvarez-Fuentes, G., Appuhamy, J. A. D. R. N., and Kebreab, E. (2016). Prediction of phosphorus output in manure and milk by lactating dairy cows. *Journal of Dairy Science*, 99(1):771–782.

[Amanlou et al., 2016] Amanlou, H., Akbari, A. P., Farsuni, N. E., and Silva-del Río, N. (2016). Effects of subcutaneous calcium administration at calving on mineral status, health, and production of Holstein cows. *Journal of Dairy Science*, 99(11):9199–9210.

[Anderson et al., 2017] Anderson, S. T., Kidd, L. J., Benvenutti, M. A., Fletcher, M. T., and Dixon, R. M. (2017). New candidate markers of phosphorus status in beef breeder cows. *Animal Production Science*, 57(11):2291–2303.

[ARC, 1980] ARC (1980). *The Nutrient requirements of ruminant livestock : technical review by an Agricultural Research Council working party*. Slough : Published on behalf of the Agricultural Research Council by Commonwealth Agricultural Bureaux, [new ed.] edition.

[Ardeshirpour et al., 2006] Ardeshirpour, L., Dann, P., Pollak, M., Wysolmerski, J., and VanHouten, J. (2006). The calcium-sensing receptor regulates PTHrP production and calcium transport in the lactating mammary gland. *Bone*, 38(6):787–793.

[Arnold and Becker, 1936] Arnold, P. T. D. and Becker, R. B. (1936). Influence of preceding dry period and of mineral supplement on lactation. *Journal of Dairy Science*, 19(4):257–266.

[Barlet et al., 1992] Barlet, J. P., Champredon, C., Coxam, V., Davicco, M. J., and Tressol, J. C. (1992). Parathyroid hormone-related peptide might stimulate calcium secretion into the milk of goats. *The Journal of Endocrinology*, 132(3):353–359.

[Becker et al., 1934] Becker, R. B., Neal, W. M., and Shealy, A. L. (1934). Effect of calcium-deficient roughages upon milk yield and bone strength in cattle. *Journal of Dairy Science*, 17(1):1–10.

[Bedere et al., 2017] Bedere, N., Disenhaus, C., Ducrocq, V., Leurent-Colette, S., and Delaby, L. (2017). Ability of dairy cows to ensure pregnancy according to breed and genetic merit for production traits under contrasted pasture-based systems. *Journal of Dairy Science*, 100(4):2812–2827.

[Beighle, 1999] Beighle, D. E. (1999). The effect of gestation and lactation on bone calcium, phosphorus and magnesium in dairy cows. *Journal of the South African Veterinary Association*, 70(4):142–146.

[Beighle et al., 1993] Beighle, D. E., Boyazoglu, P. A., and Hemken, R. W. (1993). Use of bovine rib bone in serial sampling for mineral analysis. *Journal of Dairy Science*, 76(4):1047–1052.

[Benzie et al., 1955] Benzie, D., Boyne, A. W., Dalgarno, A. C., Duckworth, J., Hill, R., and Walker, D. M. (1955). Studies of the skeleton of the sheep I. The effect of different levels of dietary calcium during pregnancy and lactation on individual bones. *The Journal of Agricultural Science*, 46(04):425–440.

[Bijl et al., 2013] Bijl, E., van Valenberg, H. J. F., Huppertz, T., and van Hooijdonk, A. C. M. (2013). Protein, casein, and micellar salts in milk: Current content and historical perspectives. *Journal of Dairy Science*, 96(9):5455–5464.

[Bilzekian et al., 2014] Bilzekian, J. P., Marcus, R., Levine, M. A., Marcocci, C., Silverberg, S. J., and Potts, J. T., editors (2014). *The Parathyroids: Basic and Clinical Concepts*. Academic Press, Amsterdam ; Boston, 3 edition.

[Blanco and Blanco, 2017a] Blanco, A. and Blanco, G. (2017a). Chapter 27 - Vitamins. In *Medical Biochemistry*, pages 645–687. Academic Press.

[Blanco and Blanco, 2017b] Blanco, A. and Blanco, G. (2017b). Chapter 29 - Essential Minerals. In *Medical Biochemistry*, pages 715–743. Academic Press.

[Bonjour et al., 2014] Bonjour, J.-P., Kohrt, W., Levasseur, R., Warren, M., Whiting, S., and Kraenzlin, M. (2014). Biochemical markers for assessment of calcium economy and bone metabolism: application in clinical trials from pharmaceutical agents to nutritional products. *Nutrition Research Reviews*, 27(2):252–267.

[Bortolussi et al., 1999] Bortolussi, G., Ternouth, J. H., and McMeniman, N. P. (1999). Phosphorus repletion of cattle previously exposed to dietary nitrogen and phosphorus deficiencies. *Australian Journal of Agricultural Research*, 50(1):93–100.

[Boudon et al., 2016] Boudon, A., Johan, M., Narcy, A., Boutinaud, M., Lamberton, P., and Hurtaud, C. (2016). Dietary cation-anion difference and day length have an effect on milk calcium content and bone accretion of dairy cows. *Journal of Dairy Science*, 99(2):1527–1538.

[Bouillon et al., 1995] Bouillon, R., Okamura, W. H., and Norman, A. W. (1995). Structure-function relationships in the vitamin D endocrine system. *Endocrine Reviews*, 16(2):200–257.

[Braithwaite, 1983a] Braithwaite, G. D. (1983a). Calcium and phosphorus requirements of the ewe during pregnancy and lactation. 1. Calcium. *The British Journal of Nutrition*, 50(3):711–722.

[Braithwaite, 1983b] Braithwaite, G. D. (1983b). Calcium and phosphorus requirements of the ewe during pregnancy and lactation. 2. Phosphorus. *The British Journal of Nutrition*, 50(3):723–736.

[Brûlé et al., 1974] Brûlé, G., Maubois, J. L., and Fauquant, J. (1974). Etude de la teneur en éléments minéraux des produits obtenus lors de l'ultrafiltration du lait sur membrane. *Le Lait*, 54(539-540):600–615.

[Bronner, 1987] Bronner, F. (1987). Intestinal calcium absorption: mechanisms and applications. *The Journal of Nutrition*, 117(8):1347–1352.

[Bronner, 2003] Bronner, F. (2003). Mechanisms of intestinal calcium absorption. *Journal of Cellular Biochemistry*, 88(2):387–393.

[Brown, 1991] Brown, E. M. (1991). Extracellular Ca2+ sensing, regulation of parathyroid cell function, and role of Ca2+ and other ions as extracellular (first) messengers. *Physiological reviews*, 71(2):371–411.

[Brown et al., 1993] Brown, E. M., Gamba, G., Riccardi, D., Lombardi, M., Butters, R., Kifor, O., Sun, A., Hediger, M. A., Lytton, J., and Hebert, S. C. (1993). Cloning and characterization of an extracellular Ca2+-sensing receptor from bovine parathyroid. *Nature*, 366(6455):575–580.

[Brown et al., 1995] Brown, E. M., Vassilev, P. M., and Hebert, S. C. (1995). Calcium ions as extracellular messengers. *Cell*, 83(5):679–682.

[Buckwalter et al., 1996] Buckwalter, J. A., Glimcher, M. J., Cooper, R. R., and Recker, R. (1996). Bone biology. I: Structure, blood supply, cells, matrix, and mineralization. *Instructional Course Lectures*, 45:371–386.

[Buitenhuis et al., 2015] Buitenhuis, B., Poulsen, N. A., Larsen, L. B., and Sehested, J. (2015). Estimation of genetic parameters and detection of quantitative trait loci for minerals in Danish Holstein and Danish Jersey milk. *BMC Genetics*, 16:52–59.

[Bullough, 2010] Bullough, P. (2010). *Orthopaedic Pathology*. Elsevier, Maryland Heights, Missouri, USA, mosby edition.

[Bushinsky, 2010] Bushinsky, D. A. (2010). Contribution of intestine, bone, kidney, and dialysis to extracellular fluid calcium content. *Clinical journal of the American Society of Nephrology: CJASN*, 5 Suppl 1:S12–22.

[Caixeta et al., 2017] Caixeta, L. S., Ospina, P. A., Capel, M. B., and Nydam, D. V. (2017). Association between subclinical hypocalcemia in the first 3 days of lactation and reproductive performance of dairy cows. *Theriogenology*, 94:1–7.

[Cardinali et al., 2003] Cardinali, D. P., Ladizesky, M. G., Boggio, V., Cutrera, R. A., and Mautalen, C. (2003). Melatonin effects on bone: experimental facts and clinical perspectives. *Journal of Pineal Research*, 34(2):81–87.

[Cerbulis and Farrell, 1976] Cerbulis, J. and Farrell, H. (1976). Composition of milks of dairy-cattle .2. Ash, calcium, magnesium, and phosphorus. *Journal of Dairy Science*, 59(4):589–593.

[Charbonneau et al., 2006] Charbonneau, E., Pellerin, D., and Oetzel, G. R. (2006). Impact of lowering dietary cation-anion difference in ,onlactating dairy cows: A meta-analysis. *Journal of Dairy Science*, 89(2):537–548.

[Chassaing et al., 2016] Chassaing, C., Sibra, C., Verbič, J., Harstad, O. M., Golecký, J., Martin, B., Ferlay, A., Constant, I., Delavaud, C., Hurtaud, C., Pongrac, V. Z., and Agabriel, C. (2016). Mineral, vitamin A and fat composition of bulk milk related to European production conditions throughout the year. *Dairy Science & Technology*, 96(5):715–733.

[Chen and Graedel, 2016] Chen, M. and Graedel, T. E. (2016). A half-century of global phosphorus flows, stocks, production, consumption, recycling, and environmental impacts. *Global Environmental Change*, 36:139–152.

[Christakos et al., 2014] Christakos, S., Lieben, L., Masuyama, R., and Carmeliet, G. (2014). Vitamin D endocrine system and the intestine. *BoneKEy Reports*, 3:496.

[Cohen, 1988] Cohen, J. (1988). *Statistical Power Analysis for the Behavioral Sciences*. Routledge, 2nd edition.

[Cornish et al., 1997] Cornish, J., Callon, K. E., Nicholson, G. C., and Reid, I. R. (1997). Parathyroid hormone-related protein-(107-139) inhibits bone resorption in vivo. *Endocrinology*, 138(3):1299–1304.

[Cunningham and Klein, 2007] Cunningham, J. G. and Klein, B. G. (2007). *Textbook of Veterinary Physiology, 4e*. Saunders, St. Louis, MO, 4th edition.

[Cutullic et al., 2009] Cutullic, E., Delaby, L., Causeur, D., Michel, G., and Disenhaus, C. (2009). Hierarchy of factors affecting behavioural signs used for oestrus detection of Holstein and Normande dairy cows in a seasonal calving system. *Animal Reproduction Science*, 113(1):22–37.

[Davicco et al., 1993] Davicco, M. J., Rouffet, J., Durand, D., Lefaivre, J., and Barlet, J.-P. (1993). Paratyhroid hormon-related peptide may increase mammary blood flow. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research*, 8(12):1519–1524.

[DeGaris and Lean, 2008] DeGaris, P. J. and Lean, I. J. (2008). Milk fever in dairy cows: A review of pathophysiology and control principles. *The Veterinary Journal*, 176(1):58–69.

[Delaby et al., 2009] Delaby, L., Faverdin, P., Michel, G., Disenhaus, C., and Peyraud, J. L. (2009). Effect of different feeding strategies on lactation performance of Holstein and Normande dairy cows. *animal*, 3(6):891–905.

[Dezetter et al., 2015] Dezetter, C., Leclerc, H., Mattalia, S., Barbat, A., Boichard, D., and Ducrocq, V. (2015). Inbreeding and crossbreeding parameters for production and fertility traits in Holstein, Montbéliarde, and Normande cows. *Journal of Dairy Science*, 98(7):4904–4913.

[Dixon et al., 2017] Dixon, R. M., Kidd, L. J., Coates, D. B., Anderson, S. T., Benvenutti, M. A., Fletcher, M. T., and McNeill, D. M. (2017). Utilising mobilisation of body reserves

to improve the management of phosphorus nutrition of breeder cows. *Animal Production Science*, 57(11):2280–2290.

[Donabedian et al., 2005] Donabedian, M., Delguste, C., Perona, G., Lebecque, P., Duboeuf, F., Lepaga, O., and Martin-Rosset, W. (2005). Third metacarpal bone mineral density assessment in the standing horse by dual X-ray absorptiometry: suitability, precision and accuracy. *Veterinary and Comparative Orthopaedics and Traumatology*, 18(1):26–30.

[Dou et al., 2002] Dou, Z., Knowlton, K. F., Kohn, R. A., Wu, Z., Satter, L. D., Zhang, G., Toth, J. D., and Ferguson, J. D. (2002). Phosphorus characteristics of dairy feces affected by diets. *Journal of Environmental Quality*, 31(6):2058–2065.

[Ducy, 2011] Ducy, P. (2011). 5-HT and bone biology. *Current Opinion in Pharmacology*, 11(1):34–38.

[Dunbar et al., 1999] Dunbar, M. E., Dann, P. R., Robinson, G. W., Hennighausen, L., Zhang, J. P., and Wysolmerski, J. J. (1999). Parathyroid hormone-related protein signaling is necessary for sexual dimorphism during embryonic mammary development. *Development (Cambridge, England)*, 126(16):3485–3493.

[Durand and Beaudeux, 2011] Durand, G. and Beaudeux, J.-L. (2011). *Biochimie médicale: Marqueurs actuels et perspectives*. Lavoisier, Paris, France.

[Ekelund et al., 2006] Ekelund, A., Spörndly, R., and Holtenius, K. (2006). Influence of low phosphorus intake during early lactation on apparent digestibility of phosphorus and bone metabolism in dairy cows. *Livestock Science*, 99(2–3):227–236.

[Elizondo Salazar et al., 2013] Elizondo Salazar, J. A., Ferguson, J. D., Beegle, D. B., Remsburg, D. W., and Wu, Z. (2013). Body phosphorus mobilization and deposition during lactation in dairy cows. *Journal of Animal Physiology and Animal Nutrition*, 97(3):502–514.

[Ender et al., 1971] Ender, F., Dishington, I. W., and Helgebostad, A. (1971). Calcium balance studies in dairy cows under experimental induction and prevention of

hypocalcaemic paresis puerperalis. *Zeitschrift für Tierphysiologie Tierernährung und Futtermittelkunde*, 28(1-5):233–256.

[Escofier and Pagès, 1994] Escofier, B. and Pagès, J. (1994). Multiple factor analysis (AFMULT package). *Computational Statistics & Data Analysis*, 18(1):121–140.

[Fantuz et al., 2012] Fantuz, F., Ferraro, S., Todini, L., Piloni, R., Mariani, P., and Salimei, E. (2012). Donkey milk concentration of calcium, phosphorus, potassium, sodium and magnesium. *International Dairy Journal*, 24(2):143–145.

[Farrell et al., 2006] Farrell, H. M., Malin, E. L., Brown, E. M., and Qi, P. X. (2006). Casein micelle structure: What can be learned from milk synthesis and structural biology? *Current Opinion in Colloid & Interface Science*, 11(2):135–147.

[Feher et al., 1992] Feher, J. J., Fullmer, C. S., and Wasserman, R. H. (1992). Role of facilitated diffusion of calcium by calbindin in intestinal calcium absorption. *American Journal of Physiology - Cell Physiology*, 262(2):C517–C526.

[Ferrand-Calmels et al., 2014] Ferrand-Calmels, M., Palhière, I., Brochard, M., Leray, O., Astruc, J. M., Aurel, M. R., Barbey, S., Bouvier, F., Brunschwig, P., Caillat, H., Douguet, M., Faucon-Lahalle, F., Gelé, M., Thomas, G., Trommenschlager, J. M., and Larroque, H. (2014). Prediction of fatty acid profiles in cow, ewe, and goat milk by mid-infrared spectrometry. *Journal of Dairy Science*, 97(1):17–35.

[Ferris et al., 2010a] Ferris, C. P., McCoy, M. A., Patterson, D. C., and Kilpatrick, D. J. (2010a). Effect of offering dairy cows diets differing in phosphorus concentration over four successive lactations: 2. Health, fertility, bone phosphorus reserves and nutrient utilisation. *Animal*, 4(4):560–571.

[Ferris et al., 2010b] Ferris, C. P., Patterson, D. C., McCoy, M. A., and Kilpatrick, D. J. (2010b). Effect of offering dairy cows diets differing in phosphorus concentration over four successive lactations: 1. Food intake, milk production, tissue changes and blood metabolites. *Animal*, 4(4):545–559.

[Flynn and Cashman, 1997] Flynn, A. and Cashman, K. (1997). Nutritional aspects of minerals in bovine and human milks. In Fox, P. F., editor, *Advanced Dairy Chemistry Volume 3*, pages 257–302. Springer US.

[Forar et al., 1982] Forar, F. L., Kincaid, R. L., Preston, R. L., and Hillers, J. K. (1982). Variation of inorganic phosphorus in blood plasma and milk of lactating cows. *Journal of Dairy Science*, 65(5):760–763.

[Gaignon et al., 2018a] Gaignon, P., Faverdin, P., Laza-Knoerr, A., Le Grand, K., Hurtaud, C., and Boudon, A. (2018a). Effects of parity and individual variability on bone accretion and resorption and milk calcium and phosphorus content during lactation in dairy cows. *Animal*, Submitted.

[Gaignon et al., 2018b] Gaignon, P., Gelé, M., Hurtaud, C., and Boudon, A. (2018b). Characterization of the nongenetic causes of variation in the calcium content of bovine milk on French farms. *Journal of Dairy Science*, 101(5):4554–4569.

[Gallego et al., 2006] Gallego, L., Landete-Castillejos, T., Garcia, A., and Sánchez, P. J. (2006). Seasonal and lactational changes in mineral composition of milk from Iberian red deer (Cervus elaphus hispanicus). *Journal of Dairy Science*, 89(2):589–595.

[Garner et al., 1990] Garner, S. C., Boass, A., and Toverud, S. U. (1990). Parathyroid hormone is not required for normal milk composition or secretion or lactation-associated bone loss in normocalcemic rats. *Journal of Bone and Mineral Research: The Official Journal of the American Society for Bone and Mineral Research*, 5(1):69–75.

[Gaucheron, 2005] Gaucheron, F. (2005). The minerals of milk. *Reproduction, Nutrition, Development*, 45(4):473–483.

[Gelé et al., 2014] Gelé, M., Minery, S., Astruc, J.-M., Brunschwig, P., Ferrand-Calmels, M., Lagriffoul, G., Larroque, H., Legarto, J., Leray, O., Martin, P., Miranda, G., Palhiere, I., Trossat, P., and Brochard, M. (2014). Phénotypage et génotypage à grande échelle de la composition fine des laits dans les filières bovine et caprine. *INRA Productions Animales*, 27(4):255–268.

[Glantz et al., 2009] Glantz, M., Lindmark Månsson, H., Stålhammar, H., Bårström, L. O., Fröjelin, M., Knutsson, A., Teluk, C., and Paulsson, M. (2009). Effects of animal selection on milk composition and processability. *Journal of Dairy Science*, 92(9):4589–4603.

[Goff, 2000] Goff, J. P. (2000). Pathophysiology of calcium and phosphorus disorders. *Veterinary Clinics of North America: Food Animal Practice*, 16(2):319–337.

[Goff, 2008] Goff, J. P. (2008). The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. *The Veterinary Journal*, 176(1):50–57.

[Goff et al., 2014] Goff, J. P., Liesegang, A., and Horst, R. L. (2014). Diet-induced pseudohypoparathyroidism: A hypocalcemia and milk fever risk factor. *Journal of Dairy Science*, 97(3):1520–1528.

[Gunther, 2005] Gunther, F. (2005). A solution to the heap problem: The doubly balanced agriculture: integration with population | Global Phosphorus Network.

[Hannon and Eastell, 2000] Hannon, R. and Eastell, R. (2000). Preanalytical variability of biochemical markers of bone turnover. *Osteoporosis international*, 11 Suppl 6:S30–44.

[Hansard et al., 1957] Hansard, S. L., Crowder, H. M., and Lyke, W. A. (1957). The biological availability of calcium in feeds for cattle. *Journal of Animal Science*, 16(2):437–443.

[Hardcastle and Dittmer, 2015] Hardcastle, M. R. and Dittmer, K. E. (2015). Fibroblast Growth Factor 23: A new dimension to diseases of calcium-phosphorus metabolism. *Veterinary Pathology*.

[Hennings et al., 1980] Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S. H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell*, 19(1):245–254.

[Henry, 2011] Henry, H. L. (2011). Regulation of vitamin D metabolism. *Best Practice & Research. Clinical Endocrinology & Metabolism*, 25(4):531–541.

[Hermansen et al., 2005] Hermansen, J. E., Badsberg, J. H., Kristensen, T., and Gundersen, V. (2005). Major and trace elements in organically or conventionally produced milk. *Journal of Dairy Research*, 72(3):362–368.

[Hernández-Castellano et al., 2017] Hernández-Castellano, L. E., Hernandez, L. L., Sauerwein, H., and Bruckmaier, R. M. (2017). Endocrine and metabolic changes in transition dairy cows are affected by prepartum infusions of a serotonin precursor. *Journal of Dairy Science*, 100(6):5050–5057.

[Hibbs and Conrad, 1983] Hibbs, J. W. and Conrad, H. R. (1983). *The Relation of Calcium and Phosphorus Intake and Digestion and the Effects of Vitamin D Feeding on the Utilization of Calcium and Phosphorus by Lactating Dairy Cows*. Ohio Agricultural Research and Development Center, Ohio State University.

[Hidiroglou and Proulx, 1982] Hidiroglou, M. and Proulx, J. G. (1982). Factors affecting the calcium, magnesium and phosphorus content of beef cow milk. *Canadian Journal of Comparative Medicine*, 46(2):212–214.

[Hill et al., 2008] Hill, S. R., Knowlton, K. F., Kebreab, E., France, J., and Hanigan, M. D. (2008). A model of phosphorus digestion and metabolism in the lactating dairy cow. *Journal of Dairy Science*, 91(5):2021–2032.

[Hiremath and Wysolmerski, 2014] Hiremath, M. and Wysolmerski, J. (2014). Role of PTHrP in mammary gland development and breast cancer. *Clinical Reviews in Bone and Mineral Metabolism*, 12(3):178–189.

[Höller et al., 1988] Höller, H., Breves, G., Gerdes, H., and Kocabatmaz, M. (1988). Flux of calcium across the sheep rumen wall in vivo and in vitro. *Quarterly Journal of Experimental Physiology*, 73(4):609–618.

[Hoden et al., 1991] Hoden, A., Peyraud, J. L., Muller, A., Delaby, L., Faverdin, P., Peccatte, J. R., and Fargetton, M. (1991). Simplified rotational grazing management of dairy cows: effects of rates of stocking and concentrate. *The Journal of Agricultural Science*, 116(3):417–428.

[Holt, 2004] 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. *European Biophysics Journal*, 33(5):421–434.

[Holt and Muir, 1979] Holt, C. and Muir, D. D. (1979). Inorganic constituents of milk: I. Correlation of soluble calcium with citrate in bovine milk. *Journal of Dairy Research*, 46(3):433–439.

[Holtenius and Ekelund, 2005] Holtenius, K. and Ekelund, A. (2005). Biochemical markers of bone turnover in the dairy cow during lactation and the dry period. *Research in Veterinary Science*, 78(1):17–19.

[Horseman and Hernandez, 2014] Horseman, N. D. and Hernandez, L. L. (2014). New concepts of breast cell communication to bone. *Trends in Endocrinology & Metabolism*, 25(1):34–41.

[Horst, 1986] Horst, R. L. (1986). Regulation of calcium and phosphorus homeostasis in the dairy cow. *Journal of Dairy Science*, 69(2):604–616.

[Horst et al., 1990] Horst, R. L., Goff, J. P., and Reinhardt, T. A. (1990). Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor. *Endocrinology*, 126(2):1053–1057.

[Horst et al., 1997] Horst, R. L., Goff, J. P., and Reinhardt, T. A. (1997). Calcium and vitamin D metabolism during lactation. *Journal of Mammary Gland Biology and Neoplasia*, 2(3):253–263.

[Horst et al., 2005] Horst, R. L., Goff, J. P., and Reinhardt, T. A. (2005). Adapting to the transition between gestation and lactation: differences between rat, human and dairy cow. *Journal of Mammary Gland Biology and Neoplasia*, 10(2):141–156.

[Hurtaud et al., 2014] Hurtaud, C., Dutreuil, M., Coppa, M., Agabriel, C., and Martin, B. (2014). Characterization of milk from feeding systems based on herbage or corn silage with or without flaxseed and authentication through fatty acid profile. *Dairy Science & Technology*, 94(2):103–123.

[Hurtaud et al., 2000] Hurtaud, C., Lemosquet, S., and Rulquin, H. (2000). Effect of graded duodenal infusions of glucose on yield and composition of milk from dairy cows. 2. Diets based on grass silage. *Journal of Dairy Science*, 83(12):2952–2962.

[Husson and Josse, 2013] Husson, F. and Josse, J. (2013). Handling missing values in multiple factor analysis. *Food Quality and Preference*, 30(2):77–85.

[Hymøller and Jensen, 2010] Hymøller, L. and Jensen, S. K. (2010). Vitamin D3 synthesis in the entire skin surface of dairy cows despite hair coverage. *Journal of Dairy Science*, 93(5):2025–2029.

[INRA, 2010] INRA (2010). *Alimentation des bovins, ovins et caprins: besoins des animaux, valeurs des aliments : tables Inra 2010*. Editions Quae, Paris, France.

[INRA, 2018] INRA (2018). *INRA feeding system for ruminants*. Wageningen Academic Publishers, Wageningen, the Netherlands.

[Institute, 2013] Institute, S. (2013). SAS Institute.

[Iwama et al., 2004] Iwama, Y., Kamiya, M., Tanaka, M., and Shioya, S. (2004). The change of dry matter intake, milk yield and bone turnover in primiparous cows compared to multiparous cows during early lactation. *Animal Science Journal*, 75(3):213–218.

[Jenness, 1979] Jenness, R. (1979). Comparative aspects of milk proteins. *Journal of Dairy Research*, 46(2):197–210.

[Jong, 1926] Jong, W. F. (1926). La substance minérale dans les os. *Recueil des Travaux Chimiques des Pays-Bas*, 45(6):445–448.

[Jubiz et al., 1972] Jubiz, W., Canterbury, J. M., Reiss, E., and Tyler, F. H. (1972). Circadian rhythm in serum parathyroid hormone concentration in human subjects: correlation with serum calcium, phosphate, albumin, and growth hormone levels. *The Journal of Clinical Investigation*, 51(8):2040–2046.

[Kamiya et al., 2010] Kamiya, Y., Kamiya, M., and Tanaka, M. (2010). The effect of high ambient temperature on Ca, P and Mg balance and bone turnover in high-yielding dairy cows. *Animal Science Journal*, 81(4):482–486.

[Kaufmann and Hagemeister, 1987] Kaufmann, W. and Hagemeister, H. (1987). Chapter 4 - Composition of Milk. In *Dairy-Cattle Production*, pages 107–171. Elsevier, Amsterdam, h.o gravert edition.

[Kebreab et al., 2008] Kebreab, E., Odongo, N. E., McBride, B. W., Hanigan, M. D., and France, J. (2008). Phosphorus utilization and environmental and economic implications of reducing phosphorus pollution from Ontario dairy cows. *Journal of Dairy Science*, 91(1):241–246.

[Keene et al., 2004] Keene, B. E., Knowlton, K. F., McGilliard, M. L., Lawrence, L. A., Nickols-Richardson, S. M., Wilson, J. H., Rutledge, A. M., McDowell, L. R., and Van Amburgh, M. E. (2004). Measures of bone mineral content in mature dairy cows. *Journal of Dairy Science*, 87(11):3816–3825.

[Khorasani et al., 1997] Khorasani, G. R., Janzen, R. A., McGill, W. B., and Kennelly, J. J. (1997). Site and extent of mineral absorption in lactating cows fed whole-crop cereal grain silage of alfalfa silage. *Journal of Animal Science*, 75(1):239–248.

[Klop et al., 2013] Klop, G., Ellis, J. L., Bannink, A., Kebreab, E., France, J., and Dijkstra, J. (2013). Meta-analysis of factors that affect the utilization efficiency of phosphorus in lactating dairy cows. *Journal of Dairy Science*, 96(6):3936–3949.

[Klop et al., 2014] Klop, G., Ellis, J. L., Blok, M. C., Brandsma, G. G., Bannink, A., and Dijkstra, J. (2014). Variation in phosphorus content of milk from dairy cattle as affected by differences in milk composition. *Journal of Agricultural Science*, 152(5):860–869.

[Kocabagli et al., 1995] Kocabagli, N., Riond, J. L., Spichiger, U. E., and Wanner, M. (1995). Parathyroid hormone-related protein and calcium homeostasis during the periparturient period of dairy cows. *American Journal of Veterinary Research*, 56(3):380–385.

[Kovacs, 2005] Kovacs, C. S. (2005). Calcium and bone metabolism during pregnancy and lactation. *Journal of Mammary Gland Biology and Neoplasia*, 10(2):105–118.

[Kovacs, 2016] Kovacs, C. S. (2016). Maternal mineral and bone metabolism during pregnancy, lactation, and post-weaning recovery. *Physiological Reviews*, 96(2):449–547.

[Kovacs and Kronenberg, 1997] Kovacs, C. S. and Kronenberg, H. M. (1997). Maternal-fetal calcium and bone metabolism during pregnancy, puerperium, and lactation. *Endocrine Reviews*, 18(6):832–872.

[Kume et al., 1998] Kume, S., Yanamoto, E., Kudo, T., Toharmat, T., and Nonaka, I. (1998). Effect of parity on mineral concentration in milk and plasma of holstein cows during early lactation. *Asian-Australasian Journal of Animal Sciences*, 11(2):133–138.

[Kume and Tanabe, 1993] Kume, S.-I. and Tanabe, S. (1993). Effect of parity on colostral mineral concentrations of Holstein cows and value of colostrum as a mineral source for newborn calves. *Journal of Dairy Science*, 76(6):1654–1660.

[Kurosaki et al., 2007] Kurosaki, N., Yamato, O., Sato, J., Naito, Y., Mori, F., Imoto, S., and Maede, Y. (2007). Biomarkers for the activation of calcium metabolism in dairy cows: Elevation of tartrate-resistant acid phosphatase activity by lowering dietary cation-anion difference is associated with the prevention of milk fever. *Journal of Veterinary Medical Science*, 69(3):265–270.

[Lakatta et al., 2010] Lakatta, E. G., Maltsev, V. A., and Vinogradova, T. M. (2010). A coupled system of intracellular Ca2+ clocks and surface membrane voltage clocks controls the timekeeping mechanism of the heart's pacemaker. *Circulation Research*, 106(4):659–673.

[Laporta et al., 2014] Laporta, J., Keil, K. P., Weaver, S. R., Cronick, C. M., Prichard, A. P., Crenshaw, T. D., Heyne, G. W., Vezina, C. M., Lipinski, R. J., and Hernandez, L. L. (2014). Serotonin regulates calcium momeostasis in lactation by epigenetic activation of hedgehog signaling. *Molecular Endocrinology*, 28(11):1866–1874.

[Laporta et al., 2015] Laporta, J., Moore, S. A. E., Weaver, S. R., Cronick, C. M., Olsen, M., Prichard, A. P., Schnell, B. P., Crenshaw, T. D., Penagaricano, F., Bruckmaier, R. M., and Hernandez, L. L. (2015). Increasing serotonin concentrations alter calcium and energy metabolism in dairy cows. *Journal of Endocrinology*, 226(1):43–55.

[Law et al., 1991] Law, F. M., Moate, P. J., Leaver, D. D., Diefenbach-Jagger, H., Grill, V., Ho, P. W., and Martin, T. J. (1991). Parathyroid hormone-related protein in milk and its correlation with bovine milk calcium. *The Journal of Endocrinology*, 128(1):21–26.

[Lean et al., 2014] Lean, I. J., DeGaris, P. J., Celi, P., McNeill, D. M., Rodney, R. M., and Fraser, D. R. (2014). Influencing the future: interactions of skeleton, energy, protein and calcium during late gestation and early lactation. *Animal Production Science*, 54(9):1177–1189.

[Lee et al., 2006] Lee, W. J., Monteith, G. R., and Roberts-Thomson, S. J. (2006). Calcium transport and signaling in the mammary gland: targets for breast cancer. *Biochimica Et Biophysica Acta*, 1765(2):235–255.

[Leno et al., 2017] Leno, B. M., Ryan, C. M., Stokol, T., Kirk, D., Zanzalari, K. P., Chapman, J. D., and Overton, T. R. (2017). Effects of prepartum dietary cation-anion difference on aspects of peripartum mineral and energy metabolism and performance of multiparous Holstein cows. *Journal of Dairy Science*, 100(6):4604–4622.

[Lenstrup, 1926] Lenstrup, E. (1926). The Phosphorus Content of Human Milk and Cow's Milk. *Journal of Biological Chemistry*, 70(1):193–202.

[Liesegang et al., 2007] Liesegang, A., Chiappi, C., Risteli, J., Kessler, J., and Hess, H. D. (2007). Influence of different calcium contents in diets supplemented with anionic salts on bone metabolism in periparturient dairy cows. *Journal of Animal Physiology and Animal Nutrition*, 91(3-4):120–129.

[Liesegang et al., 2000] Liesegang, A., Eicher, R., Sassi, M. L., Risteli, J., Kraenzlin, M., Riond, J. L., and Wanner, M. (2000). Biochemical markers of bone formation and resorption around parturition and during lactation in dairy cows with high and low standard milk yields. *Journal of Dairy Science*, 83(8):1773–1781.

[Liesegang et al., 1998] Liesegang, A., Sassi, M. L., Risteli, J., Eicher, R., Wanner, M., and Riond, J. L. (1998). Comparison of Bone Resorption Markers During Hypocalcemia in Dairy Cows. *Journal of Dairy Science*, 81(10):2614–2622.

[Little, 1972] Little, D. A. (1972). Bone biopsy in cattle and sheep for studies of phosphorus status. *Australian Veterinary Journal*, 48(12):668–670.

[Liu et al., 2012] Liu, S., Rovine, M. J., and Molenaar, P. C. M. (2012). Selecting a linear mixed model for longitudinal data: repeated measures analysis of variance, covariance pattern model, and growth curve approaches. *Psychological Methods*, 17(1):15–30.

[Lopez et al., 2004] Lopez, H., Kanitz, F. D., Moreira, V. R., Wiltbank, M. C., and Satter, L. D. (2004). Effect of dietary phosphorus on performance of lactating dairy cows: milk production and cow health. *Journal of Dairy Science*, 87(1):139–145.

[Mahieu et al., 1977] Mahieu, H., Luquet, F. M., Mouillet, L., and Normand, A. (1977). A propos de la teneur des laits individuels et de mélange en matières minérales et urée (suite). *Le Lait*, 57(561-562):55–112.

[Malacarne et al., 2014] Malacarne, M., Franceschi, P., Formaggioni, P., Sandri, S., Mariani, P., and Summer, A. (2014). Influence of micellar calcium and phosphorus on rennet coagulation properties of cows milk. *Journal of Dairy Research*, 81(2):129–136.

[Mamillapalli et al., 2013] Mamillapalli, R., VanHouten, J., Dann, P., Bikle, D., Chang, W., Brown, E., and Wysolmerski, J. (2013). Mammary-specific ablation of the calcium-sensing receptor during lactation alters maternal calcium metabolism, milk calcium transport, and neonatal calcium accrual. *Endocrinology*, 154(9):3031–3042.

[Martinez et al., 2012] Martinez, N., Risco, C. A., Lima, F. S., Bisinotto, R. S., Greco, L. F., Ribeiro, E. S., Maunsell, F., Galvão, K., and Santos, J. E. P. (2012). Evaluation of peripartal calcium status, energetic profile, and neutrophil function in dairy cows at low or high risk of developing uterine disease. *Journal of Dairy Science*, 95(12):7158–7172.

[Martín-Tereso and Martens, 2014] Martín-Tereso, J. and Martens, H. (2014). Calcium and magnesium physiology and nutrition in relation to the prevention of milk fever and tetany (dietary management of macrominerals in preventing disease). *The Veterinary Clinics of North America. Food Animal Practice*, 30(3):643–670.

[Martz et al., 1990] Martz, F. A., Belo, A. T., Weiss, M. F., Belyea, R. L., and Goff, J. P. (1990). True absorption of calcium and phosphorus from alfalfa and corn silage when fed to lactating cows. *Journal of Dairy Science*, 73(5):1288–1295.

[Matsuda et al., 2004] Matsuda, M., Imaoka, T., Vomachka, A. J., Gudelsky, G. A., Hou, Z., Mistry, M., Bailey, J. P., Nieport, K. M., Walther, D. J., Bader, M., and Horseman, N. D. (2004). Serotonin regulates mammary gland development via an autocrine-paracrine loop. *Developmental Cell*, 6(2):193–203.

[McArt and Oetzel, 2015] McArt, J. A. A. and Oetzel, G. R. (2015). A stochastic estimate of the economic impact of oral calcium supplementation in postparturient dairy cows. *Journal of Dairy Science*, 98(10):7408–7418.

[McDowell, 2017] McDowell, L. (2017). *Mineral Nutrition History: The Early Years*. First Edition Design Pub.

[McDowell, 2003] McDowell, L. R. (2003). Chapter 2 - Calcium and Phosphorus. In *Minerals in Animal and Human Nutrition (Second Edition)*, pages 33–100. Elsevier, Amsterdam.

[McNeill et al., 2002] McNeill, D. M., Roche, J. R., Stockdale, C. R., and McLachlan, B. P. (2002). Nutritional strategies for the prevention of hypocalcaemia at calving for dairy cows in pasture-based systems. *Australian Journal of Agricultural Research*, 53(7):755–770.

[Meschy, 2002] Meschy, F. (2002). Recommandations d'apport en phosphore absorbé chez les ruminants. Rencontres Recherches Rumiants (3R). 279-285.

[Meschy, 2010] Meschy, F. (2010). *Nutrition minérale des ruminants*. Editions Quae, Versailles, France.

[Meschy, 2017] Meschy, F. (2017). *Nutrition minérale des ruminants*. Editions Quae, Versailles, France.

[Meschy and Corris, 2005] Meschy, F. and Corris, R. (2005). Recommandations d'apport alimentaire en calcium et magnésium absorbables pour les ruminants. Rencontres Recherches Rumiants (3R). 221-224.

[Metson and Saunders, 2012] Metson, A. J. and Saunders, W. M. H. (2012). Seasonal variations in chemical composition of pasture. *New Zealand Journal of Agricultural Research*, 21(2):341–353.

[Miltenburg et al., 2016] Miltenburg, C. L., Duffield, T. F., Bienzle, D., Scholtz, E. L., and LeBlanc, S. J. (2016). Randomized clinical trial of a calcium supplement for improvement of health in dairy cows in early lactation. *Journal of Dairy Science*, 99(8):6550–6562.

[Moreira et al., 2009] Moreira, V. R., Zeringue, L. K., Williams, C. C., Leonardi, C., and McCormick, M. E. (2009). Influence of calcium and phosphorus feeding on markers of bone metabolism in transition cows. *Journal of Dairy Science*, 92(10):5189–5198.

[Muff et al., 1988] Muff, R., Nemeth, E. F., Haller-Brem, S., and Fischer, J. A. (1988). Regulation of hormone secretion and cytosolic Ca2+ by extracellular Ca2+ in parathyroid cells and C-cells: Role of voltage-sensitive Ca2+ channels. *Archives of Biochemistry and Biophysics*, 265(1):128–135.

[Mundy and Guise, 1999] Mundy, G. R. and Guise, T. A. (1999). Hormonal control of calcium homeostasis. *Clinical Chemistry*, 45(8 Pt 2):1347–1352.

[Murthy and Rhea, 1967] Murthy, G. K. and Rhea, U. (1967). Determination of major cations in milk by atomic absorption spectrophotometry. *Journal of Dairy Science*, 50(3):313–317.

[Nakache and Confais, 2004] Nakache, J.-P. and Confais, J. (2004). *Approche pragmatique de la classification: arbres hiérarchiques, partitionnements*. Éditions Technip, 2004, Paris, France.

[Nantapo and Muchenje, 2013] Nantapo, C. T. W. and Muchenje, V. (2013). Winter and spring variation in daily milk yield and mineral composition of Jersey, Friesian cows and their crosses under a pasture-based dairy system. *South African Journal of Animal Science*, 43(5):S17–S21.

[Neves et al., 2017] Neves, R. C., Leno, B. M., Stokol, T., Overton, T. R., and McArt, J. A. A. (2017). Risk factors associated with postpartum subclinical hypocalcemia in dairy cows. *Journal of Dairy Science*, 100(5):3796–3804.

[Neville and Peaker, 1979] Neville, M. and Peaker, M. (1979). Secretion of calcium and phosphorus into milk. *Journal of Physiology*, 290(MAY):59–67.

[Neville, 2005] Neville, M. C. (2005). Calcium secretion into milk. *Journal of Mammary Gland Biology and Neoplasia*, 10(2):119–128.

[Neville et al., 1994] Neville, M. C., Keller, R. P., Casey, C., and Allen, J. C. (1994). Calcium partitioning in human and bovine milk. *Journal of Dairy Science*, 77(7):1964–1975.

[Neville et al., 1995] Neville, M. C., Zhang, P., and Allen, J. C. (1995). CHAPTER 7 - Minerals, ions, and trace elements in milk: A. ionic interactions in milk. In *Handbook of Milk Composition*, Food Science and Technology, pages 577–592. Academic Press, San Diego.

[NRC, 2001] NRC (2001). *Nutrient requirements of dairy cattle: Seventh Revised Edition, 2001*. National Academies Press, Washington (DC).

[Odongo et al., 2007] Odongo, N. E., McKnight, D., KoekKoek, A., Fisher, J. W., Sharpe, P., Kebreab, E., France, J., and McBride, B. W. (2007). Long-term effects of feeding diets without mineral phosphorus supplementation on the performance and phosphorus excretion in high-yielding dairy cows. *Canadian Journal of Animal Science*, 87(4):639–646.

[Oetzel and Miller, 2012] Oetzel, G. R. and Miller, B. E. (2012). Effect of oral calcium bolus supplementation on early-lactation health and milk yield in commercial dairy herds. *Journal of Dairy Science*, 95(12):7051–7065.

[Onda et al., 2006] Onda, K., Sato, A., Yamaguchi, M., Matsuki, N., Ono, K., and Wada, Y. (2006). Parathyroid hormone-related protein (PTHrP) and Ca levels in the milk of lactating cows. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science*, 68(7):709–713.

[Pien, 1969] Pien, J. (1969). Dosage du phosphore dans le lait. *Le Lait*, 49(483_484):175–188.

[Pinto et al., 2015] Pinto, M. C. X., Kihara, A. H., Goulart, V. A. M., Tonelli, F. M. P., Gomes, K. N., Ulrich, H., and Resende, R. R. (2015). Calcium signaling and cell proliferation. *Cellular Signalling*, 27(11):2139–2149.

[Poulsen et al., 2015] Poulsen, N. A., Rybicka, I., Poulsen, H. D., Larsen, L. B., Andersen, K. K., and Larsen, M. K. (2015). Seasonal variation in content of riboflavin and major minerals in bulk milk from three Danish dairies. *International Dairy Journal*, 42:6–11.

[Puggaard et al., 2011] Puggaard, L., Kristensen, N. B., and Sehested, J. (2011). Effect of decreasing dietary phosphorus supply on net recycling of inorganic phosphate in lactating dairy cows. *Journal of Dairy Science*, 94(3):1420–1429.

[Puggaard et al., 2014] Puggaard, L., Lund, P., Liesegang, A., and Sehested, J. (2014). Long term effect of reduced dietary phosphorus on feed intake and milk yield in dry and lactating dairy cows. *Livestock Science*, 159:18–28.

[R Development Core Team, 2008] R Development Core Team, R. (2008). R: a language and environment for statistical computing.

[Ramberg et al., 1970] Ramberg, C., Mayer, G., Kronfeld, D., Phang, J., and Berman, M. (1970). Calcium kinetics in cows during late pregnancy, parturition, and early lactation. *American Journal of Physiology-Legacy Content*, 219(5):1166–1177.

[Ramberg et al., 1976] Ramberg, C. F., Mayer, G. P., Kronfeld, D. S., and Potts, J. T. (1976). Dietary calcium, calcium kinetics and plasma parathyroid hormone concentration in cows. *The Journal of Nutrition*, 106(5):671–679.

[Reinhardt et al., 1988] Reinhardt, T. A., Horst, R. L., and Goff, J. P. (1988). Calcium, phosphorus, and magnesium homeostasis in ruminants. *The Veterinary Clinics of North America. Food Animal Practice*, 4(2):331–350.

[Reinhardt et al., 2011] Reinhardt, T. A., Lippolis, J. D., McCluskey, B. J., Goff, J. P., and Horst, R. L. (2011). Prevalence of subclinical hypocalcemia in dairy herds. *Veterinary Journal*, 188(1):122–124.

[Reinhardt et al., 2004] Reinhardt, T. A., Lippolis, J. D., Shull, G. E., and Horst, R. L. (2004). Null mutation in the gene encoding plasma membrane Ca2+-ATPase isoform 2 impairs calcium transport into milk. *The Journal of Biological Chemistry*, 279(41):42369–42373.

[Rey et al., 2009] Rey, C., Combes, C., Drouet, C., and Glimcher, M. (2009). Bone mineral: update on chemical composition and structure. *Osteoporosis international*, 20(6):1013–1021.

[Rillema, 2002] Rillema, J. A. (2002). Effect of prolactin on phosphate transport and incorporation in mouse mammary gland explants. *American Journal of Physiology-Endocrinology and Metabolism*, 283(1):E132–E137.

[Roca-Cusachs et al., 1991] Roca-Cusachs, A., DiPette, D. J., and Nickols, G. A. (1991). Regional and systemic hemodynamic effects of parathyroid hormone-related protein: preservation of cardiac function and coronary and renal flow with reduced blood pressure. *The Journal of Pharmacology and Experimental Therapeutics*, 256(1):110–118.

[Rodríguez et al., 2017] Rodríguez, E. M., Arís, A., and Bach, A. (2017). Associations between subclinical hypocalcemia and postparturient diseases in dairy cows. *Journal of Dairy Science*, 100(9):7427–7434.

[Ross et al., 2011] Ross, A. C., Taylor, C. L., Yaktine, A. L., and Valle, H. B. D. (2011). Overview of calcium. In *Dietary Reference Intakes for Calcium and Vitamin D*. National Academies Press (US).

[Saito and Fukumoto, 2009] Saito, T. and Fukumoto, S. (2009). Fibroblast Growth Factor 23 (FGF23) and disorders of phosphate metabolism. *International Journal of Pediatric Endocrinology*, 2009.

[Sanchez et al., 2016] Sanchez, M. P., Govignon-Gion, A., Ferrand, M., Gelé, M., Pourchet, D., Amigues, Y., Fritz, S., Boussaha, M., Capitan, A., Rocha, D., Miranda, G., Martin, P., Brochard, M., and Boichard, D. (2016). Whole-genome scan to detect quantitative trait loci associated with milk protein composition in 3 French dairy cattle breeds. *Journal of Dairy Science*, 99(10):8203–8215.

[Sato et al., 2013] Sato, R., Onda, K., Kato, H., Ochiai, H., Kawai, K., Iriki, T., Kaneko, K., Yamazaki, Y., and Wada, Y. (2013). An evaluation of the effect of age and the peri-parturient period on bone metabolism in dairy cows as measured by serum

bone-specific alkaline phosphatase activity and urinary deoxypyridinoline concentration. *Veterinary Journal*, 197(2):358–362.

[Sato et al., 2014] Sato, R., Onda, K., Kazama, K., Ohnishi, M., Ochiai, H., Kawai, K., Kaneko, K., Ohashi, T., Miyamoto, T., and Wada, Y. (2014). Changes in parathyroid hormone-related protein concentrations in bovine milk from the early stage of lactation. *Livestock Science*, 159:117–122.

[Sato et al., 2011] Sato, R., Onda, K., Ochiai, H., Iriki, T., Yamazaki, Y., and Wada, Y. (2011). Serum osteocalcin in dairy cows: Age-related changes and periparturient variation. *Research in Veterinary Science*, 91(2):196–198.

[Sehested, 2004] Sehested, J. (2004). Feeding phosphorus (P) to cattle: A new basis for Nordic recommendations. *Acta Agriculturae Scandinavica*, 54(4):169–180.

[Seibel, 2000] Seibel, M. J. (2000). Molecular markers of bone turnover: biochemical, technical and analytical aspects. *Osteoporosis International*, 11(Suppl. 6):S18–S29.

[Shennan and Peaker, 2000] Shennan, D. B. and Peaker, M. (2000). Transport of milk constituents by the mammary gland. *Physiological Reviews*, 80(3):925–951.

[Shillingford et al., 1996] Shillingford, J. M., Calvert, D. T., Beechey, R. B., and Shennan, D. B. (1996). Phosphate transport via Na+-Pi cotransport and anion exchange in lactating rat mammary tissue. *Experimental Physiology*, 81(2):273–284.

[Slatopolsky et al., 1995] Slatopolsky, E., Finch, J., Denda, M., Ritter, C., Zhong, M., Dusso, A., MacDonald, P. N., and Brown, A. J. (1995). Phosphorus restriction prevents parathyroid gland growth. High phosphorus directly stimulates PTH secretion in vitro. *The Journal of Clinical Investigation*, 97(11):2534–2540.

[Soares Jr, 1995] Soares Jr, J. H. (1995). 12 - Phosphorus bioavailability. In *Bioavailability of Nutrients for Animals*, pages 257–294. Academic Press, San Diego.

[Soyeurt et al., 2009] Soyeurt, H., Bruwier, D., Romnee, J.-M., Gengler, N., Bertozzi, C., Veselko, D., and Dardenne, P. (2009). Potential estimation of major mineral contents in cow milk using mid-infrared spectrometry. *Journal of Dairy Science*, 92(6):2444–2454.

[Soyeurt et al., 2010] Soyeurt, H., Misztal, I., and Gengler, N. (2010). Genetic variability of milk components based on mid-infrared spectral data. *Journal of Dairy Science*, 93(4):1722–1728.

[Steen, 1998] Steen, I. (1998). Phosphorus avaibility in the 21st century: Management of a non-renewable ressoyrce. *Phosphorus & Potassium*, 217:25–31.

[Steenbock and Hart, 1913] Steenbock, H. and Hart, E. (1913). The influence of function on the lime requirements of animals. *Journal of biological chemistry*, 14:59–74.

[Stein et al., 2006] Stein, H. H., Boersma, M. G., and Pedersen, C. (2006). Apparent and true total tract digestibility of phosphorus in field peas (Pisum sativum L.) by growing pigs. *Canadian Journal of Animal Science*, 86(4):523–525.

[Suttle, 2010] Suttle, N. F. (2010). *Mineral Nutrition of Livestock*. CABI, Cambridge (USA).

[Taylor et al., 2008] Taylor, M. S., Knowlton, K. F., McGilliard, M. L., Seymour, W. M., and Herbein, J. H. (2008). Blood mineral, hormone, and osteocalcin responses of multiparous Jersey cows to an oral dose of 25-hydroxyvitamin D3 or vitamin D3 before parturition. *Journal of Dairy Science*, 91(6):2408–2416.

[Taylor et al., 2009] Taylor, M. S., Knowlton, K. F., McGilliard, M. L., Swecker, W. S., Ferguson, J. D., Wu, Z., and Hanigan, M. D. (2009). Dietary calcium has little effect on mineral balance and bone mineral metabolism through twenty weeks of lactation in Holstein cows. *Journal of Dairy Science*, 92(1):223–237.

[Teramura et al., 2015] Teramura, M., Wynn, S., Reshalaitihan, M., Kyuno, W., Sato, T., Ohtani, M., Kawashima, C., and Hanada, M. (2015). Supplementation with difructose anhydride III promotes passive calcium absorption in the small intestine immediately after calving in dairy cows. *Journal of Dairy Science*, 98(12):8688–8697.

[Thiede, 1994] Thiede, M. A. (1994). Parathyroid hormone-related protein: a regulated calcium-mobilizing product of the mammary gland. *Journal of Dairy Science*, 77(7):1952–1963.

[Thiede and Rodan, 1988] Thiede, M. A. and Rodan, G. A. (1988). Expression of a calcium-mobilizing parathyroid hormone-like peptide in lactating mammary tissue. *Science*, 242(4876):278–280.

[Toffanin et al., 2015a] Toffanin, V., De Marchi, M., Lopez-Villalobos, N., and Cassandro, M. (2015a). Effectiveness of mid-infrared spectroscopy for prediction of the contents of calcium and phosphorus, and titratable acidity of milk and their relationship with milk quality and coagulation properties. *International Dairy Journal*, 41:68–73.

[Toffanin et al., 2015b] Toffanin, V., Penasa, M., McParland, S., Berry, D. P., Cassandro, M., and De Marchi, M. (2015b). Genetic parameters for milk mineral content and acidity predicted by mid-infrared spectroscopy in Holstein-Friesian cows. *Animal*, 9(5):775–780.

[Tuff, 1923] Tuff, P. (1923). Ostemoalacia and its occurences in cattle in Norway. *Journal of Comparative Pathology and Therapeutics*, 36(3):143–155.

[Uemura et al., 1997] Uemura, H., Yasui, T., Yoneda, N., Irahara, M., and Aono, T. (1997). Measurement of N- and C-terminal-region fragments of parathyroid hormone-related peptide in milk from lactating women and investigation of the relationship of their concentrations to calcium in milk. *The Journal of Endocrinology*, 153(3):445–451.

[Valk et al., 2002] Valk, H., Šebek, L. B. J., and Beynen, A. C. (2002). Influence of phosphorus intake on excretion and blood plasma and saliva concentrations of phosphorus in dairy cows. *Journal of Dairy Science*, 85(10):2642–2649.

[Van Hulzen et al., 2009] Van Hulzen, K. J. E., Sprong, R. C., van der Meer, R., and van Arendonk, J. a. M. (2009). Genetic and nongenetic variation in concentration of selenium, calcium, potassium, zinc, magnesium, and phosphorus in milk of Dutch Holstein-Friesian cows. *Journal of Dairy Science*, 92(11):5754–5759.

[VanHouten et al., 2004] VanHouten, J., Dann, P., McGeoch, G., Brown, E. M., Krapcho, K., Neville, M., and Wysolmerski, J. J. (2004). The calcium-sensing receptor regulates mammary gland parathyroid hormone-related protein production and calcium transport. *The Journal of Clinical Investigation*, 113(4):598–608.

[VanHouten et al., 2003] VanHouten, J. N., Dann, P., Stewart, A. F., Watson, C. J., Pollak, M., Karaplis, A. C., and Wysolmerski, J. J. (2003). Mammary-specific deletion of parathyroid hormone-related protein preserves bone mass during lactation. *The Journal of Clinical Investigation*, 112(9):1429–1436.

[VanHouten and Wysolmerski, 2007] VanHouten, J. N. and Wysolmerski, J. J. (2007). Transcellular calcium transport in mammary epithelial cells. *Journal of Mammary Gland Biology and Neoplasia*, 12(4):223–235.

[Venjakob et al., 2016] Venjakob, P. L., Borchardt, S., Thiele, G., and Heuwieser, W. (2016). Evaluation of ear skin temperature as a cow-side test to predict postpartum calcium status in dairy cows. *Journal of Dairy Science*, 99(8):6542–6549.

[Vitti and Kebreab, 2010] Vitti, D. M. S. S. and Kebreab, E. (2010). *Phosphorus and Calcium Utilization and Requirements in Farm Animals*. CABI, Wallingford, UK, cab international edition.

[Volden, 2011] Volden, H. (2011). *NorFor - the Nordic Feed Evaluation System*. Wageningen Academic Pub.

[Walstra, 1999] Walstra, P. (1999). *Dairy Technology: Principles of Milk Properties and Processes*. CRC Press.

[Ward et al., 1972] Ward, G., Dobson, R., and Dunham, J. (1972). Influences of calcium and phosphorus intakes, vitamin D supplement, and lactation on calcium and phosphorus balances. *Journal of Dairy Science*, 55(6):768–776.

[Weaver et al., 2016] Weaver, S. R., Prichard, A. P., Endres, E. L., Newhouse, S. A., Peters, T. L., Crump, P. M., Akins, M. S., Crenshaw, T. D., Bruckmaier, R. M., and Hernandez, L. L. (2016). Elevation of circulating serotonin improves calcium dynamics in the peripartum dairy cow. *Journal of Endocrinology*, 230(1):105–123.

[Wilhelm et al., 2017] Wilhelm, A. L., Maquivar, M. G., Bas, S., Brick, T. A., Weiss, W. P., Bothe, H., Velez, J. S., and Schuenemann, G. M. (2017). Effect of serum calcium status at calving on survival, health, and performance of postpartum Holstein cows and calves under certified organic management. *Journal of Dairy Science*, 100(4):3059–3067.

[Wohlt et al., 1986] Wohlt, J. E., Ritter, D. E., and Evans, J. L. (1986). Calcium Sources for Milk Production in Holstein Cows Via Changes in Dry Matter Intake, Mineral Utilization, and Mineral Source Buffering Potential1. *Journal of Dairy Science*, 69(11):2815–2824.

[Wojcik et al., 1998] Wojcik, S. F., Schanbacher, F. L., McCauley, L. K., Zhou, H., Kartsogiannis, V., Capen, C. C., and Rosol, T. J. (1998). Cloning of bovine parathyroid hormone-related protein (PTHrP) cDNA and expression of PTHrP mRNA in the bovine mammary gland. *Journal of Molecular Endocrinology*, 20(2):271–280.

[Wolf, 2008] Wolf, G. (2008). Energy regulation by the skeleton. *Nutrition Reviews*, 66(4):229–233.

[Wu et al., 2001] Wu, Z., Satter, L. D., Blohowiak, A. J., Stauffacher, R. H., and Wilson, J. H. (2001). Milk production, estimated phosphorus excretion, and bone characteristics of dairy cows fed different amounts of phosphorus for two or three years. *Journal of Dairy Science*, 84(7):1738–1748.

[Wu et al., 2000] Wu, Z., Satter, L. D., and Sojo, R. (2000). Milk production, reproductive performance, and fecal excretion of phosphorus by dairy cows fed three amounts of phosphorus. *Journal of Dairy Science*, 83(5):1028–1041.

[Yamamoto et al., 1992] Yamamoto, M., Fisher, J. E., Thiede, M. A., Caulfield, M. P., Rosenblatt, M., and Duong, L. T. (1992). Concentrations of parathyroid hormone-related protein in rat milk change with duration of lactation and interval from previous suckling, but not with milk calcium. *Endocrinology*, 130(2):741–747.

[Zull et al., 1966] Zull, J. E., Czarnowska-Misztal, E., and DeLuca, H. F. (1966). On the relationship between vitamin D action and actinomycin-sensitive processes. *Proceedings of the National Academy of Sciences*, 55(1):177–184.

# Appendix

**Appendix 1:** Abstrat for American Dairy Science Assocation Meeting, 25-18 June 2017, Pittsburgh,PA (USA).

**Appendix 2:** Abstrat for 12th International Meeting on Montain Cheese, 20-22 June 2017, Padova (Italy).

**Appendix 3:** Poster for Journées d'Animations Scientifiques du département PHASE, Avril 2018, Rennes (France).

**Appendix 4:** Poster for International Symposium on the Nutrition of Herbivores (ISNH), 2-6 September 2018, Clermont-Ferrand (France).

**Appendix 5:** Abstract for Rencontres Recherches Ruminants (3R), 5-6 December 2018, Paris (France).

# Appendix 1

This work provided some gene-associated insights to facilitate further investigation of the mechanisms underlying lactation in dairy cows.

**Key Words:** lactation, bovine mammary gland, transcriptomics

**494    Understanding the regulatory mechanisms of milk production using integrative transcriptomic and proteomic analyses: Reducing inefficient utilization of crop by-products as forage in dairy industry.** W. Dai*[1], Q. Wang[1], F. Zhao[2], J. Liu[1], and H. Liu[1], [1]*Institute of Dairy Science, College of Animal Sciences, Zhejiang University, Hangzhou, Zhejiang, China,* [2]*Laboratory of Lactation and Metabolic Physiology, Department of Animal Science, University of Vermont, Burlington, VT.*

Milk from dairy cows is an essential nutrient for the young and human as well. Forage plays a vital role in dairy husbandry via affecting milk quality and quantity. However, the differences in mammary metabolism of dairy cows fed different forages remains elucidated. In this study, we utilized transcriptomic RNA-seq and iTRAQ proteomic techniques to investigate and integrate the differences of molecular pathways and biological processes in the mammary gland of dairy cows fed differing forages. Bovine mammary tissues were obtained from 6 healthy multiparous lactating dairy cows fed with corn stover (CS, low-quality; n = 6) and alfalfa hay (AH, high-quality; n = 6), respectively. A total of 1631 differentially expressed transcripts (DETs; 1046 upregulated and 585 downregulated) and 346 differentially expressed proteins (DEPs; 138 increased and 208 decreased) were detected in the mammary glands between the CS- and AH-fed animals. Expression patterns of 33 DEPs (18 increased and 15 decreased) were consistent with the expression of their mRNAs. The gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) analyses of the DETs and DEPs indicated that the decreased energy metabolism, increased fatty-acid oxidation, attenuated protein synthesis, enhanced protein degradation, and the lower mammary cell growth may be the prime factors contributing to the lower milk production in the CS-fed cows compared with the AH-fed cows. Moreover, 19 milk-synthesis-related genes were quantitated by real-time RT-PCR to examine the transcriptional profile and validate the proteins identified by LC-MS/MS between CS-fed and AH-fed bovine mammary gland. Four DEPs were further verified by Western blot analysis. These results provide the biological understanding of insights into mammary metabolism alterations affected by differing foraged and will be beneficial in developing highly efficient strategies for utilization of low-quality forages.

**Key Words:** dairy cow, mammary gland, forage

**495    Characterization of the non-genetic causes of variation of bovine milk calcium concentrations on French farms.** P. Gaignon*[1,2], M. Gele[3], C. Hurtaud[1], and A. Boudon[1], [1]*PEGASE, INRA, Agrocampus Ouest, Saint-Gilles, France,* [2]*CMI, 18 avenue F. Roosevelt, Saint-Malo, France,* [3]*Institut de l'élevage, Angers, France.*

Calcium concentration (CaC) in bovine milk has often been described as independent of feeding strategy and mainly dependent on cow genetics and lactation stage. However, isolated experiments showed that variations in milk CaC could be linked to the diet of cows. Our objective was to identify and quantify non-genetic factors of variation in CaC in milk samples collected from about a thousand French dairy farms with contrasting feeding strategies and cow breeds. This study was based on the PhénoFinlait program that consisted of a survey performed between 2009 and 2010 in 924 dairy farms located in the major French milk production areas. The breeds used in the investigated farms were Holstein,

Normande and Montbeliarde. Each farm was visited on average 4 to 6 times during the year. Each time, information about cow diets and production were gathered and individual milk samples were collected to extract their mid-infrared (MIR) spectra. More than 200,000 MIR spectra were measured. Nearly 10,000 milk samples were also frozen and stored in a bank for further analyses. We estimated CaC in milk samples from their MIR spectra using a predictive equation. This equation was established from 300 milk samples extracted from the bank and chosen to represent the diversity of investigated dairy systems. From the composition of the cow diets collected at each survey, we characterized 7 feeding strategies using multiple factorial analyses across 3 periods: winter, early and late summer. For each breed, the variations in milk CaC were quantified by ANOVA with a model including the effects of feeding strategies, stage of lactation, parity, and calendar month as fixed effects and the cow as random effect. The feeding strategy affected milk CaC with the constant fact that the diets based on fresh or conserved grass induced lower milk CaC whatever the month of the year ($P < 0.05$). The difference in CaC can be up to 100 mg/kg between 2 extreme diets at a given month, which is as important as the drop in CaC observed at the beginning of lactation. This study reinforces the idea that the diet of cows has an influence on milk CaC.

**Key Words:** calcium, milk, feeding strategies

**496    Milk fat globule size is regulated by phosphatidylethanolamine-dependent fusion: In vitro model.** N. Argov-Argaman*[1], B.-C. Cohen[1], and A. Shamay[2], [1]*Hebrew University, Rehovot, Israel,* [2]*The Volcani Center, The Ministry of Agriculture, Rehovot, Israel.*

Milk fat is secreted in a unique structure, termed milk fat globule (MFG) which consists of a triglyceride core covered with 3 layers of phospholipids (MFG membrane; MFGM). MFG are secreted in a wide range of sizes; from the nanometer length scale to over 15 μm, and their size is tightly associated with their lipid composition. Particularly, higher MFGM content is found in small compared with large globules. MFG size is determined by the size of its precursors — the intracellular lipid droplets (LD) which are produced and secreted by the mammary epithelial cells (MEC). Fusion is one of the suggested mechanisms controlling LD size. Nevertheless, what controls the extent of fusion and how dominant this mechanism is in controlling LD size is still illusive, especially in mammalian cells. We hypothesized that LD fusion is controlled by the stability of their membrane, which is modulated by the content and mass ratio between 2 main phospholipids - phosphatidylethanolamine (PE) and phosphatidylcholine (PC). We used primary MEC culture, treated with oleic or palmitic acid, to study the role of membrane stability in determining LD size. Results show that 22% of MEC treated with oleic acid had large LD (>2.5 μm) compared with only 4% of the cells treated with palmitic acid. The increased LD size in the oleic acid treatment was associated with 63% increase in PE, and 7 fold increase in LD fusion. Adding $NaN_3$+NaF to the oleic acid treatment decreased PE content by 19%, concomitantly with 8 fold decrease in the number of large LD. Interestingly, the addition of $NaN_3$+NaF to oleic acid treatment did not change the cellular triglyceride content. In contrast, adding 3-deazaadenosine to palmitic acid treatment tended to increase PE content by 29%, and consequently increased the number of large LD by 3 fold, relative to cells treated with palmitic acid alone. Our findings have uncovered a defining role for LD fusion in determining their size in MEC, which is independent of triglycerides content of the cells. Understanding the mechanisms controlling LD size in mammalian cells is of great importance, especially in MEC due to the effect of LD size on milk composition.

**Key Words:** milk fat globule, size, fusion

# Appendix 2

**Characterization of the non-genetic causes of variation of bovine milk calcium concentrations in French farms**

P. Gaignon [1-2]*, C. Hurtaud[1], M. Gelé [3], A. Boudon[1]

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**Abstract**
Our objective was to identify and quantify non-genetic factors of variation in CaC (Calcium Concentration) in milk samples collected from a significant pool of French dairy farms. This study was based on program PhénoFinLait that consisted in a survey performed between 2009 and 2010 in about a thousand dairy farms located in the major French milk production areas, with three breeds: Holstein, Normande and Montbeliarde. Information about cows' diet was gathered and individual milk samples were collected in order to extract their mid-infrared (MIR) spectra. More than 200,000 MIR spectra were measured. We estimated CaC in milk samples from their MIR spectra. From the composition of the cow diets collected, we characterized 7 feeding strategies. The feeding strategy affected milk Ca with the constant fact that the diets based on fresh or conserved grass induced lower milk CaC regardless of the month of the year ($p < 0.05$). The difference in CaC can be up to 100 mg/kg between two extreme diets at a given month, which was as important as the drop in CaC observed at the beginning of lactation. This study reinforced the idea that the diet of cows has an influence on milk CaC.

Keywords: calcium, milk, feeding strategies

**Introduction**
The main factors of variation of calcium concentration of cow milk reported in the literature are the genetics of the cow and their stage of lactation (Alais, 1984; van Hulzen *et al*., 2009). However, milk calcium concentration (CaC) is considered to be little affected by cow's diet, diets low in calcium driving to decreased milk production rather than milk CaC reduction(Alais, 1984; Suttle, 2010). However, experiments run at the UMR PEGASE showed that milk CaC can vary significantly according to the nature of the diets (Hurtaud *et al*., 2013). The aim of this study was to evaluate the effect of the cow feeding strategy on the annual dynamics of milk CaC content in several areas of France with the 3 main breeds i.e. Holstein, Montbeliarde and Normande. We assumed that cow's diet affects CaC in bovine milk but may not be the only explanation of the seasonal variation of milk CaC content.

**Material and methods**
*Field prospection*
The data were issued from the program PhénoFinLait, which consisted in a survey performed through the major areas of milk production in France. Between October 2009 and October 2010, 945 farms were enquired. During this period, several visits (5 on average) were performed in each farm, to follow evolution of herd diets during a complete year. During each visit, interviewers collected data about dairy cows (parity, stage of lactation, stage of gestation, etc…) and their diet (description of the composition of the diets based on 54 variables). They also collected individual milk samples and MIR spectra were measured for a part of the herd (Foss FTS).Within each enquired farms, interviewers selected as much as possible the same cows, visit after visit. The survey resulted into 252,519 milk spectra from 63,818 dairy cows, divided between the 3 main breeds in France: Holstein, Montbeliarde and

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# Appendix 2

Normande, spread over 13 departments. Program PhénoFinLait has been fully described by Gelé *et al.* (2014).

*Prediction of milk calcium using MIR spectrum*

A prediction equation, specific to our study, was estimated to predict CaC in milk from MIR spectra. To achieve this, milk calcium contents of 495 frozen milk samples taken from the bank of samples of program PhénoFinlait were analyzed by atomic absorption spectrometry after diluting the samples with nitric acid (Brûlé *et al.*, 1974). Those samples were chosen to represent the diversity of the whole bank of samples of program PhénoFinLait (parity, lactation stage, breed, department, cow diet, etc.…). The calibration was performed with a partial least square regression using the PLS procedure of SAS.

*Characterization of feeding strategy*

For each visit in each farm, a mean diet was estimated by averaging the proportions of each feed in the diet. Feeding strategies of farms were characterized over 3 periods: winter season (from 15ᵗʰ November to end of March), early summer season (from 1ˢᵗ April to 15ᵗʰ June), and late summer season (from 16ᵗʰ June to 15ᵗʰ October). Only farms that were investigated every season were used to characterize the feeding strategies. As a feeding strategy consisted in a characterization of the evolution of the diet over the year, each season had to bring the same amount of information. A multiple factor analyses (MFA) was therefore performed to characterize feeding strategies (Escofier and Pagès, 1994) with R (R Core Team, 2013) and package *FactoMineR*. A MFA is a generalization of principal component analysis for comparison of multiple data tables (Abdi *et al.*, 2013). An ascending hierarchical classification was then performed on the factor scores using package *FactoMineR*.

*Factors of variations of milk calcium concentration*

An ANOVA with a mixed model was performed using PROC MIXED with SAS to characterize factors of variations of CaC. The selected model was:

Milk Calcium Content= Month of Lactation + parity + Calendar Month*Feeding Strategy + Individual +ε,

where all explanatory variables were considered as qualitative factors. All explanatory variables were included as fixed factors, excepting "Individual" factor that was a random factor. The model was run independently for each breed. Within breed, some strategies were removed when they had not enough data or when they were unbalanced throughout the year.

**Result and discussion**

Milk CaC was clearly affected by the stage of lactation for the 3 considered breeds (p < 0.05, Figure 1). CaC decreased sharply after the first month and lowest values were observed between 2ⁿᵈ and 5ᵗʰ month of lactation. From the 5th month, CaC increased gradually until the end of lactation. Milk CaC was higher for Normande compared with Montbeliarde or Holstein, with lower range of variation during lactation. Milk CaC was remained higher for Montbeliarde compared with Holstein, with similar dynamics during the lactation.
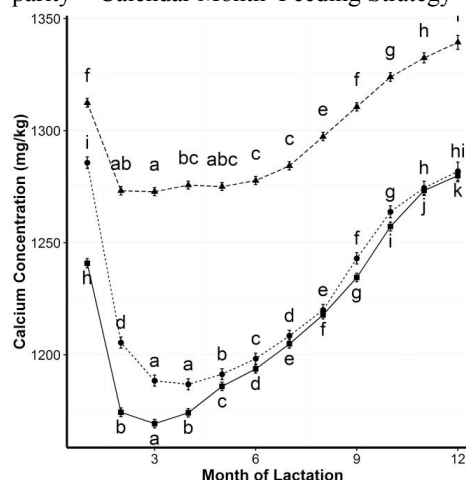


*Figure 2 : Effect of stage of lactation on calcium Concentration*

●*: Montbeliarde,* ▲*: Normande,* ■*: Holstein. Letters are results of post-Hoc and group difference analyses for each breed. (Adjusted means corrected from all effects included in the model except the stage of lactation)*

# Appendix 2

The MFA and classification resulted into 7 feeding strategies: The nature of the forage used was the major factor of strategy characterization. Strategies were named according to the relative importance of the main forages and the distribution of their contribution to the diet during the year (Figure 2). The strategies '*Grazing & FC Hay*' and '*Grazing & BD Hay*' represented feed systems based on grazed pasture during spring and summer and on field cured hay (FC) or barn-dried (BD) hay in winter. The strategy '*Max Grazing*' consisted in a maximal use of grazing in spring and summer, and in diets based on maize silage in winter. The strategy '*Grazing and Maize Silage*' was based on maize silage but with a part of grass when possible. The strategy '*Maize Silage*' was based on this forage for all seasons. The strategy '*Grazed temp pasture*' was based on non-permanent pasture for spring and summer and on maize silage in winter.
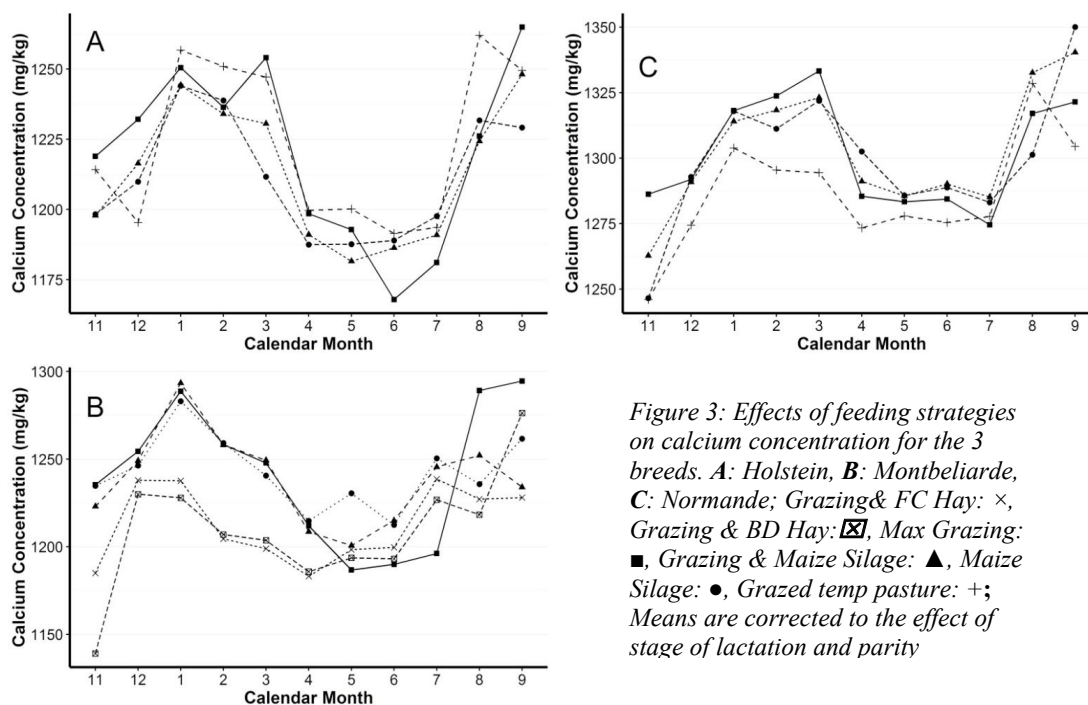
*Figure 2: Evolution of main forages for the 7 feeding strategies during the investigated period; Grazing& FC Hay: ×, Grazing & BD Hay:⊠, Max Grazing: ■, Grazing & Maize Silag : ▲, Maize Silage: ●, Grazed temp pasture:+*

*Figure 3: Effects of feeding strategies on calcium concentration for the 3 breeds. **A**: Holstein, **B**: Montbeliarde, **C**: Normande; Grazing& FC Hay: ×, Grazing & BD Hay:⊠, Max Grazing: ■, Grazing & Maize Silage: ▲, Maize Silage: ●, Grazed temp pasture: +; Means are corrected to the effect of stage of lactation and parity*

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# Appendix 2

When performing the analyses of the effect the feeding strategies on the milk CaC, the data of strategies under-represented, i.e. less than 2,000 data within strategy and breed, or unevenly distributed throughout the year had to be removed for some breed. The strategies '*Grazing & FC hay*' and '*Grazing & BD Hay*' were only kept for Montbeliarde because they were underrepresented within Hostein and Normande. Indeed, those 2 feeding strategies were very specific to the areas of production where Montbeliarde is the predominant breed. Similarly, the strategy '*Grazed temp pasture*' was under represented within Montbeliard breed because it was very specific to the west of France where Montbeliarde is less represented.

The feeding strategy clearly affected the annual dynamics of milk CaC (Figure 3; effect of the interaction calendar month x feeding strategy: $p < 0.05$). CaC in milk has a huge drop in spring for each breed, i.e. between March and May. This could be related to the fact that for most of strategies, cows started grazing at this period. For Holstein, considering the summer months, CaC was higher for strategies based on higher proportion of maize silage. For Montbeliarde, the difference between strategies was more marked in winter, with higher CaC with strategies based on maize silage, and lower CaC with those based on hay. The difference in CaC between both types of strategies was high up to 100 mg/kg more calcium in milk obtained with strategies based on maize silage rather than hay (Graph B, Figure 3). For Normande, the strategy effect was less clear that for the 2 others breeds.

When considering the 3 breeds, the variability linked to the diet was important, with about 150 mg/kg of difference over the year between strategies. This difference was comparable to the maximal difference in CaC between the 3 breeds within a same feeding strategy and a similar stage of lactation.

**Conclusions**

This study clearly showed that the feeding strategy of the dairy system affected the annual variation of milk CaC. Variations in CaC seemed to be, at least partially, explained by the nature of the forages fed to the cows. Maize silage led to higher CaC in milk, in comparison with grass or hay. This was observable when comparing the strategies within breed and when considering the drop of CaC observed when cows started grazing in spring. The nature of the forage fed to the cows seems to be a factor explaining an important part of the variability of CaC, comparable to the variability explained by breed.

**Acknowledgements**

**References**

Abdi H., Williams L.J., Valentin D. 2013. Wiley Interdiscip. Rev. Comput. Stat. 5: 149-179.
Alais C. *Science du lait : principes des techniques laitières*.1984. Edition SEPAIC.
Brûlé G., Maubois J.L., Fauquant J. 1974. 54: 600-615. Escofier, B., and J. Pagès. 1994. Comput. Stat. Data Anal. 18: 121-140.
Gelé M., Minery S., Astruc J.-M., Brunschwig P., Ferrand-Calmels M., Lagriffoul G., Larroque H., Legarto J., Leray O., Martin P., Miranda G., Palhiere I., Trossat P., Brochard M. 2014. INRA Prod. Anim., 27: 255-268.
Van Hulzen K.J.E., R.C. Sprong, R. van der Meer, J. a. M. van Arendonk. 2009. J. Dairy Sci., 92:5754-5759.
Hurtaud C., M. Dutreuil, M. Coppa, C. Agabriel, B. Martin. 2013. Dairy Sci. Technol., 94: 103-123.
Suttle N.F. *Mineral Nutrition of Livestock*. 2010. CABI.

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# Appendix 3

## ETUDE DE L'EFFET DE LA PARITÉ ET DE LA VARIABILITÉ INDIVIDUELLE SUR LES DYNAMIQUES DE MOBILISATION ET DE RECONSTITUTION OSSEUSE AU COURS DE LA LACTATION EN RELATION AVEC LA TENEUR EN CALCIUM DU LAIT

### Contexte

PIERRE GAIGNON (1), PHILIPPE FAVERDIN (1), DAVID SIDANER (1), CATHERINE HURTAUD (1), ANNE BOUDON (1)

En début de lactation, la vache laitière subit une forte augmentation de ses besoins en Ca, nécessitant une adaptation physiologique notamment par la mobilisation de ses réserves osseuses qui seront reconstituées plus tard dans la lactation. Les méthodes actuelles de suivi de la mobilisation/reconstitution osseuse sont difficiles à mettre en œuvre sur de gros effectifs d'animaux. Il a été démontré chez la souris que la glande mammaire peut répondre à une chute de la calcémie, en permettant une baisse de la teneur en Ca dans du lait et une augmentation de la résorption osseuse.

### Objectif

Analyser les effets de la parité et de la variabilité individuelle sur la dynamique de mobilisation osseuse au cours de la lactation et les liens entre les dynamiques de mobilisation osseuse et de teneur en calcium du lait.
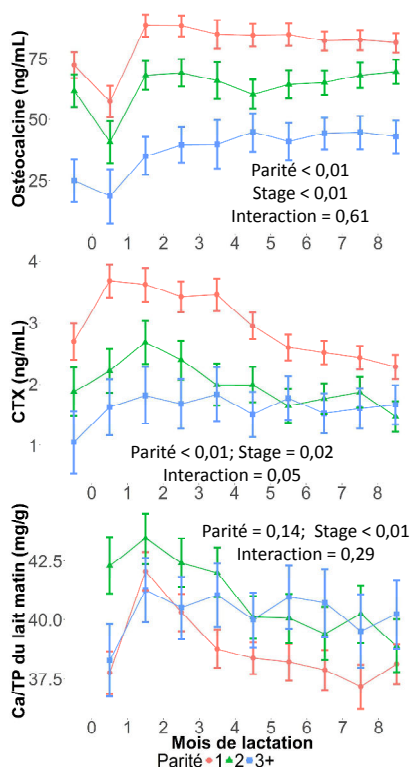
### Matériel et méthodes

- **33 vaches laitières Holstein, suivies sur toute la lactation**. Une ration unique prévue pour couvrir les besoins en UF, PDI, P et Ca de milieu de lactation offerte *ad libitum*.
- **Prélèvements** de plasma à jeun le matin et de lait lors de la traite du matin et du soir, 15 jours avant vêlage, 15 jours après vêlage puis toutes les 4 semaines.
- **Analyses** des teneurs plasmatiques en ostéocalcine (accrétion osseuse), CTX (résorption osseuse), Ca et P, et des teneurs en Ca et P du lait

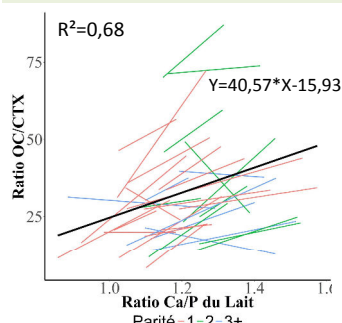ANOVA : $Y = \mu + Parité + Stade\ de\ lactation + Interaction + \varepsilon$

### Résultats

- **Teneurs plasmatiques en OC et CTX plus élevées chez les primipares que les multipares.**
  => plus fort remodelage osseux (Fig 1)

- **Plus forte augmentation de la teneur plasmatique en CTX lors de la première moitié de lactation chez les primipares.**
  => plus de mobilisation osseuse (Fig 1)

- Ratio Ca/protéines du lait affecté par le stade, mais pas par la parité ni par l'interaction (Fig 1).

- **Pas de lien en inter-individu entre la dynamique du ratio Ca/protéines du lait (matin ou soir) et celle des biomarqueurs de l'os au cours de la lactation** (Fig 1).

- **Une relation en intra-individu entre les ratios OC/CTX plasmatique et Ca/P du lait du matin (Fig 2).** Néanmoins ce dernier ne permet pas de prédire la plus forte mobilisation osseuse des primipares (interaction stade * parité, NS).



**Figure 1 –** Effets de la parité et du stade de lactation sur les teneurs plasmatiques en OC et CTX et sur le ratio Ca/TP du lait matin

**Figure 2 –** Relation intra-individu entre le ratio des biomarqueurs plasmatiques de l'os et le ratio Ca/P du lait

### Conclusion

Cette étude confirme **un plus fort remodelage osseux chez les animaux les plus jeunes**. Elle démontre aussi pour la première fois chez les vaches laitières **une plus forte mobilisation osseuse en début de lactation chez les primipares** par rapport aux multipares. Ces effets de la parité ne sont pas traduits par des variations différentes entre parité de la teneur en Ca/protéines ou en Ca/P du lait suggérant que ces ratios ne peuvent pas être de bons indicateurs de la dynamique de mobilisation osseuse à l'échelle de la lactation. De ce fait, des études visant à caractériser l'influence des cycles de mobilisation/reconstitution osseuse des vaches sur leur longévité restent encore difficiles à envisager à ce jour.

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# Appendix 4

## EFFECT OF PARITY ON DYNAMICS OF MILK CALCIUM (Ca) CONTENT & BLOOD BIOMARKERS OF BONE ACCRETION AND RESORPTION THROUGHOUT LACTATION IN DAIRY COWS

GAIGNON P.[1] , FAVERDIN P.[1] , SIDANER D.[1] , LAZA-KNOERR A.[2] , LEGRAND K.[2] ,HURTAUD C.[1] , BOUDON A.[1]
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### Context

At the beginning of lactation, dairy cows face a huge increase in their Ca requirements due to the important increase in their milk production. Consequently, they mobilize Ca from their bone and Ca reserves in bone can be restored later during lactation. The amplitude of the cycles of bone mobilization/reconstitution are difficult to quantify on important number of cows. For that reason, its consequences on cows' health remain to be determined.

### Objective

= to determine if a link could exist between individual dynamics of milk Ca content and amplitude of cycles of bone mobilization/reconstitution. The existence of such a link could constitute a way to indirectly and quickly quantify the amplitude of the cycles of bone mobilization/reconstitution during lactation on important number of cows.
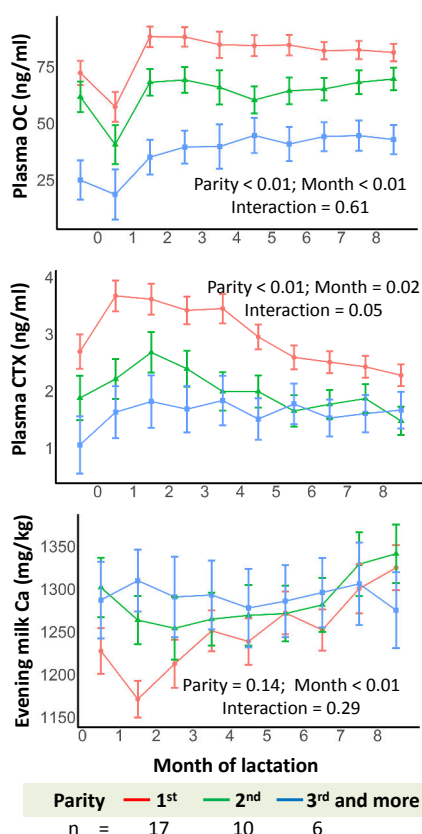


**Fig 1** – Effects of parity and month of lactation on plasma OC and CTX and milk Ca
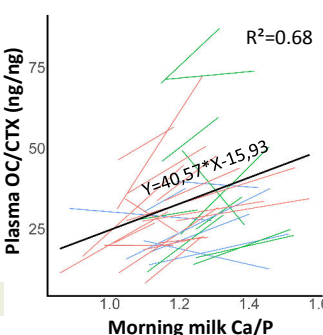


**Fig 2** – Within cow relationship between plasma OC/CTX ratio and milk Ca/P ratio

### Material & methods

- **33 Holstein dairy cows / a unique total mixed ration** (*ad libitum*, calculated to cover nutritional requirements at middle of lactation).
- **Sampling**: **Blood,** before morning feeding, in the caudal vein. **Milk** at each milking. **15 d before calving, 15 d after calving and every months after**.
- **Analyses**: plasma concentrations of **osteocalcin** (**OC, blood biomarker of bone accretion**), **CTX (blood biomarker of bone resorption)**, Ca and Pi, **milk contents of Ca and P**. ANOVA : $Y = \mu + Parity + Month of lactation + Interaction + \varepsilon$, cows as random, repeated measurements.

### Results

- A tendency for higher plasma Ca in primiparous cows ($P = 0.09$, data not shown)

- **Both plasma OC and CTX higher in young cows** (Fig 1) => higher bone remodeling

- **Higher increase in plasma CTX during the 1st half of lactation in young cows, no effect of parity on OC dynamics (Fig 1)** => more bone mobilization in young cows ?

- **Decrease in milk Ca for primiparous cows at the beginning of lactation (Fig 1)**=> a possible role of the mammary gland parallel to that of the bone for plasma Ca regulation ?

- Between cows, no link between the dynamics of milk Ca during lactation and that of CTX and OC (data not shown).

- Within cows, a positive relationship between plasma OC/CTX and milk Ca/P **(Fig 2). =>** maybe milk Ca/P could be an indicator of the equilibrium between bone accretion & resorption during lactation ?

### Conclusion

This study confirms **a higher bone remodeling in younger cows**. It also suggests **a higher bone mobilization at the beginning of lactation with primiparous compared with multiparous cows** accompanied by **a concomitant higher decrease of milk Ca**.

Dynamics of milk Ca/ P ratio may be a gross predictor of plasma OC to CTX ratio and thus of the dynamics of the equilibrium between bone accretion and resorption throughout lactation but this requires confirmation in experiments with higher variability of those dynamics. Milk Ca alone cannot be used as a predictor of bone resorption.

# Appendix 5

**Effet d'une restriction des apports en calcium en début de lactation sur la production laitière, la composition du lait et les dynamiques de mobilisation et de reconstitution osseuses au cours de la lactation chez la vache laitière**

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**RESUME –** Les objectifs de cet essai étaient d'évaluer les conséquences d'une augmentation de la mobilisation osseuse de vache laitière en début de lactation sur les teneurs en Ca et en P du lait et la dynamique de reconstitution osseuse en fin de lactation. Quinze vaches laitières Holstein multipares ont été réparties en trois lots différents 5 semaines avant la date planifiée de vêlage. Chaque lot recevait un traitement différent, consistant à différentier l'alimentation des vaches entre 5 jours et 10 semaines de lactation. Le traitement témoin (TEM) consistait en une ration couvrant 100 % des besoins en Ca, les traitements BCa et BCaBE consistaient en des rations couvrant 70% des besoins en Ca, le bilan alimentaire cation-anion étant de 200 mEq/Kg MS pour TEM et BCa, et 0 pour BCaBE. Les traitements BCa et BCaBE ont eu tendance à diminuer légèrement la rétention corporelle de Ca à 3 semaines de lactation (P< 0.09) par rapport au traitement TEM mais n'ont eu d'effet ni sur la dynamique des teneurs en biomarqueurs d'accrétion et de résorption osseuse au cours des 32 semaines de lactation, ni sur la rétention corporelle du Ca à 17 semaines de lactation. Les vaches ont presque entièrement compensé la baisse des apports en Ca par une élévation de l'absorption apparente du Ca à 3 semaines de lactation (P = 0.03). Les traitements n'ont pas eu d'effet sur la composition du lait. Par contre la production laitière a eu tendance (P = 0.09) à être plus faible sur l'ensemble de la lactation avec les régimes BCa et BCaBE par rapport à TEM, avec une différence moyenne de 2 kg/j.

## Effect of low Ca intake in early lactation on milk production, milk quality and dynamics of bone mobilization and resorption throughout lactation in dairy cows

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**SUMMARY –** The objective of this experiment was to evaluate the consequences of an increase of bone mobilization in early lactation, on the milk contents of Ca and P and the bone reconstitution dynamics in late lactation. Fifteen multiparous Holstein cows have been spread between three treatments 5 weeks before the expected calving date. Those treatments consisted in differentiating the cow's diets between 5 days and 10 weeks of lactation. The control treatment (TEM) consisted in a diet covering 100% of the Ca requirements, the treatments BCa and BCaBE consisted in a diet covering 70% of the Ca requirements, the dietary anion-cation difference was 200 mEq/Kg DM for TEM and BCa and 0 for BCaBE. The treatments BCa and BCaBE induced a small decrease of the body retention of Ca at 3 weeks of lactation compared with the treatment TEM (P< 0.09) but did not affect either the dynamics of blood biomarkers of bone accretion and resorption during the 32 weeks of lactation or the body retention of Ca at 17 weeks of lactation. Cows almost entirely compensated the decrease of Ca supply in BCa and BCaBE treatments by an increase of the apparent absorption of Ca at 3 weeks of lactation (P = 0.03). Treatments did not clearly affect either the milk composition. Nevertheless, milk yield tended (P = 0.09) to be lower throughout the lactation with treatments BCa and BCaBE compared with TEM with a mean difference of 2 kg/d between TEM treatment and BCa and BCaBE treatments.

### Introduction

L'organisme des vaches laitières fait face à un important flux d'excrétion de Ca pendant la lactation en raison de la teneur élevée du lait en Ca (Horst et al., 1997). Ceci explique l'existence de cycles de mobilisation osseuse et de reconstitution pendant la lactation afin de maintenir les teneurs plasmatiques en Ca dans des limites physiologiques (Braithwaite, 1983; Taylor et al., 2009; Elizondo-Salazar et al., 2013). Des questions demeurent quant aux conséquences de l'amplitude et de la complétude de ces cycles sur la santé et la productivité des vaches. On peut suspecter à partir de la littérature qu'une reconstitution osseuse incomplète à la fin de la lactation puisse entraîner une sensibilité plus grande des vaches à un apport limité en P lors de la lactation suivante avec des performances de production sous-optimales (Dixon et al., 2017) ou une sensibilité accrue à la fièvre vitulaire au début de la lactation suivante (McNeill et al. 2002). La confirmation de ces soupçons aurait des conséquences sur la définition des besoins en Ca et en P de ces animaux. Les recommandations actuelles reposent sur le principe que les excrétions quotidiennes de Ca et de P permettent un certain niveau de production avec des pertes fécales et urinaires minimales qui doivent être remplacées par une ingestion

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équivalente quotidienne de ces éléments. Ce principe ne considère pas que la mobilisation osseuse en début de lactation et la reconstitution en fin de lactation puissent constituer un apport ou un besoin supplémentaire.

Le nombre d'expériences publiées reste trop limité pour permettre de définir une stratégie optimale de supplémentation en Ca et en P à l'échelle de la lactation (AFRC, 1991; NRC, 2001; INRA, 2010). Le manque de méthodes rapides et peu onéreuses pour évaluer l'amplitude et éventuellement la complétude des cycles de mobilisation et de reconstitution osseuses lors de la lactation et de la gestation des vaches constitue une limite majeure pour répondre à ces questions. VanHouten et al. (2004) ont montré qu'une diminution de l'apport en Ca induisait chez les mammifères une excrétion plus faible de Ca dans le lait et une résorption osseuse plus élevée, régulées par le récepteur CaSR dans la glande mammaire. Le suivi de la teneur en lait du calcium pendant la lactation pourrait donc être un moyen peu coûteux d'estimer indirectement la dynamique de la résorption osseuse.

L'objectif de cette expérience était d'induire une mobilisation osseuse chez la vache laitière grâce à des traitements alimentaires et d'en déterminer les conséquences sur (1) la dynamique des teneurs en Ca et en P du lait, des concentrations sanguines en biomarqueurs de l'accrétion et de la résorption osseuse et des rétentions corporelles en Ca et P, (2) sur la dynamique de reconstitution osseuse en fin de lactation. La mobilisation osseuse devait être induite soit par une restriction des apports alimentaires en Ca par rapport aux recommandations françaises (INRA, 2010) soit par une restriction des apports alimentaires en Ca couplée à une diminution du bilan alimentaire cation anion (BACA).

## 1. MATERIEL ET METHODES
### 1.1 ANIMAUX ET SCHEMA EXPERIMENTAL
Les traitements ont consisté en trois supplémentations minérales distribuées en complément d'une même ration de base entre 5 jours et 10 semaines de lactation. La teneur en Ca de la ration était de 8,3 g/kg MS pour le traitement TEM pour un BACA de 219 mEq/kg de MS, de 6,0 g/kg MS pour un BACA de 279 mEq/kg de MS pour le traitement BCa et de 5,8 g/kg MS pour un BACA de 0 mEq/kg de MS pour le traitement BCaBE. Quinze vaches Holstein multipares ont été réparties en trois groupes et suivies depuis 5 semaines avant leur date de vêlage prévue, jusqu'à 31 semaines de lactation selon un schéma expérimental totalement randomisé, les groupes assignés à chaque traitement étant homogènes en terme de parité moyenne, de production laitière sur la lactation précédente et de date attendue de vêlage. Les vaches étaient logées en stabulation libre pendant l'essai, à l'exception des périodes de mesure des rétentions en Ca et P pendant lesquelles elles étaient maintenues en stabulation entravée pendant 3 semaines. Elles étaient traites deux fois par jour. La production laitière était enregistrée tous les jours et les taux protéiques et butyreux du lait étaient mesurés deux fois par semaine.

### 1.2 ALIMENTATION
L'alimentation des vaches selon les traitements a été différenciée entre 5 jours et 10 semaines de lactation. Pendant les 3 semaines qui précédaient le vêlage, toutes les vaches ont reçu la même ration de préparation au vêlage et pendant les 5 premiers jours de lactation, elles ont reçu la

ration du traitement TEM. Les rations ont été formulées pour couvrir les besoins des vaches laitières selon les recommandations, exceptés pour le Ca et la BACA (INRA, 2010). La ration de base jusqu'à 10 semaines de lactation se composait de 72 % MS d'ensilage de maïs, 16 % MS de concentré énergétique, 11 % MS de tourteaux tannés et 1 % MS d'urée. Au bout de 10 semaines, toutes les vaches recevaient une même ration couvrant 100 % de leurs besoins en Ca avec un BACA positif, composée de 74% MS d'ensilage de maïs, 11% MS de concentré énergétique, 14 % MS de tourteau de soja 48 et moins d'un pourcent d'urée, hors aliments minéraux. L'apport d'aliment minéraux était de 3,5, 2.8 et 3,4% MS pour les traitements TEM, BCa et BCaBE, le Ca étant avant tout apporté par du carbonate de Ca. Pendant la lactation, les vaches étaient nourries *ad libitum*.

**Tableau 1 -** Caractéristiques des rations

|  | 5 – 70 jours de lactation | | | Après 70 jours |
|---|---|---|---|---|
|  | TEM | BCa | BCaBE |  |
| Ca[1] | 8,3 | 5,9 | 5,8 | 7,8 |
| Ca$_{abs}$[1] | 3,4 | 2,4 | 2,4 | 3,1 |
| P[1] | 4,1 | 4,0 | 3,9 | 4,0 |
| P$_{abs}$[1] | 2,8 | 2,7 | 2,7 | 2,8 |
| PDIE/UFL[2] | 115 | 115 | 115 | 95 |
| PDIN/UFL[2] | 116 | 116 | 116 | 86 |
| BACA[3] | 219 | 279 | 0 | 220 |

[1] : g/kg de MS
[2] : g/UFL
[3] :BACA = Na +K –Cl -S (où Na, K, Cl et S sont les teneurs de la ration en ces éléments exprimées en mEq/kg MS)

### 1.3 PRELEVEMENTS ET ANALYSES
Neuf prélèvements de sang ont été réalisés entre 3 semaines avant la date estimée de vêlage et 31 semaines de lactation afin de suivre les évolutions des teneurs plasmatiques en Ca, P inorganique (Pi), ostéocalcine (OC, biomarqueur de l'accrétion osseuse) et télopeptide C-terminal du collagène de type I (CTX, biomarqueur de la résorption osseuse). Quatorze prélèvements de lait ont été réalisés entre 1 et 31 semaines après vêlage pour suivre les teneurs en Ca et P, à la traite du matin et du soir.

Trois semaines avant vêlage et à 3 et 17 semaines de lactation, les vaches ont été placées en stalle entravée pendant 3 semaines afin de mesurer l'ingestion et l'excrétion journalières de Ca et de P sur 4 jours par collecte complète de l'urine et des fèces.

### 1.5 ANALYSES STATISTIQUES
Un modèle linéaire généralisé a été utilisé à l'aide de la PROC GLIMMIX de SAS. Il comportait les effets des régimes et du stade de lactation :

$$Y_{ijk} = Régime_i + Stade\ de\ lactation_j + Régime{:}Stade_{ij} + Vache_{k(i)}$$

où *Y* représente la variable réponse d'intérêt. La vache était incluse en effet aléatoire et une matrice de covariance entre les stades de lactation a été choisie sur un critère d'AIC.

## 2 RESULTATS
### 2.1 DES APPORTS EN Ca EN DEBUT DE LACTATION CONFORMES A CE QUI ETAIT ATTENDU
Les apports alimentaires de Ca ont été moins importants avec les traitements BCa et BCaBE par rapport à TEM entre

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5 jours et 10 semaines de lactation et n'ont pas été affectés par les traitements conformément à ce qui était attendu (Figure 1). La matière sèche ingérée a été un peu plus élevée avec le traitement BCa par rapport aux deux autres à partir de 7 semaines de lactation (+ 1,0 kg en moyenne, P<0,01). Cette différence est attribuable en partie à des vaches un peu plus lourdes sur ce traitement (623 kg pour TEM, 704 kg pour BCa et 667 kg pour BCaBE).

**Figure 1 :** Effets des traitements sur l'ingestion de Ca et de MS au cours de la lactation



Traitement —— TEM – – BCa ···· BCaBE
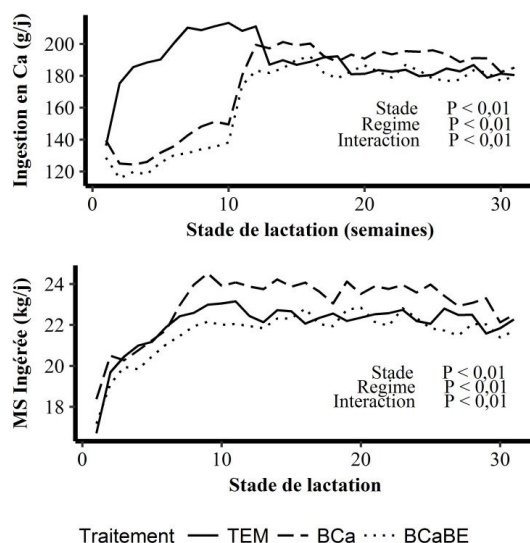
## 2.2 PEU D'EFFETS DES TRAITEMENTS SUR LA MOBILISATION OSSEUSE EN DEBUT DE LACTATION

Les dynamiques des teneurs plasmatiques en OC et CTX n'ont été affectées ni par les traitements, ni par l'interaction entre les traitements et le stade de lactation (P > 0,30 ; Figure 2). L'accrétion osseuse (OC) a chuté après le vêlage et a augmenté au cours de la lactation, fortement jusqu'à 8 semaines de lactation et plus lentement ensuite (P < 0,01). La résorption osseuse (CTX) a augmenté pour ensuite diminuer continuellement (P < 0,01). La rétention corporelle du Ca, c'est-à-dire la différence entre l'ingestion du Ca et son excrétion, qui peut dans le cas du Ca être estimée comme l'opposé de la mobilisation osseuse, a eu tendance à être plus faible avec les traitements BCa et BCaBE par rapport à TEM à 3 semaines de lactation (-2, 0 et 8 g/j, pour les traitements BCa, BCaBE et TEM, P = 0,09, Figure 3) mais n'a pas été affectée par les traitements à 17 semaines (P > 0,70). Les traitements n'ont eu aucun effet sur les teneurs en Ca plasmatique (P = 0,60). Une seule vache a été en hypocalcémie après le vêlage (76 mg/L).

## 2.3 UNE CAPACITE D'ABSORPTION DIGESTIVE DU Ca AUGMENTEE AVEC LES TRAITEMENTS BCa ET BCaBE

Le coefficient d'absorption apparent (CAA) du Ca a été nettement plus élevé pour les traitements BCa et BCaBE que TEM à 3 semaines de lactation (P = 0,03, Figure 3) alors qu'il n'était pas affecté par les traitements aux autres stades (P > 0,54). Le CAA dépassait 37 % pour ces deux traitements alors qu'il était de 30 % pour TEM. Le CAA du Ca a été plus élevé en lactation, à 3 ou 17 semaines de lactation, qu'avant vêlage où il n'était que de 21 % en moyenne (P < 0,01). Le

flux journalier d'absorption apparente de Ca a été de 56,9, 48,1 et 51,5 g/j pour les traitements TEM, BCa et BCaBE respectivement (P = 0,31) à trois semaines après vêlage.

**Figure 2 :** Effets des traitements sur les teneurs plasmatiques en OC (biomarqueur de l'accrétion osseuse) et en CTX (biomarqueur de la résorption)



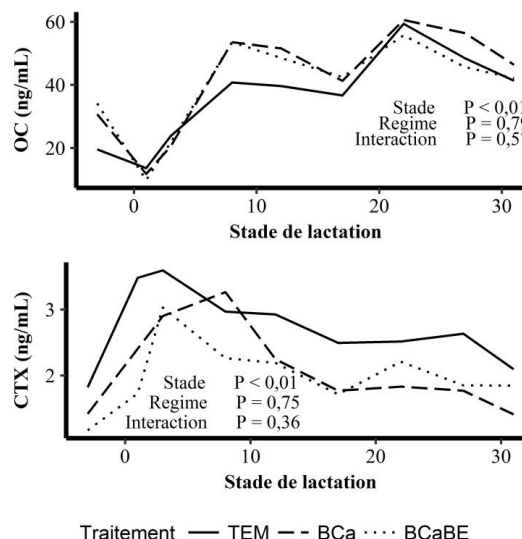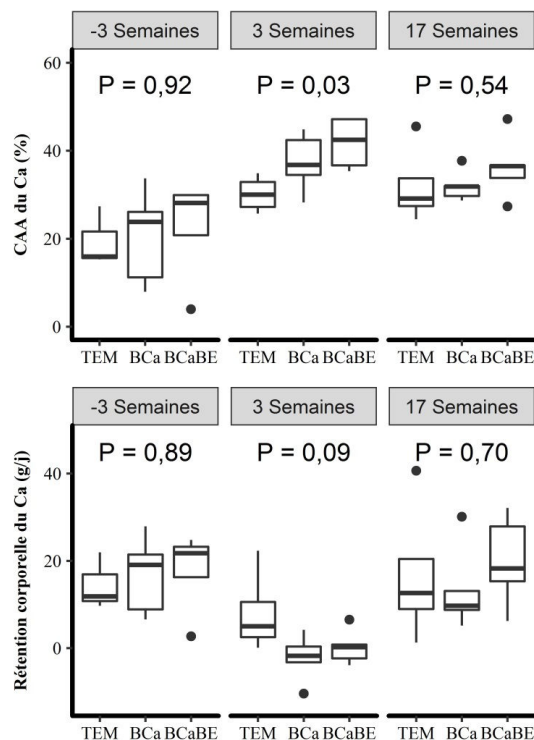Traitement —— TEM – – BCa ···· BCaBE

**Figure 3 :** Effets des traitements sur la digestibilité apparente du Ca et la rétention en Ca 3 semaines avant la date de vêlage à 3 et 17 semaines de lactation.

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## 2.4 PAS D'EFFET DES TRAITEMENTS SUR LA TENEUR EN Ca DU LAIT MAIS UN POSSIBLE EFFET SUR LA PRODUCTION LAITIERE

La teneur en Ca du lait n'a pas été plus faible pour les traitements BCa et BCaBE par rapport à TEM (P = 0,40, Figure 4). Elle était même plus élevée en fin de lactation (interaction traitement × stade de lactation, P < 0,01 le matin). La production laitière a eu tendance à être plus faible sur toute la lactation avec les traitements BCa et BCaBE par rapport au traitement TEM (P = 0,09, Figure 4), avec un écart moyen de 2 kg/j, qui a atteint plus de 5 kg au moment du pic de lactation.

**Figure 4 :** Effet des traitements sur la teneur en Ca du lait et la production laitière
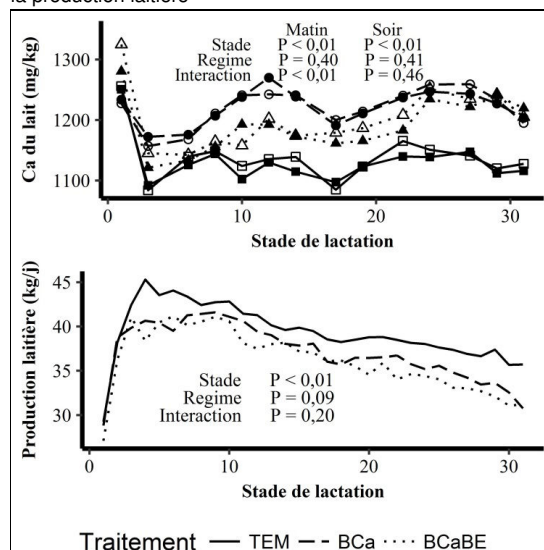


## 3. DISCUSSION

### 3.1 UNE RELATIVE ABSENCE D'EFFET D'UN APPORT RESTREINT EN Ca EN DEBUT DE LACTATION SUR LA MOBILISATION OSSEUSE INATTENDUE

L'objectif des traitements BCa et BCaBE était d'induire une mobilisation osseuse accrue au cours des 10 premières semaines de lactation. Avec une restriction des apports en Ca comparable à celle que nous avons pratiquée dans l'essai, certaines études ont mis en évidence une diminution claire de la rétention corporelle de Ca au début de la lactation chez la vache laitière, cette dernière atteignant des valeurs clairement négatives (Taylor et al., 2009 ; Wholt, 1986) ou une augmentation de la concentration sérique de pyridinoline qui est un biomarqueur de la résorption osseuse (Moreira et al., 2009). Pour augmenter les chances d'induire une mobilisation osseuse dans notre expérience, le BACA a également été diminué pour le traitement BCaBE à une valeur proche de 0, qui est la limite maximale en dessous de laquelle on pouvait attendre à un effet positif sur la mobilisation osseuse (Charbonneau et al., 2006). Pourtant, nous n'avons obtenu que des effets ténus des traitements.

La dynamique des biomarqueurs osseux sanguins au cours de la lactation que nous avons observée était cohérente avec celles précédemment observées (Liesegang et al., 2000; Ekelund et al., 2006; Puggaard et al., 2014) avec une forte diminution de l'OC au vêlage et une augmentation du CTX en début de lactation. Ces résultats ont été cohérents avec les

mesures de rétention du Ca montrant une rétention de Ca plus faible en début de lactation comparée à celle mesurée avant le vêlage ou à 17 semaines de lactation, illustrant une reconstitution osseuse nette à ces moments.

## 3.2 UNE AUGMENTATION PLUS IMPORTANTE QUE PREVUE DU CAA DU Ca AVEC UN APPORT RESTREINT EN Ca EN DEBUT DE LACTATION

Dans notre expérience, l'évolution de la calcémie au cours du cycle lactation-gestation n'a pas été affectée par l'apport alimentaire en Ca. Ceci suggère que, si la mobilisation osseuse n'était pas le principal effecteur mobilisé pour la régulation de la calcémie lorsque l'apport de Ca a été réduit, d'autres flux de Ca doivent avoir permis cette régulation. Nos résultats ont clairement montré que la diminution de la consommation de Ca avec le traitement BCa et BCaBE a été presque entièrement compensée au niveau de l'organisme par une diminution équivalente de la quantité de Ca excrétée dans les fèces, avec un CAA du Ca particulièrement élevé (moyenne supérieure à 40 % pour BCaBE). Ces résultats contrastent avec ceux de Taylor et al. (2009) et Moreira et al. (2009) qui ont observé une digestibilité apparente plus faible du Ca à un stade similaire de lactation. Une explication est peut-être qu'une proportion significative de Ca alimentaire a été fournie par de l'ensilage de maïs ou du foin de luzerne dans ces études alors qu'il était principalement fourni par un aliment minéral dans notre essai. On sait que le Ca de la luzerne est moins disponible pour l'absorption digestive chez les ruminants (INRA, 2010). Il est probable que ces auteurs n'ont pas pu observer une augmentation du CAA autour de 3 semaines de lactation avec un faible apport en Ca, contrairement à nous, car les vaches ne pouvaient pas augmenter leur absorption apparente de Ca en raison de la faible disponibilité en Ca alimentaire. Dans notre expérience, les vaches ont peut-être privilégié une augmentation de l'absorption digestive plutôt qu'une mobilisation du Ca de l'os pour réguler la calcémie, car le Ca alimentaire était plus disponible pour l'absorption. Cette hypothèse nécessiterait une confirmation.

Nous avons clairement observé un effet important du stade physiologique des vaches sur le CAA du Ca. L'augmentation de la capacité d'absorption du Ca par le tube digestif entre la gestation et la lactation est cohérente avec l'augmentation de la libération de PTH et de la synthèse de 1,25-(OH)$_2$D$_3$ au début de la lactation (Horst et al., 2005).

### 3.4 RELATION ENTRE LA TENEUR EN Ca DU LAIT ET LA DYNAMIQUE DE MOBILISATION OSSEUSE AU COURS DE LA LACTATION

Notre hypothèse était que le faible apport en Ca induirait à la fois une augmentation de la résorption osseuse et une diminution de la sécrétion de Ca dans le lait. Cependant, le faible apport en Ca dans notre expérience n'a eu qu'un effet limité sur la mobilisation osseuse au début de la lactation. Cette expérience n'a donc pas permis de tester complètement notre hypothèse. Les vaches ayant reçu les traitements BCa et BCaBE ont eu tendance à avoir une teneur en Ca dans le lait plus élevée après 10 semaines de lactation, ce qui n'était pas conforme à notre hypothèse pour deux raisons. Tout d'abord, cette différence est apparue lorsque les rations ont cessé d'être différenciées en fonction du traitement. Deuxièmement, une teneur plus faible de Ca

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dans le lait était attendue pour ces traitements. Comme on sait que la génétique de la vache est un déterminant majeur de la teneur en Ca du lait chez les vaches (Van Hulzen et al., 2009) et que la teneur en Ca du lait n'a pas été mesurée lors de la lactation précédente, il ne peut être exclu que les vaches des traitements BCa et BCaBE avaient des teneurs en Ca de lait plus élevées en raison de leur génétique, à l'origine des teneurs en Ca du lait plus élevées pour ces traitements.

### 3.3 UN EFFET POSSIBLE DE L'APPORT LIMITE EN Ca SUR LA PRODUCTION DE LAIT ET LA LONGEVITE DES VACHES ?

L'écart de 2 kg/j de production laitière avec les traitements BCa et BCaBE par rapport à TEM est un résultat inattendu qui ne peut pas être attribué aux caractéristiques pré-expérimentales des vaches. Cet écart est apparu environ deux semaines après la différenciation des rations en fonction du traitement et a duré jusqu'à la fin de l'expérience, c'est-à-dire largement après 10 semaines de lactation correspondant au moment à partir duquel toutes les vaches ont reçu le même régime. On peut imaginer que les traitements BCa et BCaBE ont pu altérer le potentiel de sécrétion de la glande mammaire au pic de lactation, en modifiant soit la prolifération des cellules épithéliales mammaires, soit leur exfoliation. Wohlt et al. (1986) ont également observé une diminution de la production de lait chez les vaches ayant un apport plus faible en Ca dans des proportions comparables aux nôtres mais ni Taylor et al. (2009), ni Moreira et al. (2009) n'ont observé de telles diminutions. Il est possible que le fait d'avoir utilisé une ration riche en PDI, avec du tourteau de soja tanné, chez des vaches multipares, ait maximisé le potentiel laitier, le Ca étant devenu alors un facteur limitant.

Une autre observation inattendue dans notre expérience a été que le taux de réforme avant le prochain vêlage a été numériquement nettement plus élevé avec les traitements BCa et BCaBE par rapport au traitement TEM. Avec le traitement BCa, 3 des 5 vaches ont été réformées avant la lactation suivante, une en raison de l'absence de détection d'œstrus, deux en raison de problèmes de pieds. Avec le traitement BCaBE, 1 des 5 vaches a été éliminée en raison d'échecs à l'insémination. Aucune vache n'a été réformée avec le traitement TEM. Nos résultats suggèrent qu'une restriction des apports de Ca pendant les premières semaines de lactation pourrait avoir eu un effet néfaste sur la reproduction et la santé des vaches, mais cela reste à démontrer avec plus d'animaux.

### CONCLUSION

Un apport alimentaire de Ca réduit de 30 % par rapport aux recommandations en début de lactation n'a induit que de faibles différences de mobilisation osseuse au cours de la lactation car il a été compensé par une augmentation de la capacité d'absorption du Ca par le tube digestif. Ce résultat, un peu en contradiction avec la bibliographie est peut-être à mettre en relation avec des apports en Ca en grande partie assurés par un aliment minéral et donc relativement absorbables. Un effet inattendu de cet apport réduit en Ca a été une altération du potentiel laitier des vaches et une suspicion de moindre performance de reproduction. Ces résultats nécessitent néanmoins d'être confirmés. Cet essai ne permet de mettre en relation l'évolution de la teneur en Ca du lait au cours de la lactation avec celle de la résorption osseuse.

**AFRC, 1991,** Nutrition Abstract and Reviews, 61, 573-612

**BRAITHWAITE, G.D., 1983,** J Mammary Gland Biol Neoplasia, 50, 711-722

**CHARBONNEAU, E., PELLERIN, D., OETZEL, G.G., 2006**, J. Dairy. Sci, 89, 537-548

**ELIZONDO SALAZAR, J.A.A, FERDUSON, J.D., BEEGLE, D.B., REMSBURG, D.W., WU, Z., 2013**, J Anim Physiol Anim Nutr (Berl), 97, 502-514

**DIXON, R.M., KIDD, L.J., COATES, D.B., ANDERSON, S.T., BENVENUTTI, M.A., FLETCHER, M.T., MCNEILL, D.M., 2017**, Anim. Prod. Sci., 57, 2280-2290

**EKELUND, A., SPORNDLY, R, HOLTENIUS, K, 2006**, Livest Sci, 9, 227-236

**HORST, R.L., GOFF, J.P., REINHARDT, T.A.A, 1997**, J Mammary Gland Biol Neoplasia, 2,253-263

**HORST, R.L., GOFF, J.P., REINHARDT, T.A.A, 2005**, J Mammary Gland Biol Neoplasia, 10, 141-156

**INRA, 2010**, Editions Quae, Alimentation des bovins, ovins et caprins : besoins des animaux, valeurs des aliments : tables Inra 2010, Paris, France

**LIESEGANG, A., EICHER, R., SASSI, M.L., RISTELI, J., KRAENZLIN, M., RIOND, J.L., WANNER, M., 2000**, J. Dairy Sci., 83, 1773-1781

**MCNEILL, D.M., ROCHE, J.R., STOCKDALE, C.R., MCLACHLAN, B.P., 2002**, Aust. J. Agric. Res., 53, 755-770

**MOREIRA, V.R., ZERINGUE, L.K., WILLIAMS, C.C., LEONADI, C., MCCORMICK, M.E., 2009**, J Dairy Sci, 92, 5189-5198

**NRC, 2001,** National Academic Press, Nutrient requirements of dairy cattle::Seventh Revised Edition, Washington, USA

**PUGGAARD, L., LUND, P., LIESEGANG, A., SEHESTED, J., 2014**, Livest Sci, 159, 18-25

**TAYLOR M.S., KNOWLTON, K.F., MCGILLIARD, M.L., FERGUSON, J.D., WU, Z., HANIGAN, M.D., 2009**, J Dairy Sci, 92, 223-237

**VANHOUTEN, J., DANN, P., MCGEOCH, G. BROWN, E., KRAPCHO, K., NEVILLE, M., WYSOLMERSKI, J. 2004,** J. Clin. Invest, 113, 598-608

**VAN HULZEN, K.J.E., SPRONG, R.C., VAN DER MEER, R., VAN ARENDONK, J.A.M., 2009**, J. Dairy Sci., 92, 5754-5759

**WOHLT, J.E., RITTER, D.E., EVANS, J.L., 1986**, J Dairy Sci., 69, 2815-2824

**Titre :** L'évolution de la teneur en calcium du lait au cours de la lactation peut-elle être un indicateur de l'effet de l'alimentation sur les réserves osseuses des vaches laitières ?

**Mots clés :** Calcium, vache laitière, mobilisation osseuse, composition du lait ?

**Résumé :** Au cours de leur lactation, les vaches laitières font face à d'importants besoins en calcium du fait de la production laitière. L'augmentation très rapide de ces besoins en début de lactation fait que l'organisme des vaches peut difficilement s'adapter par une hausse de l'ingestion et de l'absorption digestive de calcium. L'organisme doit puiser dans les réserves osseuses, qui seront reconstituées plus tard en fin de lactation. Ces cycles de mobilisation et reconstitution osseuses restent cependant impossibles à quantifier chez la vache laitière sur de larges effectifs et les conséquences d'une mauvaise reconstitution osseuse sur les lactations suivantes restent inconnues.

Cette thèse avait pour objectif de développer un indicateur des phénomènes de mobilisation et reconstitution osseuse au cours de la lactation. Plusieurs facteurs de mobilisation osseuse ont pu être identifiés, comme la race, la parité ou l'alimentation. Cependant, les cycles de mobilisation et de reconstitution osseuse n'ont pas pu être reliés à des variations de la composition du lait en Ca et P. Il a cependant pu être montré, que contrairement à ce qui est décrit dans la littérature, un apport insuffisant en Ca en début de lactation n'est pas toujours accompagné d'une augmentation de la mobilisation osseuse, mais peut être compensé par une augmentation des capacités d'absorption digestive. Cette thèse montre aussi la nécessité de quantifier les conséquences d'un apport insuffisant de calcium alimentaire sur les performances de production et la santé des vaches laitières sur l'ensemble de la lactation.

**Title :** Can the dynamic of milk Ca content throughout lactation be an indicator of the effects of management system and diets on bone mobilization in dairy cows ?

**Keywords :** Calcium, dairy cow, bone mobilization, milk composition

**Abstract :** During lactations, dairy cows faces huge calcium requirements due to milk production. Because of the fast increase in those requirements at the beginning of the lactation, the organism of dairy cows can hardly adapt by increasing intake and digestive calcium absorption. The organism must mobilize calcium from its storage pool, bones, which will replenish later in lactation. Those cycles of bone mobilization and reconstitution remain impossible to quantify for important number of dairy cows and the consequences of an incomplete bone reconstitution on following lactation remain unknown.

The aim of this PhD was to develop an indicator of the phenomenon of bone mobilization and reconstitution during lactation. Several factors increasing bone mobilization have been identified, such as breed, parity or diet. However, the cycles of bone mobilization and reconstitution could not be related to variation in milk Ca and P content.

However, it has been showed that, an insufficient Ca supply in early lactation does not always induce a higher bone mobilization, as it has been described in the literature, but can be compensated by an increase in digestive absorption capacity. The thesis also showed the need to quantify the consequences on insufficient dietary calcium supply on dairy cows' milk production and health throughout lactation.