MINERAL AND ENERGY IMBALANCES OF THE TRANSITION PERIOD IN DAIRY COWS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Luciano Souza Caixeta

May 2017

MINERAL AND ENERGY IMBALANCES OF THE TRANSITION PERIOD IN DAIRY COWS

Luciano Souza Caixeta, DVM, Ph. D.

Cornell University 2017

The mineral and energy imbalances of the transition period of dairy cows have a negative impact in the dairy industry because they are prevalent, contribute to economic losses and are an animal welfare issue. This dissertation aimed to advance our understanding of the physiological adaptations characteristic of this period. A series of experiments were conducted to: advance the understanding of body reserves mobilization around parturition, characterize the dynamics of blood calcium concentration after calving and evaluate its association with reproductive performance, and advance the understanding of the regulation of fibroblast growth factor 21 in the dairy cow.

Chapter 2 reviews the current information available regarding the physiological adaptations necessary to overcome the mineral and energy challenges faced by dairy cows in the periparturient period, with an emphasis in subclinical hypocalcemia and fibroblast growth factor 21. In Chapter 3, it is reported that blood calcium concentration is the lowest in the first day post-partum, independently of parity, but normal levels are regained by day 3 of lactation. Additionally, the interaction between subclinical hypocalcemia, disease occurrence, and increased levels of metabolites surrogates of negative energy balance significantly influence the loss of body weight in parity $\geq$ 3 animals. Chapter 4 introduces a new concept of subclinical hypocalcemia that considers not only the blood calcium concentration at a given time but also how many days post-partum blood calcium concentration is below the established cut-off point. In this chapter, it is reported that approximately 1/3 of the dairy cows have low blood calcium

concentration during the first 3 days in lactation. Additionally, chronic subclinical hypocalcemia was associated with impaired reproductive performance of these animals.

Chapter 5 aims to identify objective measurements to assess body condition in dairy cows. In this chapter, sequential measurement of body weight and the measurement of back-fat thickness were compared to the traditional visual body condition scoring system. It was determined that body weight has the potential to be used to predict milk production throughout lactation, but other variables (i.e. disease and negative energy balance) can significant interfere with this measurement and further investigation of this methodology is necessary prior to the diffusion of this technique through the dairy industry at large.

Finally in Chapter 6, we demonstrated the importance of elevated plasma non-esterified fatty acids in hepatic fibroblast growth factor 21 production and consequently the increased circulating levels of this this hormone.

In summary, this dissertation contributes to the current knowledge regarding various aspects of dairy cows adaptation to milk production. Nonetheless, further research is needed to advance our knowledge on the epidemiology of subclinical hypocalcemia and its influence in production outcomes. As well as to better understand the effects of elevated fibroblast growth factor 21 concentrations in early lactation dairy cows.

BIOGRAPHICAL SKETCH

Luciano Souza Caixeta was born on July 12th of 1984 in Goiânia, Goiás, Brazil. Son of a veterinarian, Cairo Caixeta, and a school teacher, Glauce Mônica Vilela Souza, he grew up with two sisters, Lívia and Marina. Growing up, Luciano spent his school vacations shadowing his father which highly influenced his decision to be a bovine veterinarian.

In March 2008, Luciano graduated with a degree in veterinary medicine from his hometown veterinary school – Universidade Federal de Goiás – 34 years after his father got his veterinary medicine degree from the same veterinary school. Prior to his graduation, Luciano spent 6 months at Cornell University under the supervision of Dr. Robert Gilbert, Klibs Galvão, and Marial Lucia Gambarini. During this period, Luciano got involved with several research projects and also had the opportunity to understand the dairy industry of the United States.

After graduation, Luciano moved back to Ithaca, NY, USA, where he worked as a research assistant in Dr. Bicalho's laboratory before starting his residency program in production medicine. During his clinical training under the supervision of Dr. Charles Guard, Luciano's interest in preventive medicine grew and he decided to pursue further academic training. After his residency, Luciano was accepted in the Clinical Fellows program at the Hospital for Animals at Cornell University. During this time Luciano worked closely with Dr. Daryl Nydam and Dr. Yves Boisclair developing experiments to understand the metabolic adaptations characteristics of the early lactation period in dairy cows. Immediately after his fellowship program, Luciano was accepted to the graduate program at the Animal Science Department where he continued to study dairy cows' metabolism under Dr. Boisclair supervision. In 2015, Luciano was hired by the Clinical Sciences Department at the College of Veterinary Medicine and Biomedical Science at Colorado State University as a Dairy Population Health Management Instructor. During the following two years, Luciano managed to finish his graduate school work while teaching senior veterinary student and providing clinical service to the Colorado state dairy industry.

This dissertation is dedicated to my family and friends.

“Todas as coisas na Terra passam.
Os dias de dificuldades passarão.
Passarão também os dias de amargura e solidão.
As dores e as lágrimas passarão.
As frustrações que nos fazem chorar, um dia passarão.
A saudade do ser querido que se vai, na mão da morte, passará.
Os dias de glórias e triunfos mundanos, em que nos julgamos maiores e melhores que os outros, igualmente passarão.
A vaidade interna, que nos faz sentir como o centro do universo, um dia passará.”

Chico Xavier

ACKNOWLEDGMENTS

To begin with, I would like sincerely thank my PhD advisor, Dr. Yves Boisclair, for his mentorship and patience. Also, I am immensely grateful for Dr. Boisclair's understanding and willingness to accommodate all the extra-curricular activities that I signed up for during my graduate school training -- even though it deviated from his plans for a PhD student. I am especially thankful for being able to finish my PhD dissertation away from Cornell in order to assume a faculty position in Colorado. More importantly, I am thankful for the opportunity to learn how to be a researcher under the supervision of an extremely accomplished, intelligent and professional scientist. It is my hope that throughout my career I can emulate Dr. Boisclair's scientific rigor and research excellence.

I would like to extend my appreciation to my committee members, Drs. Daryl Nydam, Mike van Amburgh and Julio Giordano. Your guidance and examples throughout my professional training was extremely valuable and has influenced my scientific and clinical thinking more that you can imagine.

I am also thankful to all my lab mates for all the help during my experiments, especially when I had biopsies or sample collections between 2am and 6 am: Manolo Ramos-Nieves, Kristen Davis, Chris Krumm, Phillip Godinho, Sarah Giesy, and Katie Schoenberg. I am very thankful to all my Animal Sciences and Veterinary School fellow graduate students for all the help with my experiments and for the opportunity to help them with theirs.

A big thanks go to everyone in the Cornell Ambulatory Clinic for opening their doors to a naïve Brazilian visiting student. More than just work colleagues, you were my family in Ithaca for almost a decade. I would like to send a special thanks to Dr. Chuck Guard (a.k.a. Papa Chuck) for his wise advices and for being always believing in my capabilities. I am sure that I would not be where I am today without Chuck's support.

More important than letters after my name, Cornell/Ithaca gave me real friends that I will have with me for my whole life: Vinícius Machado, Eila Machado, André Gustavo Teixeira,

Rodrigo Bicalho, Marcela Bicalho, Felipe Bicalho, Rafael Bicalho, Luiz Santos, Rafael Neves, Rafael Bisinotto and many others that were in Ithaca for few months or many years.

Above all, I am incredibly thankful to my parents, Cairo e Glauce, for their love, sacrifice, and for their support especially when I moved far away from home. I am thankful for all the opportunities they provided me growing up and for their sacrifice to support me during my first few months in Cornell. If I had success throughout my life, it certainly happened because of the values they thought me. Special thanks go to my sisters Livia and Marina, and all my immediate relatives. Finally, an immense thank you goes to my fiancée Emily Barrell for making me a better person and for making me view the world through a much more understanding lens. Without Emily's love and support this journey would have been much harder.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1: Introduction

For the last few decades the dairy industry has selected dairy cows for milk production at the expense of other traits (Lassen et al., 2003, Berry et al., 2016). As a consequence the metabolic challenge of meeting the nutrient requirements of early lactation has been exacerbated (Bell, 1995, DeGaris and Lean, 2008), ultimately increasing the likelihood of disease occurrence during the periparturient period, and hampering milk production and reproductive performance (LeBlanc et al., 2005, Duffield et al., 2009, Ospina et al., 2010a, b, Chapinal et al., 2012, Martinez et al., 2012, McArt et al., 2012). During the transition period, traditionally defined as the period from 3 weeks before to 3 weeks after calving, calcium and energy requirements are increased by 65% and 300%, respectively, to support lactogenesis (Bell, 1995, Drackley, 1999, Reynolds et al., 2003, DeGaris and Lean, 2008). Therefore, physiological adaptations triggered by key metabolic hormones are essential to coordinate the mobilization of lipid and mineral reserves that will be used to overcome these deficits. Amongst the potential mineral imbalances of the transition period, low blood calcium concentration or hypocalcemia, has been extensively studied. Scientists have shown that various adaptations are triggered to re-establish calcium homeostasis in early lactation including increased absorption of dietary calcium, increased mobilization from bone, and enhanced renal re-absorption (DeGaris and Lean, 2008, Goff, 2008). Furthermore, by using efficient nutritional management protocols scientists were able to maximize the results of the aforementioned adaptations leading to a decrease in the incidence of clinical cases of hypocalcemia to 1% or less (DeGaris and Lean, 2008, Goff, 2008, Reinhardt et al., 2011). However, it takes up to 48 hours for this adaptation to be fully effective (Goff, 2008, Oetzel, 2013). Thus, a period of moderately low blood calcium concentration, also known as subclinical hypocalcemia (**SHPC**), can be found in a substantial proportion of dairy cows post-partum (Goff, 2008, Reinhardt et al., 2011, Oetzel, 2013, Caixeta et al., 2015).

In parallel, energy requirements to support milk production are also elevated during the periparturient period. Yet, voluntary feed intake is depressed and not sufficient to cover the nutrient demands of this period. Hence, dairy cows face a period of negative energy balance (**NEB**) in early lactation (Bell, 1995). This physiological state leads to the mobilization of adipose tissue and muscle in the form of non-esterified fatty acids (**NEFA**) and amino acids to be used as alternative fuel sources for various tissues (Bell and Bauman, 1997, Drackley, 1999). The mobilization of adipose tissue is an essential source of energy for dairy cows in early lactation, but when in excess, it has been associated with decreased milk production and reproductive performance, and increased risk of disease occurrence (Duffield et al., 2009, Ospina et al., 2010a, b, Chapinal et al., 2011, McArt et al., 2012).

Previous research have produced vast knowledge that has been applied to mitigate the mineral and energy imbalances characteristic of the transition from late gestation to early lactation in the modern dairy cow. However, the physiological adaptations during this period are not fully understood, and several questions remain to be answered.

The mobilization of body reserves to maintain milk production requirements have been usually assessed using body condition scoring systems that indirectly measure the mobilization of adipose tissue (Edmonson et al., 1989, Ferguson et al., 1994, Schroder and Staufenbiel, 2006, Thorup et al., 2013). Yet, the relationship between the loss of body condition score and the concurrent mineral imbalances have not been addressed. Can hypocalcemia exacerbate mobilization of body reserves in the modern dairy cow? Can mineral imbalances and excessive mobilization of body reserves act synergistically to hinder productivity?

Also, SHPC has traditionally been defined as a single low blood calcium (< 8.0 ng/dL) measurement within the first 48 hours after calving (Goff, 2008, Reinhardt et al., 2011, Martinez

et al., 2012). Nevertheless, the physiological adaptations to overcome this challenge may take longer than 48 hours (Goff, 2008, Martin-Tereso and Verstegen, 2011). Thus, using a single blood calcium measurement during the first two days of lactation to define hypocalcemia might be overestimating the number of hypocalcemic animals. Given the time necessary for complete adaptation to this new physiological state after calving, are multiple blood calcium measurements necessary to determine SHPC cases? What is the proportion of animals that present low blood calcium concentration for more than 48 hours? Do animals that adapt faster and re-establish normal blood calcium concentration within 48 hours perform better than animals that take longer to re-establish normal blood calcium concentrations?

Other methods of assessing body reserve mobilization and body condition have been used less frequently throughout the years (Edmonson et al., 1989, Ferguson et al., 1994, Schroder and Staufenbiel, 2006, Thorup et al., 2013). A visual body condition scoring system has been commonly used to determine the extent of adipose tissue energy reserves even though undesirable inter and intra-observer variance and low repeatability is observed when this measurement is performed by not highly trained personnel (Ferguson et al., 1994, Kristensen et al., 2006). Despite different scales, changes in the body condition measured by any of the aforementioned methods (i.e. body condition score, backfat thickness, and body weight) have been associated with differences in the amount of milk produced and reproductive performance (Mosenfechtel et al., 2002, Berry et al., 2003, Lopez-Gatius et al., 2003, Roche et al., 2007, Sakaguchi, 2009). These different methods, however, have not been compared in the same experiment. Would an objective (sequential body weight and back-fat thickness measurements) measurement behave in a similar fashion as the traditional method (body condition score) during early lactation? Which of the current methods available can be more predictive of production

outcomes? Can new approaches eliminate the inherent variance and low repeatability of methods based on a visual appraisal of fatness?

Lastly, the change in body condition during early lactation is a consequence of several physiological adaptations that are in place to fulfil energy requirements for milk production. Among these adaptations, increased plasma levels of NEFA and glucagon are a hallmark of this period (Herdt, 2000, Bobe et al., 2003). Interestingly, these adaptations are also observed in other species in periods when energy availability is decreased. Moreover, increased NEFA and glucagon have been reported to stimulate the liver expression of fibroblast growth factor 21 (**FGF21**) in humans and rodents (Inagaki et al., 2007, Badman et al., 2009, Cyphert et al., 2012, Arafat et al., 2013). Likewise, the plasma concentration of this protein hormone has been reported to peak at calving and remain elevated throughout the first five weeks of lactation (Schoenberg et al., 2011), which coincides with the period of elevated plasma NEFA and glucagon. Therefore, it is important to understand if such adaptations are also responsible for triggering FGF21 expression in dairy cows. Does elevated plasma fatty acids and glucagon induce FGF21 expression in the liver of dairy cows?

The overall objective of this dissertation is to better understand metabolic adaptations occuring during the transition period of dairy cows. It is also my goal that the knowledge generated by this research will ultimately contribute to the development of new strategies that can enhance animal productivity, dairy farming profitability, and most importantly animal health and well-being.

REFERENCES

Arafat, A. M., P. Kaczmarek, M. Skrzypski, E. Pruszynska-Oszmalek, P. Kolodziejski, D. Szczepankiewicz, M. Sassek, T. Wojciechowicz, B. Wiedenmann, A. F. Pfeiffer, K. W. Nowak, and M. Z. Strowski. 2013. Glucagon increases circulating fibroblast growth factor 21 independently of endogenous insulin levels: a novel mechanism of glucagon-stimulated lipolysis? Diabetologia 56(3):588-597.

Badman, M. K., A. Koester, J. S. Flier, A. Kharitonenkov, and E. Maratos-Flier. 2009. Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis. Endocrinology 150(11):4931-4940.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 73(9):2804-2819.

Bell, A. W. and D. E. Bauman. 1997. Adaptations of glucose metabolism during pregnancy and lactation. Journal of mammary gland biology and neoplasia 2(3):265-278.

Berry, D. P., F. Buckley, P. Dillon, R. D. Evans, M. Rath, and R. F. Veerkamp. 2003. Genetic relationships among body condition score, body weight, milk yield, and fertility in dairy cows. Journal of dairy science 86(6):2193-2204.

Berry, D. P., N. C. Friggens, M. Lucy, and J. R. Roche. 2016. Milk Production and Fertility in Cattle. Annual review of animal biosciences 4:269-290.

Bobe, G., R. N. Sonon, B. N. Ametaj, J. W. Young, and D. C. Beitz. 2003. Metabolic responses of lactating dairy cows to single and multiple subcutaneous injections of glucagon. Journal of dairy science 86(6):2072-2081.

Caixeta, L. S., P. A. Ospina, M. B. Capel, and D. V. Nydam. 2015. The association of subclinical hypocalcemia, negative energy balance and disease with bodyweight change during the first 30 days post-partum in dairy cows milked with automatic milking systems. Veterinary journal (London, England : 1997) 204(2):150-156.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. Journal of dairy science 94(10):4897-4903.

Chapinal, N., S. J. Leblanc, M. E. Carson, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. Journal of dairy science 95(10):5676-5682.

Cyphert, H. A., X. Ge, A. B. Kohan, L. M. Salati, Y. Zhang, and F. B. Hillgartner. 2012. Activation of the farnesoid X receptor induces hepatic expression and secretion of fibroblast growth factor 21. The Journal of biological chemistry 287(30):25123-25138.

DeGaris, P. J. and I. J. Lean. 2008. Milk fever in dairy cows: a review of pathophysiology and control principles. Veterinary journal (London, England : 1997) 176(1):58-69.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? Journal of dairy science 82(11):2259-2273.

Duffield, T. F., K. D. Lissemore, B. W. McBride, and K. E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. Journal of dairy science 92(2):571-580.

Edmonson, A., I. Lean, L. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. Journal of dairy science 72(1):68-78
.
Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. Journal of dairy science 77(9):2695-2703
.
Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Veterinary journal (London, England : 1997) 176(1):50-57.

Herdt, T. H. 2000. Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. The Veterinary clinics of North America. Food animal practice 16(2):215-230, v.

Inagaki, T., P. Dutchak, G. Zhao, X. Ding, L. Gautron, V. Parameswara, Y. Li, R. Goetz, M. Mohammadi, V. Esser, J. K. Elmquist, R. D. Gerard, S. C. Burgess, R. E. Hammer, D. J. Mangelsdorf, and S. A. Kliewer. 2007. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell metabolism 5(6):415-425.

Kristensen, E., L. Dueholm, D. Vink, J. E. Andersen, E. B. Jakobsen, S. Illum-Nielsen, F. A. Petersen, and C. Enevoldsen. 2006. Within- and across-person uniformity of body condition scoring in Danish Holstein cattle. Journal of dairy science 89(9):3721-3728
.
Lassen, J., M. Hansen, M. K. Sorensen, G. P. Aamand, L. G. Christensen, and P. Madsen. 2003. Genetic analysis of body condition score in first-parity Danish Holstein cows. Journal of dairy science 86(12):4123-4128.

LeBlanc, S. J., K. E. Leslie, and T. F. Duffield. 2005. Metabolic predictors of displaced abomasum in dairy cattle. Journal of dairy science 88(1):159-170.

Lopez-Gatius, F., J. Yaniz, and D. Madriles-Helm. 2003. Effects of body condition score and score change on the reproductive performance of dairy cows: a meta-analysis. Theriogenology 59(3-4):801-812.

Martin-Tereso, J. and M. W. Verstegen. 2011. A novel model to explain dietary factors affecting hypocalcaemia in dairy cattle. Nutrition research reviews 24(2):228-243.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao, and J. E. Santos. 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.

McArt, J. A., D. V. Nydam, and G. R. Oetzel. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. Journal of dairy science 95(9):5056-5066.

Mosenfechtel, S., M. Hoedemaker, U. J. Eigenmann, and P. Rusch. 2002. Influence of back fat thickness on the reproductive performance of dairy cows. The Veterinary record 151(13):387-388.

Oetzel, G. R. 2013. Oral calcium supplementation in peripartum dairy cows. The Veterinary clinics of North America. Food animal practice 29(2):447-455.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010a. Association between the proportion of sampled transition cows with increased nonesterified fatty acids and beta-hydroxybutyrate and disease incidence, pregnancy rate, and milk production at the herd level. Journal of dairy science 93(8):3595-3601.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010b. Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. Journal of dairy science 93(4):1596-1603.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Veterinary journal (London, England : 1997) 188(1):122-124.

Reynolds, C. K., P. C. Aikman, B. Lupoli, D. J. Humphries, and D. E. Beever. 2003. Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of dairy science 86(4):1201-1217.

Roche, J. R., J. M. Lee, K. A. Macdonald, and D. P. Berry. 2007. Relationships among body condition score, body weight, and milk production variables in pasture-based dairy cows. Journal of dairy science 90(8):3802-3815.

Sakaguchi, M. 2009. Differences between body condition scores and body weight changes in postpartum dairy cows in relation to parity and reproductive indices. The Canadian veterinary journal. La revue veterinaire canadienne 50(6):649-656.

Schoenberg, K. M., S. L. Giesy, K. J. Harvatine, M. R. Waldron, C. Cheng, A. Kharitonenkov, and Y. R. Boisclair. 2011. Plasma FGF21 is elevated by the intense lipid mobilization of lactation. Endocrinology 152(12):4652-4661.

Schroder, U. J. and R. Staufenbiel. 2006. Invited review: Methods to determine body fat reserves in the dairy cow with special regard to ultrasonographic measurement of backfat thickness. Journal of dairy science 89(1):1-14.

Thorup, V. M., S. Hojsgaard, M. R. Weisbjerg, and N. C. Friggens. 2013. Energy balance of individual cows can be estimated in real-time on farm using frequent liveweight measures even in the absence of body condition score. Animal : an international journal of animal bioscience 7(10):1631-1639.

CHAPTER 2: Literature Review

INTRODUCTION

The transition from late gestation to early lactation is an extremely challenging period for the modern dairy cow because of the rapid increase in energy and mineral demands for colostrum and milk production (Bell, 1995, DeGaris and Lean, 2008). The pursuit of a more efficient production system led the dairy industry to prioritize selection for milk yield over other traits exacerbating the metabolic problems faced by dairy cows (Lassen et al., 2003, Berry et al., 2016). As a consequence dairy cows are at greatest risk of developing diseases during the periparturient period (LeBlanc et al., 2005).

In early lactation, energy demands are increased by 300% and calcium requirements are increased over 65% in order to support lactogenesis (Bell, 1995, Drackley, 1999, Reynolds et al., 2003, DeGaris and Lean, 2008). Cows do not have sufficient appetite after parturition to cover these requirements leading to a period of negative balance for both energy and major minerals, including magnesium and phosphorus in addition to calcium (Herdt, 2000). Thus, homeorhetic and homeostatic adaptations are essential during this period to coordinate the mobilization of lipid and mineral reserves (Bauman and Currie, 1980).

The increased mineral demands during the periparturient period, specially increased calcium demands, have been thoroughly studied. Low blood calcium concentration during early lactation have been associated to negative effects on health and production outcomes in dairy cows (DeGaris and Lean, 2008, Chapinal et al., 2011, Chapinal et al., 2012, Martinez et al., 2012, Overton and Yasui, 2014, Wang et al., 2014). Likelihood and severity of these imbalances increase with parity. Meta-analysis studies found that the risk of milk fever increases by nine percentage points at each successive lactation (Lean et al., 2006). Increased risk of hypocalcemia in older animals is explained by decreased capacity to mobilize calcium from

bones (van Mosel et al., 1993), decreased capacity to absorb calcium in the small intestines (Horst et al., 1990), and higher milk production.

Along with increased mineral demands, energy demands increases in the periparturient period. Lower concentrations of circulating insulin and increased lipid mobilization are important adaptations necessary to supply energy for milk production while feed intake is insufficient (Bauman and Currie, 1980, Bell, 1995, Drackley, 1999, Komatsu et al., 2005). When excessive, however, lipid mobilization is associated with the occurrence of diseases, decreased milk production, impaired reproductive performance, and increased culling rates in early lactation (Goff and Horst, 1997, Herdt, 2000, LeBlanc et al., 2005, Walsh et al., 2008, Duffield et al., 2009, Ospina et al., 2010b, a, Chapinal et al., 2011, McArt et al., 2012). Different strategies have been studied to alleviate lipid mobilization in early lactation, with limited success.

The transition period has been defined as the period 3 weeks before and after parturition (Grummer, 1995); nevertheless, metabolic changes can start earlier and have carryover effects beyond this period. An efficient transition into lactation is essential to determine the success of the modern dairy cow in the current production systems (Drackley, 1999), and yet ineffective adaptation to the new physiological state remains common. Therefore, it is important to understand the adaptations happening in the periparturient period to develop knowledge and strategies that can improve animal performance and welfare.

MINERAL IMBALANCES IN THE TRANSITION PERIOD

The interaction between macro and micro minerals has been extensively studied throughout the years and is a key determinant of occurrence of clinical diseases and negative downstream outcomes related to mineral imbalances in dairy cows. Hypocalcemia, hypophosphatemia, and hypomagnesemia have been reported as the most common mineral imbalances faced by dairy cows. Abnormal concentrations of these minerals in early lactation are involved in the development of the down cow syndrome which is characterized by recumbency and incapacity to rise (DeGaris and Lean, 2008, Martin-Tereso and Verstegen, 2011, Goff, 2014). The understanding and prevention of the occurrence of such syndrome is important not only for production aspects but also for animal welfare, since euthanasia is indicated for animals that are non-ambulatory for more than 24 hours (Green et al., 2008). Advances in the field of dairy nutrition and physiology have been paramount to the development of management strategies that decreased the occurrence of clinical hypocalcemia in the modern dairy cows (Reinhardt et al., 2011, Oetzel and Miller, 2012).

Despite the importance of all mineral imbalances during the periparturient period, hypocalcemia has been studied more frequently and described as the major mineral imbalance affecting dairy cows.

Hypocalcemia

Definition and Incidence

Low blood calcium concentration, also known as hypocalcemia, has been reported as a problem in early lactation dairy cows for over two centuries (Murray et al., 2008). Depending on its severity hypocalcemia is classified as either clinical or subclinical. Clinical hypocalcemia is

characterized by the development of clinical signs, such as recumbency, lethargy, hypothermia, and rumen atony and is associated with total blood calcium concentrations lower than 5.6 mg/dL. Subclinical hypocalcemia (**SHPC**) corresponds to low blood calcium concentration without visible clinical signs, with traditional cut off points of 5.6 to 8.0 mg/dL for total blood calcium (DeGaris and Lean, 2008, Goff, 2008, Reinhardt et al., 2011). Nevertheless, multiple total blood calcium cut-off, varying from 8.0 mg/dL to 8.8 mg/dL have been proposed to define SCHP on the basis of results of multiple clinical trials and epidemiological assessments (Chapinal et al., 2011, Martinez et al., 2012).

Effective nutritional management during the dry period and early lactation, and increased understanding of transition cow period physiology has led to a decrease in the incidence of milk fever to 1% or less (Reinhardt et al., 2011, Oetzel and Miller, 2012). Conversely, the incidence of SHPC remains high during the first 3 DIM averaging 25% for first lactation cows, 48% in older animals with as many as 73% of animals of parity $\geq$ 3 presenting SCHP (Reinhardt et al., 2011, Caixeta et al., 2015).

Calcium Homeostasis

Upon initiation of lactation, daily calcium requirements increase from 30 g before calving to over 50 g after parturition (DeGaris and Lean, 2008). Adaptations triggered to meet this nutritional challenge include increased absorption of dietary calcium, increased mobilization from bone, and enhanced renal re-absorption (DeGaris and Lean, 2008, Goff, 2008).

The lower blood calcium concentrations characteristic of early lactation increase the secretion of parathyroid hormone (**PTH**) by the parathyroid glands, which further induces calcitriol production by the kidneys. The combined actions of these two hormones are essential to the re-establishment of physiological concentration of extracellular calcium in periparturient dairy

cows. In addition to inducing calcitriol production, PTH initiates bone calcium mobilization and enhances renal tubular reabsorption of calcium as long as intestinal absorption of calcium is insufficient (Martin-Tereso and Verstegen, 2011). On the other hand, calcitriol stimulates efficient absorption of dietary calcium in the intestine and sustains bone mobilization in the presence of PTH (DeGaris and Lean, 2008, Goff, 2008). In brief, calcitriol stimulates enterocytes to enhance their calcium transport competence during their early stages of differentiation. Intestinal and bone adaptations take 24 to 48 hours to develop, a time period nearly identical to duration of SHPC after parturition (Goff, 2008, Oetzel, 2013).

Extracellular total calcium (**tCa**) concentration is maintained between 8.5 – 10.0 mg/dL (Goff, 2008); therefore intestinal capacity to absorb dietary calcium, bone mobilization, and renal resorption or excretion of calcium are dynamically changing throughout lactation in order to maintain this equilibrium (Martin-Tereso and Verstegen, 2011). The interaction between PTH and calcitriol normalize calcium concentrations in early lactation by predominantly stimulating absorption of dietary calcium over bone mobilization or renal reabsorption (Martin-Tereso and Verstegen, 2011).

It is important to highlight that only part of the blood tCa pool is free and readily available for biological activities. This calcium pool is referred to as ionized calcium (**iCa**), and is mainly transported in blood bonded to albumin (Sava et al., 2005). In humans, iCa has been shown to correspond to half of tCa circulating under normal conditions (Forman and Lorenzo, 1991, Kragh-Hansen and Vorum, 1993). However, during periods of abnormal calcium states such association is not maintained and measurement of iCa is necessary to improve calcium status diagnostics accuracy (Ong et al., 2012). Similarly, a slight change in the iCa-tCa ratio, high iCa:tCa due to increased percentage of tCa being ionized, was observed in dairy cows

immediately after parturition (Sweeney et al., 2014). Despite this discrepancy, measurements of tCa were considered adequate when predicting neutrophil function, and therefore acceptable as an index of calcium status in periparturient dairy cow (Sweeney et al., 2014). Ionized Ca represents the bioactive calcium in blood, but its determination is complicated and costly. Moreover iCa does not predict functional outcomes significantly better than tCa. Accordingly, tCa is commonly measured in dairy cattle research.

Prevention of Hypocalcemia

Nutritional strategies are commonly used to prevent clinical and subclinical hypocalcemia. An effective nutritional strategy is the utilization of low calcium diets during the pre-partum period. Theoretically, low calcium diets activate osteoclastic bone resorption and stimulate enterocytes to efficiently transport calcium into the blood prior to calving, thus avoiding hypocalcemia (Green et al., 1981). Nevertheless, formulation of low calcium diets is extremely difficult because many forages exceed the minimum calcium concentration necessary to achieve this effect (Martin-Tereso and Verstegen, 2011).

Another effective nutritional strategy proposed to prevent hypocalcemia is the supplementation of dairy cows with anionic salts pre-partum leading to a reduction of the dietary cation-anion difference (**DCAD**). The use of anionic salts, low-DCAD diets, cause a drop in blood pH which is counteracted by a low grade calcium release from bones into the extracellular fluid to balance the excessive anions in circulation (Goff and Horst, 2003). The mobilized calcium is excreted by the kidneys until parturition when it is used to fulfil the elevated milk calcium demands of lactation (DeGaris and Lean, 2008, Martin-Tereso and Verstegen, 2011). Therefore, the beneficial effects of low-DCAD diets in early lactating dairy cows is explained by enhanced capacity to mobilize calcium from bones and the maintenance of PTH actions. The use

of low-DCAD diets has been reported to help prevent hypocalcemia at calving by numerous research groups (Oetzel et al., 1988, Horst et al., 1994, Moore et al., 2000, Ramos-Nieves et al., 2009, Grunberg et al., 2011).

The prophylactic use of oral calcium supplementation in early lactation has also been proposed as a strategy to overcome calcium deficit. Calcium supplementation after parturition has relevant economic impact in the dairy industry because it is associated with increased health and production in high yielding dairy cows, especially for greater parity and lame animals (Oetzel and Miller, 2012, McArt and Oetzel, 2015). The use of oral calcium bolus establish a more sustained elevation of blood calcium concentration when compared to traditional intravenous treatments (Oetzel and Miller, 2012, Blanc et al., 2014).

Impact of Hypocalcemia in Production Outcomes

Traditionally, low calcium concentration in the periparturient period has been associated with occurrence of dystocia, uterine prolapse, retained placenta, mastitis, decreased feed intake, and decreased rumen and abomasum motility (Curtis et al., 1983, Risco et al., 1984, Goff, 2008, Seifi et al., 2011, Sepulveda-Varas et al., 2015). The association between hypocalcemia and the aforementioned diseases is mostly related to the importance of calcium for smooth muscle contraction. Low blood calcium concentration is associated with decreased myometrial contractility which in turn leads to increased likelihood of uterine prolapses and delayed uterine involution that subsequently affect conception (Hansen et al., 2003, Whiteford and Sheldon, 2005, Goff, 2008, Heppelmann et al., 2015). Similarly, rumen and abomasal motility are reduced when calcium concentration is lower which increases the risk of abomasal displacement (Chapinal et al., 2011). Additionally, low blood calcium is associated with reduction of cytosolic calcium concentration in immune cells affecting the ability of these cells to mount a strong

response to infections during the periparturient period (Kimura et al., 2006). This immunosuppression is associated with increased risk of metritis, mastitis, and possibly retained placenta (Melendez et al., 2004, Martinez et al., 2012).

In addition, there is growing evidence that hypocalcemia can impair reproductive performance by altering ovarian activity. For example hypocalcemic animals have smaller ovulatory follicle, smaller corpus luteum after ovulation, and lower plasma progesterone after the first ovulation during the voluntary waiting period than their eucalcemic counterparts resulting in decreased reproductive performance (Kamgarpour et al., 1999, Wilde, 2006, Chapinal et al., 2012). Overall hypocalcemia after parturition is associated with increased disease incidence within the first 30 DIM, decreased reproductive performance, decreased milk production, and increased culling rates.

Other Macromineral Disorders

Hypomagnesemia

Magnesium (**Mg**) is a major intracellular cation necessary as co-factor for a multitude of metabolic pathways and maintenance of normal levels in plasma depends on dietary absorption (Goff, 2008). The blood Mg concentration is determined by the difference between dietary absorption and the renal clearance of this mineral. An Mg shortage leading to concentrations below 1.80 mg/dL is defined as hypomagnesemia (Goff, 2008, Martin-Tereso and Martens, 2014). Clinical signs of hypomagnesemia resembles those of hypocalcemia and include recumbency, reduced feed intake, ataxia, and tetanic muscle spasms. Animals affected by hypomagnesemia, however, rarely present severe clinical signs.

Even though isolated cases of hypomagnesemia are easily treated by correcting magnesium deficiency (Reynolds et al., 1984), low blood magnesium concentration affects Ca metabolism. Hypomagnesemia reduces PTH secretion which in turn impairs Ca homeostasis. Moreover magnesium is essential for the activation of PTH receptors with the consequence that hypomagnesemia reduces tissue sensitivity to PTH, bone Ca resorption and renal production of 1,25-dihydroxyvitamin D (Goff, 2008). Therefore, hypomagnesemic dairy cows have a lower ability to restore Ca blood concentration.

Hypophosphatemia

Low blood phosphorus concentration is frequently observed in dairy cows in early lactation, especially in anorexic cows (Grunberg, 2014). Hypophosphatemia, defined by phosphorus blood concentration below 5.6 mg/dL culminates in clinical presentation similar to hypocalcemia and hypomagnesemia, including recumbency and decreased feed intake. PTH increases phosphorus excretion in saliva and urine (Goff, 2004). Hypophosphatemia is commonly associated with hypocalcemia because of the higher PTH concentration required to re-establish Ca concentrations. Accordingly, hypophosphatemia is usually not an isolated metabolic disorder but rather a consequence of hypocalcemia. Oral and intravenous solutions can be used to treat hypophosphatemic cows, but in general phosphorus blood concentration rise following hypocalcemia treatment and reduction of PTH (Goff, 2004, Grunberg, 2014).

ENERGY IMBALANCES IN THE TRANSITION PERIOD

In periparturient cows, voluntary feed intake is insufficient to cover nutrient demands associated with colostrum production and the considerable increase in the amount of milk produced (Bell, 1995). In order to overcome this challenge, dairy cows mount several

adaptations in the periparturient period including mobilization of body reserves as alternative fuel sources. The mobilization of adipose tissue is of extreme importance for the dairy cow, but jeopardize well-being and productivity if excessive.

Lipids are mobilized from adipose tissue and higher concentration of circulating NEFA are detected in dairy cows during early lactation (Herdt, 2000, Ospina et al., 2010c, McArt et al., 2012). Circulating NEFA can be used by various tissues as energy source and, as a source of preformed fatty acids by mammary gland, thus, the higher concentration of fat in milk of early lactation animals (Duffield et al., 1997, Drackley, 1999, Herdt, 2000, Reynolds et al., 2003, Ospina et al., 2013). Nonetheless the majority of the circulating NEFA, approximately 25%, is taken up by liver where it can be completely oxidized in the tricarboxylic acid cycle to produce ATP or partially oxidized to ketone bodies that can be used as energy source by extrahepatic tissues (Grummer, 1993, Drackley, 1999, Herdt, 2000). NEFA taken in excess of liver oxidative capacity are repackaged into TG which are then exported at inherently low rates as very low density lipoprotein (**VLDL**) or stored in liver leading to the occurrence of fatty liver (Drackley, 1999, Bobe et al., 2004).

NEFA and β-hydroxybutyrate (**BHB**) have been used as surrogates of NEB during early lactation. Despite the interchangeable use of these two parameters, concentrations of NEFA and BHB during early lactation have a weak relationship and caution should be used when extrapolating the relationships between the two metabolites (McCarthy et al., 2016). Nevertheless, extreme lipid mobilization during periods of excessive NEB leads to elevated concentrations of NEFA and BHB. The elevated concentration of NEB surrogates has been associated with negative downstream outcomes by various epidemiological studies (Duffield, 2000, Ospina et al., 2010b, Chapinal et al., 2011, Ospina et al., 2013). Lately, McArt et al.

(2014) have determined that the total cost per hyperketonemia, defined as BHB $\geq$ 1.2 mmol/L, case in the modern dairy farm is on average $289, highlighting the importance of adequate nutrition and management during the periparturient period of dairy cows. Despite that, hyperketonemia still has a relative high incidence in early lactation. In an effort to decrease economical losses due to hyperketonemia cases, a combined testing-and-treating strategy has been developed. This strategy consists of testing approximately 20 cows, every other week, between 3 and 14 DIM, for blood hyperketonemia using a cow-side test. Animals with BHB concentrations $\geq$ 1.2 mmol/L are deemed positive for hyperketonemia. Frequency of hyperketonemia determines the recommended intervention: if less than 15%, herd level prevalence should be monitored; if 15 to 40% all animals should be monitored twice between 3 and 9 DIM and all positives individuals should be treated with 300 mL of propylene glycol for 5 days; if more than 40% all cows should be treated with propylene glycol starting on 3 DIM for 5 days. Herds with elevated hyperketonemia prevalence should revise management and nutritional protocols in order to achieve acceptable prevalence rates and disease prevalence should be re-assessed within a month (Ospina et al., 2013, McArt et al., 2014).

The increased levels of NEFA circulating during the periparturient period also leads to TG infiltration of the liver. Excessive accumulation of TG leads to the development of a condition known as fatty liver and decreased metabolic function (Grummer, 1993, Drackley, 1999, Bobe et al., 2004, McCarthy et al., 2015). Fatty liver can be categorized based on the extent of TG accumulation into normal liver (< 1% of wet weight), mild fatty liver (1 to 5% of wet weight), moderate fatty liver (5 to 10% of wet weight), and severe fatty liver (> 10% of wet weight) (Reid, 1980). The accumulation of TG in liver has been associated with decreased capacity for urea synthesis, and glucose synthesis from propionate (Grummer, 1993, Strang et al., 1998,

McCarthy et al., 2015). Liver accumulation of lipids affects up to 50% of early lactation dairy cows varying from 20% to 65% of the animals having moderate cases and 5% to 20% of the early lactation dairy cows presenting severe cases of fatty liver (Bobe et al., 2004). Substantial economic losses due to impaired reproductive performance, exacerbation of metabolic problems, and decreased milk production because of the decreased hepatic gluconeogenesis have been associated with moderate and severe cases of fatty liver (Veenhuizen et al., 1991, Jorritsma et al., 2000, Jorritsma et al., 2003, McCarthy et al., 2015).

Several nutritional and management strategies have been tested to treat, prevent, or alleviate fatty liver with limited success. Increasing nutrient density of transition diets in order to increase propionate production in the rumen, as well as supplementing dietary fat to increase dietary energy density are strategies that have been proposed to prevent fatty liver (Grummer and Carroll, 1991). Nonetheless, increasing energy density of pre-partum diet has little effects on liver accumulation of TG after calving (Rabelo et al., 2005). Additionally, feed additives that decrease adipose tissue lipolysis (i.e. propylene glycol, monensin, chromium, and niacin), enhance hepatic VLDL secretion (i.e. choline and methionine), and alter hepatic fatty acid metabolism (i.e. carnitine and tallow) have been suggested as nutritional strategies to prevent and treat fatty liver (Grummer, 2008). Among the dietary supplements tested only choline and propylene glycol repeatedly reduced TG in liver (Grummer, 2008). Management strategies such as feeding one diet during the entire dry period and shortening the dry period have been proposed but insufficient data are available to assess the effectiveness of such strategies in reducing lipid accumulation in liver (Grummer, 2008).

Hormonal Adaptations of Early Lactation

Major changes in the plasma concentration of metabolic hormones occur during the periparturient period. They include decreased levels of circulating insulin, leptin, and insulin-like growth factor 1 (**IGF-I**)(Block et al., 2001, Ohtani et al., 2012, Mann et al., 2016), and increased levels of glucagon and growth hormone (**GH**) (De Koster and Opsomer, 2013, Mann et al., 2016). In addition, periparturient dairy cows develop resistance to the actions of GH and insulin. These hormonal adaptations are essential to maintain glucose availability for fetus growth and colostrum production in the pre-partum period, and to support the copious amount of milk produced after calving (Bauman and Currie, 1980).

Insulin and glucagon are two important players of glucose homeostasis during the periparturient period. During this period, the physiological decrease in insulin levels is associated with decreased uptake of glucose by peripheral tissue and increased lipolysis in adipose tissue (Bauman and Currie, 1980, Bell, 1995, McNamara and Murray, 2001, Komatsu et al., 2005). Lower plasma insulin directly contributes to increased hepatic glucose production because the repressive effects of insulin on key gluconeogenic enzymes is reduced. Lower plasma insulin indirectly contributes to hepatic glucose production by increasing availability of endogenously derived precursors such as glycerol (De Koster and Opsomer, 2013).

Early lactation is also characterized by elevated concentrations of circulating glucagon. Glucagon increases gene expression of gluconeogenic and ureagenic enzymes, which in turn increases hepatic glucose synthesis and output, mainly through utilization of non-essential amino acids (Hanigan et al., 2004, Bobe et al., 2009). Glucagon activity in early lactation dairy cows is also important to enhance fatty acids oxidation and ketones production in order to provide alternative energy source for peripheral tissues (Bobe et al., 2003). Insulin inhibits glucagon

gene transcription in various species (Bansal and Wang, 2008, Zarrin et al., 2015), and therefore the hypoinsulinemia of early lactation increases glucagon production. Hence, increased levels of circulating NEFA and BHB are a hallmark of the transition period in dairy cows.

Leptin is produced by adipose tissue with gene expression and circulating concentration reflecting adiposity as well as energy balance status. A marked decrease in leptin concentrations reflects negative energy balance, decreased energy intake, and decreased glucose concentrations (Rosenbaum and Leibel, 2014, Park and Ahima, 2015). Similarly, in early lactation dairy cows present a reduction of plasma concentration of leptin signaling the energy deficiency associated with NEB during this period (Block et al., 2001, Liefers et al., 2003, Janovick et al., 2011, Schoenberg et al., 2011, Ehrhardt et al., 2016). During fasting and periods of decreased feed intake, such as early lactation, the low circulating concentrations of leptin triggers centrally regulated responses such as increased food intake and reduced energy expenditure (Park and Ahima, 2015). These responses seek to reverse the energy deficient state by favoring appetite and depletion of body energy stores (Ingvartsen and Boisclair, 2001, Rosenbaum and Leibel, 2014, Park and Ahima, 2015). Additionally, lower concentrations of leptin reduce thyroid hormones and decrease response of peripheral tissues to insulin, promoting partitioning of glucose to milk production (Ehrhardt et al., 2016). The combination of physiological adaptations promoted by low leptin concentrations in early lactation are important to sustain energy demands for milk production in the energy deficient dairy cow.

Simultaneously, GH concentration is elevated during the transition from late gestation to early lactation (Bell, 1995, Block et al., 2001, Ohtani et al., 2012). Metabolic and physiological changes determined by GH can occur directly through stimulation of GH receptors in various tissues, and indirectly via stimulation of IGF-I production in the liver and other tissues. Despite

elevated concentration of GH, IGF-I transcription in liver is not increased during periods of NEB. This state of GH resistance is explained by reduced expression of the liver specific GH receptor, which is itself a consequence of the hypoinsulenemia of early lactation (Radcliff et al., 2003, Kim et al., 2005, Boisclair et al., 2006, Rhoads et al., 2007). The uncoupling of the GH-IGF axis during early lactation is important to successfully adapt dairy cows to lactation because the anabolic effects of IGF-I are limited. Moreover, it is possible that non-hepatic tissue also have a similar decrease in GH responsiveness during early lactation promoting catabolic adaptations (Boisclair et al., 2006). Taken together, this adaptations lead to a decrease glucose uptake by non-mammary tissues and increase availability of glucose for milk production.

The combination of the actions of these hormones in different tissues promotes appetite, increased gluconeogenesis, increased lipolysis, and decreased glucose uptake by peripheral tissues. A novel hormone known as fibroblast growth factor 21 (**FGF21**) has recently been identified and shown to regulate metabolic processes during various nutritional and physiological challenges in other species.

Fibroblast Growth Factor 21

FGF21 is a novel protein reported to mediate metabolic adaptations in periods of decreased energy availability in various species (Kharitonenkov et al., 2005, Badman et al., 2007, Inagaki et al., 2007, Lundasen et al., 2007). FGF21 is a secreted protein of 181 amino acids in humans and 182 amino acids in mice, with approximately 120 amino acid conserved core region with 75% identity between species (Nishimura et al., 2000). FGF21 is an atypical member of the FGF superfamily because the absence of the heparin-binding domain enables this protein to leave the site of synthesis and circulate in plasma (Itoh and Ornitz, 2008, Kharitonenkov, 2009). This

protein is produced mainly in the liver, but white adipose tissue (**WAT**), brown adipose tissue, muscle experiencing mitochondrial dysfunction, and pancreas have been reported as meaningful production sites in rodents (Antonellis et al., 2014). In dairy cows, FGF21 is produced almost exclusively by the liver, with little or no contribution by other tissues (Schoenberg et al., 2011).

FGF receptors (**FGFR**) are expressed in virtually every tissue and can be divided into seven FGFR proteins with distinct ligand-binding specificity, FGFR1b, 1c, 2b, 2c, 3b, 3c, and 4 (Itoh and Ornitz, 2004). FGF21 predominantly activates FGFR1c, but FGFR2c, 3c, and FGFR4 can be also activated (Ogawa et al., 2007, Kharitonenkov et al., 2008, Suzuki et al., 2008). Schoenberg et al. (2011) showed that FGFR2c accounts for over 50% of relevant FGFR transcripts in dairy cows liver, followed by FGFR4 and FGFR1c, whereas FGFR1c was the only receptor expressed at meaningful level in subcutaneous WAT. Even though expression of FGFR have been reported in various tissues, FGF21 is not capable of triggering metabolic actions by itself. FGF21 signaling depends on the presence of a transmembrane co-receptor known as β-klotho (**KLB**), to form signaling receptor complexes (Inagaki et al., 2007, Ogawa et al., 2007, Kharitonenkov et al., 2008, Yie et al., 2009, Itoh, 2010, Ding et al., 2012, Inagaki, 2015). Accordingly, FGF21 target tissues are those with meaningful KLB expression (Ogawa et al., 2007). In dairy cows, KLB is expressed in liver and WAT with the increased expression in KLB and FGFR during early lactation in liver, but not in WAT (Schoenberg et al., 2011).

Physiology of FGF21

Increased serum FGF21 in rodents has been reported as an adaptation to metabolic states characterized by increased lipid mobilization from WAT (Badman et al., 2007, Inagaki et al., 2007, Kharitonenkov et al., 2007, Badman et al., 2009). Increased plasma concentrations of fatty acids have been reported to induce liver expression of FGF21 in rodents and humans via

activation of the nuclear receptor peroxisome proliferator-activated receptor α (**PPARα**) (Badman et al., 2007, Inagaki et al., 2007, Lundasen et al., 2007, Cyphert et al., 2012). Increased hepatic FGF21 gene transcription further potentiates energy homeostasis processes triggered by PPARα (Inagaki et al., 2007). The hormone glucagon has also been shown to increase FGF21 production directly via a cAMP dependent posttranscriptional mechanism and indirectly by promoting adipose tissue lipolysis and increased plasma fatty acids (Berglund et al., 2010, Cyphert et al., 2012, Arafat et al., 2013).

Dairy cows undergo a period of high glucagon and increased plasma NEFA around parturition (Bell, 1995, McNamara and Murray, 2001), this combination fulfills the description of stimuli necessary for activation of FGF21 production in liver. Interestingly, dairy cattle data have shown that FGF21 plasma concentration and hepatic mRNA concentration peak around calving and remain elevated throughout the first few weeks of lactation when cows are in NEB (Schoenberg et al., 2011, Schlegel et al., 2012). Thus, it is possible that elevated glucagon and NEFA are responsible for inducing FGF21 production in the liver of dairy cows.

Increased expression of FGF21 promotes adaptations associated with decreased energy availability. In rodents, FGF21 has been shown to enhance hepatic gluconeogenesis, lipolysis in adipose tissue, and enhanced fatty acid oxidation and ketogenesis capacity in liver (Kharitonenkov et al., 2005, Badman et al., 2007, Inagaki et al., 2007, Potthoff et al., 2009, Chau et al., 2010, Chen et al., 2011, Vernia et al., 2014). Similarly, gluconeogenesis, fatty acids oxidation, and ketogenesis have been reported to be up-regulated in the liver of early lactating dairy cows (Schlegel et al., 2012). Furthermore, it has been reported that FGF21 alleviates fasting induced accumulation of fat in liver by increasing TG clearance (Badman et al., 2007,

Inagaki, 2015) and that systemic administration of FGF21 decreases TG accumulation in liver of obese rodents and primates (Coskun et al., 2008).

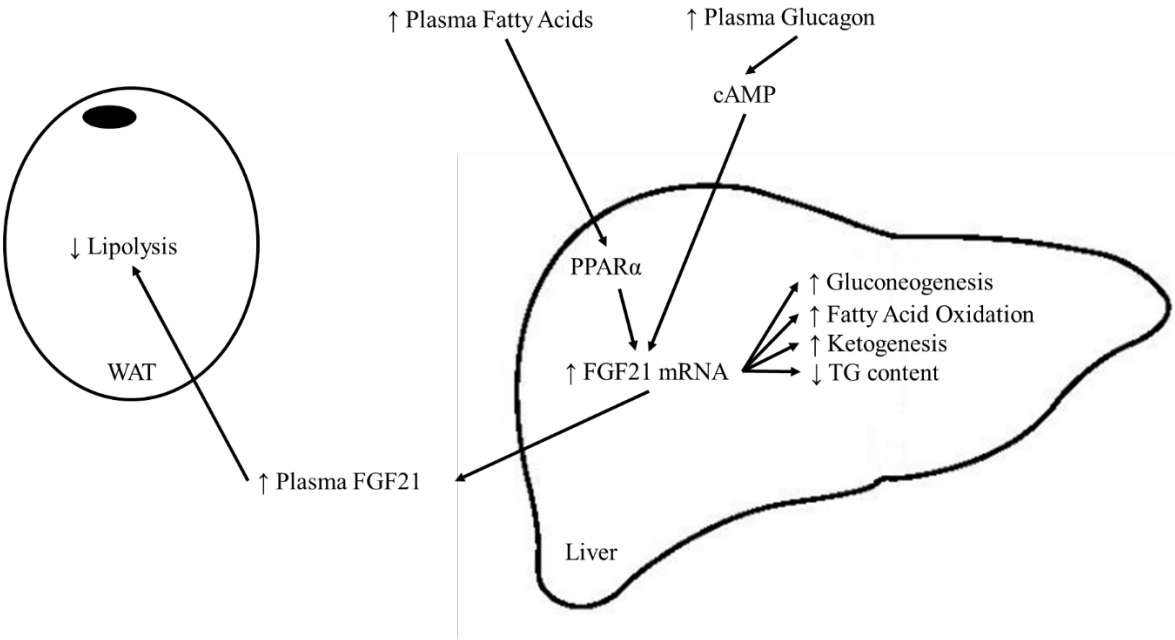

Figure 2.1. Regulation and function of FGF21 signaling representing a possible link between FGF21 and liver and WAT.

Taken together FGF21 actions in other animal models suggest that it could reduce hepatic accumulation of lipids under a variety of conditions. Therefore, this hepatokine may contribute to a better coordination between liver and WAT functions ameliorating the NEB state endured by dairy cows in early lactation.

CONCLUSION

In conclusion, the transition from late gestation to early lactation is a challenging period for dairy cows because of the inability to fulfill their nutritional demands. A complex network of metabolic adaptations take place during this period to overcome the mineral and energy imbalances. These metabolic adaptations, however, fail in a portion of the animals leading to development of diseases and loss of productivity. Despite all the knowledge generated throughout the years, the incidence of metabolic problems remains high in transition dairy cows,

contributing to economic losses. Thus, the need to develop new strategies to improve animal welfare during the transition period of dairy cows.

REFERENCES

Antonellis, P. J., A. Kharitonenkov, and A. C. Adams. 2014. Physiology and Endocrinology Symposium: FGF21: Insights into mechanism of action from preclinical studies. Journal of animal science 92(2):407-413.

Arafat, A. M., P. Kaczmarek, M. Skrzypski, E. Pruszynska-Oszmalek, P. Kolodziejski, D. Szczepankiewicz, M. Sassek, T. Wojciechowicz, B. Wiedenmann, A. F. Pfeiffer, K. W. Nowak, and M. Z. Strowski. 2013. Glucagon increases circulating fibroblast growth factor 21 independently of endogenous insulin levels: a novel mechanism of glucagon-stimulated lipolysis? Diabetologia 56(3):588-597.

Badman, M. K., A. Koester, J. S. Flier, A. Kharitonenkov, and E. Maratos-Flier. 2009. Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis. Endocrinology 150(11):4931-4940.

Badman, M. K., P. Pissios, A. R. Kennedy, G. Koukos, J. S. Flier, and E. Maratos-Flier. 2007. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. Cell metabolism 5(6):426-437.

Bansal, P. and Q. Wang. 2008. Insulin as a physiological modulator of glucagon secretion. American journal of physiology. Endocrinology and metabolism 295(4):E751-761.

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63(9):1514-1529.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 73(9):2804-2819.

Berglund, E. D., L. Kang, R. S. Lee-Young, C. M. Hasenour, D. G. Lustig, S. E. Lynes, E. P. Donahue, L. L. Swift, M. J. Charron, and D. H. Wasserman. 2010. Glucagon and lipid interactions in the regulation of hepatic AMPK signaling and expression of PPARalpha and FGF21 transcripts in vivo. American journal of physiology. Endocrinology and metabolism 299(4):E607-614.

Berry, D. P., N. C. Friggens, M. Lucy, and J. R. Roche. 2016. Milk Production and Fertility in Cattle. Annual review of animal biosciences 4:269-290.

Blanc, C. D., M. Van der List, S. S. Aly, H. A. Rossow, and N. Silva-del-Rio. 2014. Blood calcium dynamics after prophylactic treatment of subclinical hypocalcemia with oral or intravenous calcium. Journal of dairy science 97(11):6901-6906.

Block, S. S., W. R. Butler, R. A. Ehrhardt, A. W. Bell, M. E. Van Amburgh, and Y. R. Boisclair. 2001. Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. The Journal of endocrinology 171(2):339-348.

Bobe, G., R. N. Sonon, B. N. Ametaj, J. W. Young, and D. C. Beitz. 2003. Metabolic responses of lactating dairy cows to single and multiple subcutaneous injections of glucagon. Journal of dairy science 86(6):2072-2081.

Bobe, G., J. C. Velez, D. C. Beitz, and S. S. Donkin. 2009. Glucagon increases hepatic mRNA concentrations of ureagenic and gluconeogenic enzymes in early-lactation dairy cows. Journal of dairy science 92(10):5092-5099.

Bobe, G., J. W. Young, and D. C. Beitz. 2004. Invited review: pathology, etiology, prevention, and treatment of fatty liver in dairy cows. Journal of dairy science 87(10):3105-3124.

Boisclair, Y., S. Wesolowski, J. Kim, and R. Ehrhardt. 2006. Roles of growth hormone and leptin in the periparturient dairy cow. Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress (ed. K Sejrsen, T Hvelplund and MO Nielsen):327-346.

Caixeta, L. S., P. A. Ospina, M. B. Capel, and D. V. Nydam. 2015. The association of subclinical hypocalcemia, negative energy balance and disease with bodyweight change during the first 30 days post-partum in dairy cows milked with automatic milking systems. Veterinary journal (London, England : 1997) 204(2):150-156.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. Journal of dairy science 94(10):4897-4903.

Chapinal, N., S. J. Leblanc, M. E. Carson, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. Journal of dairy science 95(10):5676-5682.

Chau, M. D., J. Gao, Q. Yang, Z. Wu, and J. Gromada. 2010. Fibroblast growth factor 21 regulates energy metabolism by activating the AMPK-SIRT1-PGC-1alpha pathway. Proceedings of the National Academy of Sciences of the United States of America 107(28):12553-12558.

Chen, W., R. L. Hoo, M. Konishi, N. Itoh, P. C. Lee, H. Y. Ye, K. S. Lam, and A. Xu. 2011. Growth hormone induces hepatic production of fibroblast growth factor 21 through a mechanism dependent on lipolysis in adipocytes. The Journal of biological chemistry 286(40):34559-34566.

Coskun, T., H. A. Bina, M. A. Schneider, J. D. Dunbar, C. C. Hu, Y. Chen, D. E. Moller, and A. Kharitonenkov. 2008. Fibroblast growth factor 21 corrects obesity in mice. Endocrinology 149(12):6018-6027.

Curtis, C. R., H. N. Erb, C. J. Sniffen, R. D. Smith, P. A. Powers, M. C. Smith, M. E. White, R. B. Hillman, and E. J. Pearson. 1983. Association of parturient hypocalcemia with eight periparturient disorders in Holstein cows. Journal of the American Veterinary Medical Association 183(5):559-561.

Cyphert, H. A., X. Ge, A. B. Kohan, L. M. Salati, Y. Zhang, and F. B. Hillgartner. 2012. Activation of the farnesoid X receptor induces hepatic expression and secretion of fibroblast growth factor 21. The Journal of biological chemistry 287(30):25123-25138.

De Koster, J. D. and G. Opsomer. 2013. Insulin resistance in dairy cows. The Veterinary clinics of North America. Food animal practice 29(2):299-322.

DeGaris, P. J. and I. J. Lean. 2008. Milk fever in dairy cows: a review of pathophysiology and control principles. Veterinary journal (London, England : 1997) 176(1):58-69.

Ding, X., J. Boney-Montoya, B. M. Owen, A. L. Bookout, K. C. Coate, D. J. Mangelsdorf, and S. A. Kliewer. 2012. betaKlotho is required for fibroblast growth factor 21 effects on growth and metabolism. Cell metabolism 16(3):387-393.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? Journal of dairy science 82(11):2259-2273.

Duffield, T. 2000. Subclinical ketosis in lactating dairy cattle. The Veterinary clinics of North America. Food animal practice 16(2):231-253, v.

Duffield, T. F., D. F. Kelton, K. E. Leslie, K. D. Lissemore, and J. H. Lumsden. 1997. Use of test day milk fat and milk protein to detect subclinical ketosis in dairy cattle in Ontario. The Canadian veterinary journal. La revue veterinaire canadienne 38(11):713-718.

Duffield, T. F., K. D. Lissemore, B. W. McBride, and K. E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. Journal of dairy science 92(2):571-580.

Ehrhardt, R. A., A. Foskolos, S. L. Giesy, S. R. Wesolowski, C. S. Krumm, W. R. Butler, S. M. Quirk, M. R. Waldron, and Y. R. Boisclair. 2016. Increased plasma leptin attenuates adaptive metabolism in early lactating dairy cows. The Journal of endocrinology 229(2):145-157.

Forman, D. and L. Lorenzo. 1991. Ionized calcium: its significance and clinical usefulness. Annals of Clinical & Laboratory Science 21(5):297-304.

Goff, J. P. 2004. Macromineral disorders of the transition cow. The Veterinary clinics of North America. Food animal practice 20(3):471-494, v.

Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Veterinary journal (London, England : 1997) 176(1):50-57.

Goff, J. P. 2014. Calcium and magnesium disorders. The Veterinary clinics of North America. Food animal practice 30(2):359-381, vi.

Goff, J. P. and R. L. Horst. 1997. Physiological changes at parturition and their relationship to metabolic disorders. Journal of dairy science 80(7):1260-1268.

Goff, J. P. and R. L. Horst. 2003. Role of acid-base physiology on the pathogenesis of parturient hypocalcaemia (milk fever)--the DCAD theory in principal and practice. Acta veterinaria Scandinavica. Supplementum 97:51-56.

Green, A. L., J. E. Lombard, L. P. Garber, B. A. Wagner, and G. W. Hill. 2008. Factors associated with occurrence and recovery of nonambulatory dairy cows in the United States. Journal of dairy science 91(6):2275-2283.

Green, H. B., R. L. Horst, D. C. Beitz, and E. T. Littledike. 1981. Vitamin D metabolites in plasma of cows fed a prepartum low-calcium diet for prevention of parturient hypocalcemia. Journal of dairy science 64(2):217-226.

Grummer, R. R. 1993. Etiology of lipid-related metabolic disorders in periparturient dairy cows. Journal of dairy science 76(12):3882-3896.

Grummer, R. R. 1995. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. Journal of animal science 73(9):2820-2833.

Grummer, R. R. 2008. Nutritional and management strategies for the prevention of fatty liver in dairy cattle. Veterinary journal (London, England : 1997) 176(1):10-20.

Grummer, R. R. and D. J. Carroll. 1991. Effects of dietary fat on metabolic disorders and reproductive performance of dairy cattle. Journal of animal science 69(9):3838-3852.

Grunberg, W. 2014. Treatment of phosphorus balance disorders. The Veterinary clinics of North America. Food animal practice 30(2):383-408, vi.

Grunberg, W., S. S. Donkin, and P. D. Constable. 2011. Periparturient effects of feeding a low dietary cation-anion difference diet on acid-base, calcium, and phosphorus homeostasis and on intravenous glucose tolerance test in high-producing dairy cows. Journal of dairy science 94(2):727-745.

Hanigan, M. D., L. A. Crompton, C. K. Reynolds, D. Wray-Cahen, M. A. Lomax, and J. France. 2004. An integrative model of amino acid metabolism in the liver of the lactating dairy cow. Journal of theoretical biology 228(2):271-289.

Hansen, S. S., J. Y. Blom, A. Ersboll, and R. J. Jorgensen. 2003. Milk fever control in Danish dairy herds. Acta veterinaria Scandinavica. Supplementum 97:137-139.

Heppelmann, M., K. Krach, L. Krueger, P. Benz, K. Herzog, M. Piechotta, M. Hoedemaker, and H. Bollwein. 2015. The effect of metritis and subclinical hypocalcemia on uterine involution in dairy cows evaluated by sonomicrometry. The Journal of reproduction and development 61(6):565-569.

Herdt, T. H. 2000. Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. The Veterinary clinics of North America. Food animal practice 16(2):215-230, v.

Horst, R. L., J. P. Goff, and T. A. Reinhardt. 1990. Advancing age results in reduction of intestinal and bone 1,25-dihydroxyvitamin D receptor. Endocrinology 126(2):1053-1057.

Horst, R. L., J. P. Goff, and T. A. Reinhardt. 1994. Calcium and vitamin D metabolism in the dairy cow. Journal of dairy science 77(7):1936-1951.

Inagaki, T. 2015. Research Perspectives on the Regulation and Physiological Functions of FGF21 and its Association with NAFLD. Frontiers in endocrinology 6:147.

Inagaki, T., P. Dutchak, G. Zhao, X. Ding, L. Gautron, V. Parameswara, Y. Li, R. Goetz, M. Mohammadi, V. Esser, J. K. Elmquist, R. D. Gerard, S. C. Burgess, R. E. Hammer, D. J. Mangelsdorf, and S. A. Kliewer. 2007. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell metabolism 5(6):415-425.

Ingvartsen, K. L. and Y. R. Boisclair. 2001. Leptin and the regulation of food intake, energy homeostasis and immunity with special focus on periparturient ruminants. Domestic animal endocrinology 21(4):215-250.

Itoh, N. 2010. Hormone-like (endocrine) Fgfs: their evolutionary history and roles in development, metabolism, and disease. Cell and tissue research 342(1):1-11.

Itoh, N. and D. M. Ornitz. 2004. Evolution of the Fgf and Fgfr gene families. Trends in genetics : TIG 20(11):563-569.

Itoh, N. and D. M. Ornitz. 2008. Functional evolutionary history of the mouse Fgf gene family. Developmental dynamics : an official publication of the American Association of Anatomists 237(1):18-27.

Janovick, N. A., Y. R. Boisclair, and J. K. Drackley. 2011. Prepartum dietary energy intake affects metabolism and health during the periparturient period in primiparous and multiparous Holstein cows. Journal of dairy science 94(3):1385-1400.

Jorritsma, R., H. Jorritsma, Y. H. Schukken, and G. H. Wentink. 2000. Relationships between fatty liver and fertility and some periparturient diseases in commercial Dutch dairy herds. Theriogenology 54(7):1065-1074.

Jorritsma, R., T. Wensing, T. A. Kruip, P. L. Vos, and J. P. Noordhuizen. 2003. Metabolic changes in early lactation and impaired reproductive performance in dairy cows. Veterinary research 34(1):11-26.

Kamgarpour, R., R. C. Daniel, D. C. Fenwick, K. McGuigan, and G. Murphy. 1999. Post partum subclinical hypocalcaemia and effects on ovarian function and uterine involution in a dairy herd. Veterinary journal (London, England : 1997) 158(1):59-67.

Kharitonenkov, A. 2009. FGFs and metabolism. Current opinion in pharmacology 9(6):805-810.

Kharitonenkov, A., J. D. Dunbar, H. A. Bina, S. Bright, J. S. Moyers, C. Zhang, L. Ding, R. Micanovic, S. F. Mehrbod, M. D. Knierman, J. E. Hale, T. Coskun, and A. B. Shanafelt. 2008. FGF-21/FGF-21 receptor interaction and activation is determined by betaKlotho. Journal of cellular physiology 215(1):1-7.

Kharitonenkov, A., T. L. Shiyanova, A. Koester, A. M. Ford, R. Micanovic, E. J. Galbreath, G. E. Sandusky, L. J. Hammond, J. S. Moyers, R. A. Owens, J. Gromada, J. T. Brozinick, E. D. Hawkins, V. J. Wroblewski, D. S. Li, F. Mehrbod, S. R. Jaskunas, and A. B. Shanafelt. 2005. FGF-21 as a novel metabolic regulator. The Journal of clinical investigation 115(6):1627-1635.

Kharitonenkov, A., V. J. Wroblewski, A. Koester, Y. F. Chen, C. K. Clutinger, X. T. Tigno, B. C. Hansen, A. B. Shanafelt, and G. J. Etgen. 2007. The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21. Endocrinology 148(2):774-781.

Kim, H., E. Barton, N. Muja, S. Yakar, P. Pennisi, and D. Leroith. 2005. Intact insulin and insulin-like growth factor-I receptor signaling is required for growth hormone effects on skeletal muscle growth and function in vivo. Endocrinology 146(4):1772-1779.

Kimura, K., T. A. Reinhardt, and J. P. Goff. 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. Journal of dairy science 89(7):2588-2595.

Komatsu, T., F. Itoh, S. Kushibiki, and K. Hodate. 2005. Changes in gene expression of glucose transporters in lactating and nonlactating cows. Journal of animal science 83(3):557-564.

Kragh-Hansen, U. and H. Vorum. 1993. Quantitative analyses of the interaction between calcium ions and human serum albumin. Clinical chemistry 39(2):202-208.

Lassen, J., M. Hansen, M. K. Sorensen, G. P. Aamand, L. G. Christensen, and P. Madsen. 2003. Genetic analysis of body condition score in first-parity Danish Holstein cows. Journal of dairy science 86(12):4123-4128.

Lean, I. J., P. J. DeGaris, D. M. McNeil, and E. Block. 2006. Hypocalcemia in dairy cows: meta-analysis and dietary cation anion difference theory revisited. Journal of dairy science 89(2):669-684.

LeBlanc, S. J., K. E. Leslie, and T. F. Duffield. 2005. Metabolic predictors of displaced abomasum in dairy cattle. Journal of dairy science 88(1):159-170.

Liefers, S. C., R. F. Veerkamp, M. F. te Pas, C. Delavaud, Y. Chilliard, and T. van der Lende. 2003. Leptin concentrations in relation to energy balance, milk yield, intake, live weight, and estrus in dairy cows. Journal of dairy science 86(3):799-807.

Lundasen, T., M. C. Hunt, L. M. Nilsson, S. Sanyal, B. Angelin, S. E. Alexson, and M. Rudling. 2007. PPARalpha is a key regulator of hepatic FGF21. Biochemical and biophysical research communications 360(2):437-440.

Mann, S., F. A. Yepes, M. Duplessis, J. J. Wakshlag, T. R. Overton, B. P. Cummings, and D. V. Nydam. 2016. Dry period plane of energy: Effects on glucose tolerance in transition dairy cows. Journal of dairy science 99(1):701-717.

Martin-Tereso, J. and H. Martens. 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.

Martin-Tereso, J. and M. W. Verstegen. 2011. A novel model to explain dietary factors affecting hypocalcaemia in dairy cattle. Nutrition research reviews 24(2):228-243.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao, and J. E. Santos. 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.

McArt, J. A., D. V. Nydam, and G. R. Oetzel. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. Journal of dairy science 95(9):5056-5066.

McArt, J. A., D. V. Nydam, G. R. Oetzel, and C. L. Guard. 2014. An economic analysis of hyperketonemia testing and propylene glycol treatment strategies in early lactation dairy cattle. Preventive veterinary medicine 117(1):170-179.

McArt, J. A. and G. R. Oetzel. 2015. A stochastic estimate of the economic impact of oral calcium supplementation in postparturient dairy cows. Journal of dairy science 98(10):7408-7418.

McCarthy, M. M., M. S. Piepenbrink, and T. R. Overton. 2015. Associations between hepatic metabolism of propionate and palmitate in liver slices from transition dairy cows. Journal of dairy science 98(10):7015-7024.

McCarthy, M. M., T. Yasui, M. J. Felippe, and T. R. Overton. 2016. Associations between the degree of early lactation inflammation and performance, metabolism, and immune function in dairy cows. Journal of dairy science 99(1):680-700.

McNamara, J. P. and C. E. Murray. 2001. Sympathetic nervous system activity in adipose tissues during pregnancy and lactation of the rat. Journal of dairy science 84(6):1382-1389.

Melendez, P., G. A. Donovan, C. A. Risco, and J. P. Goff. 2004. Plasma mineral and energy metabolite concentrations in dairy cows fed an anionic prepartum diet that did or did not have retained fetal membranes after parturition. American journal of veterinary research 65(8):1071-1076.

Moore, S. J., M. J. VandeHaar, B. K. Sharma, T. E. Pilbeam, D. K. Beede, H. F. Bucholtz, J. S. Liesman, R. L. Horst, and J. P. Goff. 2000. Effects of altering dietary cation-anion difference on calcium and energy metabolism in peripartum cows. Journal of dairy science 83(9):2095-2104.

Murray, R. D., J. E. Horsfield, W. D. McCormick, H. J. Williams, and D. Ward. 2008. Historical and current perspectives on the treatment, control and pathogenesis of milk fever in dairy cattle. The Veterinary record 163(19):561-565.

Nishimura, T., Y. Nakatake, M. Konishi, and N. Itoh. 2000. Identification of a novel FGF, FGF-21, preferentially expressed in the liver. Biochimica et biophysica acta 1492(1):203-206.

Oetzel, G. R. 2013. Oral calcium supplementation in peripartum dairy cows. The Veterinary clinics of North America. Food animal practice 29(2):447-455.

Oetzel, G. R. and B. E. Miller. 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.

Oetzel, G. R., J. D. Olson, C. R. Curtis, and M. J. Fettman. 1988. Ammonium chloride and ammonium sulfate for prevention of parturient paresis in dairy cows. Journal of dairy science 71(12):3302-3309.

Ogawa, Y., H. Kurosu, M. Yamamoto, A. Nandi, K. P. Rosenblatt, R. Goetz, A. V. Eliseenkova, M. Mohammadi, and M. Kuro-o. 2007. BetaKlotho is required for metabolic activity of fibroblast growth factor 21. Proceedings of the National Academy of Sciences of the United States of America 104(18):7432-7437.

Ohtani, Y., T. Takahashi, K. Sato, A. Ardiyanti, S. H. Song, R. Sato, K. Onda, Y. Wada, Y. Obara, K. Suzuki, A. Hagino, S. G. Roh, and K. Katoh. 2012. Changes in circulating adiponectin and metabolic hormone concentrations during periparturient and lactation periods in Holstein dairy cows. Animal science journal = Nihon chikusan Gakkaiho 83(12):788-795.

Ong, G. S., J. P. Walsh, B. G. Stuckey, S. J. Brown, E. Rossi, J. L. Ng, H. H. Nguyen, G. N. Kent, and E. M. Lim. 2012. The importance of measuring ionized calcium in characterizing calcium status and diagnosing primary hyperparathyroidism. The Journal of clinical endocrinology and metabolism 97(9):3138-3145.

Ospina, P. A., J. A. McArt, T. R. Overton, T. Stokol, and D. V. Nydam. 2013. Using nonesterified fatty acids and beta-hydroxybutyrate concentrations during the transition period for herd-level monitoring of increased risk of disease and decreased reproductive and milking performance. The Veterinary clinics of North America. Food animal practice 29(2):387-412.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010a. Association between the proportion of sampled transition cows with increased nonesterified fatty acids and beta-hydroxybutyrate and disease incidence, pregnancy rate, and milk production at the herd level. Journal of dairy science 93(8):3595-3601.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010b. Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. Journal of dairy science 93(4):1596-1603.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010c. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. Journal of dairy science 93(2):546-554.

Overton, T. R. and T. Yasui. 2014. Practical applications of trace minerals for dairy cattle. Journal of animal science 92(2):416-426.

Park, H. K. and R. S. Ahima. 2015. Physiology of leptin: energy homeostasis, neuroendocrine function and metabolism. Metabolism: clinical and experimental 64(1):24-34.

Potthoff, M. J., T. Inagaki, S. Satapati, X. Ding, T. He, R. Goetz, M. Mohammadi, B. N. Finck, D. J. Mangelsdorf, S. A. Kliewer, and S. C. Burgess. 2009. FGF21 induces PGC-1alpha and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. Proceedings of the National Academy of Sciences of the United States of America 106(26):10853-10858.

Rabelo, E., R. L. Rezende, S. J. Bertics, and R. R. Grummer. 2005. Effects of pre- and postfresh transition diets varying in dietary energy density on metabolic status of periparturient dairy cows. Journal of dairy science 88(12):4375-4383.

Radcliff, R. P., B. L. McCormack, B. A. Crooker, and M. C. Lucy. 2003. Growth hormone (GH) binding and expression of GH receptor 1A mRNA in hepatic tissue of periparturient dairy cows. Journal of dairy science 86(12):3933-3940.

Ramos-Nieves, J. M., B. J. Thering, M. R. Waldron, P. W. Jardon, and T. R. Overton. 2009. Effects of anion supplementation to low-potassium prepartum diets on macromineral status and performance of periparturient dairy cows. Journal of dairy science 92(11):5677-5691.

Reid, I. M. 1980. Incidence and severity of fatty liver in dairy cows. The Veterinary record 107(12):281-284.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Veterinary journal (London, England : 1997) 188(1):122-124.

Reynolds, C. K., P. C. Aikman, B. Lupoli, D. J. Humphries, and D. E. Beever. 2003. Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of dairy science 86(4):1201-1217.

Reynolds, C. K., M. C. Bell, and M. H. Sims. 1984. Changes in plasma, red blood cell and cerebrospinal fluid mineral concentrations in calves during magnesium depletion followed by repletion with rectally infused magnesium chloride. The Journal of nutrition 114(7):1334-1341.

Rhoads, R. P., J. W. Kim, M. E. Van Amburgh, R. A. Ehrhardt, S. J. Frank, and Y. R. Boisclair. 2007. Effect of nutrition on the GH responsiveness of liver and adipose tissue in dairy cows. The Journal of endocrinology 195(1):49-58.

Risco, C. A., J. P. Reynolds, and D. Hird. 1984. Uterine prolapse and hypocalcemia in dairy cows. Journal of the American Veterinary Medical Association 185(12):1517-1519.

Rosenbaum, M. and R. L. Leibel. 2014. 20 years of leptin: role of leptin in energy homeostasis in humans. The Journal of endocrinology 223(1):T83-96.

Sava, L., S. Pillai, U. More, and A. Sontakke. 2005. Serum calcium measurement: Total versus free (ionized) calcium. Indian journal of clinical biochemistry : IJCB 20(2):158-161.

Schlegel, G., R. Ringseis, J. Keller, F. J. Schwarz, W. Windisch, and K. Eder. 2012. Expression of fibroblast growth factor 21 in the liver of dairy cows in the transition period and during lactation. Journal of animal physiology and animal nutrition.

Schoenberg, K. M., S. L. Giesy, K. J. Harvatine, M. R. Waldron, C. Cheng, A. Kharitonenkov, and Y. R. Boisclair. 2011. Plasma FGF21 is elevated by the intense lipid mobilization of lactation. Endocrinology 152(12):4652-4661.

Seifi, H. A., S. J. Leblanc, K. E. Leslie, and T. F. Duffield. 2011. Metabolic predictors of post-partum disease and culling risk in dairy cattle. Veterinary journal (London, England : 1997) 188(2):216-220.

Sepulveda-Varas, P., D. M. Weary, M. Noro, and M. A. von Keyserlingk. 2015. Transition diseases in grazing dairy cows are related to serum cholesterol and other analytes. PloS one 10(3):e0122317.

Strang, B. D., S. J. Bertics, R. R. Grummer, and L. E. Armentano. 1998. Relationship of triglyceride accumulation to insulin clearance and hormonal responsiveness in bovine hepatocytes. Journal of dairy science 81(3):740-747.

Suzuki, M., Y. Uehara, K. Motomura-Matsuzaka, J. Oki, Y. Koyama, M. Kimura, M. Asada, A. Komi-Kuramochi, S. Oka, and T. Imamura. 2008. betaKlotho is required for fibroblast growth factor (FGF) 21 signaling through FGF receptor (FGFR) 1c and FGFR3c. Molecular endocrinology (Baltimore, Md.) 22(4):1006-1014.

Sweeney, B., E. Martens, M. Felippe, and T. Overton. 2014. Impacts and Evaluation of Subclinical Hypocalcemia in Dairy Cattle. in Proc. Cornell Nutrition Conference.

van Mosel, M., A. T. van't Klooster, F. van Mosel, and J. van der Kuilen. 1993. Effects of reducing dietary [(Na+ + K+) - (Cl- + SO4=)] on the rate of calcium mobilisation by dairy cows at parturition. Research in veterinary science 54(1):1-9.

Veenhuizen, J. J., J. K. Drackley, M. J. Richard, T. P. Sanderson, L. D. Miller, and J. W. Young. 1991. Metabolic changes in blood and liver during development and early treatment of experimental fatty liver and ketosis in cows. Journal of dairy science 74(12):4238-4253.

Vernia, S., J. Cavanagh-Kyros, L. Garcia-Haro, G. Sabio, T. Barrett, D. Y. Jung, J. K. Kim, J. Xu, H. P. Shulha, M. Garber, G. Gao, and R. J. Davis. 2014. The PPARalpha-FGF21 hormone axis contributes to metabolic regulation by the hepatic JNK signaling pathway. Cell metabolism 20(3):512-525.

Walsh, S., F. Buckley, K. Pierce, N. Byrne, J. Patton, and P. Dillon. 2008. Effects of breed and feeding system on milk production, body weight, body condition score, reproductive performance, and postpartum ovarian function. Journal of dairy science 91(11):4401-4413.

Wang, B., S. Y. Mao, H. J. Yang, Y. M. Wu, J. K. Wang, S. L. Li, Z. M. Shen, and J. X. Liu. 2014. Effects of alfalfa and cereal straw as a forage source on nutrient digestibility and lactation performance in lactating dairy cows. Journal of dairy science 97(12):7706-7715.

Whiteford, L. C. and I. M. Sheldon. 2005. Association between clinical hypocalcaemia and postpartum endometritis. The Veterinary record 157(7):202-203.

Wilde, D. 2006. Influence of macro and micro minerals in the peri-parturient period on fertility in dairy cattle. Animal reproduction science 96(3-4):240-249.

Yie, J., R. Hecht, J. Patel, J. Stevens, W. Wang, N. Hawkins, S. Steavenson, S. Smith, D. Winters, S. Fisher, L. Cai, E. Belouski, C. Chen, M. L. Michaels, Y. S. Li, R. Lindberg, M. Wang, M. Veniant, and J. Xu. 2009. FGF21 N- and C-termini play different roles in receptor interaction and activation. FEBS letters 583(1):19-24.

Zarrin, M., O. Wellnitz, and R. M. Bruckmaier. 2015. Conjoint regulation of glucagon concentrations via plasma insulin and glucose in dairy cows. Domestic animal endocrinology 51:74-77.

CHAPTER 3: The association of subclinical hypocalcemia, negative energy balance and disease with bodyweight change during the first 30 days post-partum in dairy cows milked with automatic milking systems*

* Caixeta LS, Ospina PA, Capel MB, Nydam DV. The association of subclinical hypocalcemia, negative energy balance and disease with bodyweight change during the first 30 days post-partum in dairy cows milked with automatic milking systems. Veterinary Journal. 2015;204:150-156.

ABSTRACT

In a prospective cohort study, the daily bodyweight (**BW**) and milk production of 92 cows were recorded using automatic milking systems. The objectives were to characterize calcium serum concentration variability on days 1–3 post-partum and to evaluate the association between subclinical hypocalcemia (**SHPC**) and change in BW over the first 30 days in milk (**DIM**) in Holstein dairy cows, while controlling for concurrent disease and negative energy balance (**NEB**). SHPC was defined as total serum calcium concentration between 6 and 8 mg/dL, NEB was inferred by non-esterified fatty acids (**NEFA**) > 0.7 mEq/L or β-hydroxybutyrate (**BHB**) ≥ 1.2 mmol/L.

The peak incidence of SHPC was at 1 DIM for all groups (11%, 42% and 60% for parities 1, 2, and ≥ 3, respectively). All parity groups lost weight (21, 33, and 34 kg) during the first 30 DIM. Parity 1 animals with disease compared with those without disease lost the most weight (2.6 kg/day BW loss vs. < 1.9 kg/day, respectively). Normocalcemic parity 2 animals with either NEB or disease lost the most weight (> 5 kg/day) compared with those in the SHPC group (≤ 4.5 kg/day). In parity ≥ 3 animals, SHPC was an important factor for BW loss; SHPC animals lost the most weight (> 3.7 kg/day) vs. normocalcemic cows (≤ 3.3 kg/day) regardless of NEB or disease status. Even though all animals lost weight during early lactation the effect of disease, NEB, and SHPC on BW loss was different in each parity group.

Key Words: Automatic milking systems, bodyweight, dairy cows, subclinical hypocalcemia

INTRODUCTION

The transition period is challenging for both cows and producers due to the rapid increase in energy and mineral demands to support milk production after parturition (Bell, 1995, DeGaris and Lean, 2008). Although homeorhetic and homeostatic adaptations occur in an effort to maintain a physiological mineral balance (Bauman and Currie, 1980) and metabolic changes take place to ensure there is sufficient energy to support maintenance requirements and the physiological state of lactation, cows are at greatest risk of developing diseases early in the post-partum period (LeBlanc et al., 2005).

Despite these adaptations, dairy cows frequently experience a decrease in calcium concentration early post-partum. Abnormal physiological calcium concentrations can be classified into clinical (Ca < 5.6 mg/dL; DeGaris and Lean, 2008) and subclinical hypocalcemia (SHPC; Ca 6 to 8 mg/dL; Reinhardt et al., 2011). The incidence of clinical hypocalcemia is low: 1% in first parity animals and 6% in older animals (Reinhardt et al., 2011); however, the incidence of SHPC has been reported to be much higher; 25% for first lactation and 48% in older animals (Reinhardt et al., 2011). Although there are no clinical signs associated with subclinical hypocalcemia (**SHPC**), the increased disease incidence (e.g. displaced abomasum, metritis) associated with low blood Ca concentration within the first 30 days in milk (**DIM**) (Chapinal et al., 2012, Martinez et al., 2012) makes it an important factor.

In addition to the diseases associated with SHPC, the ability to mobilize energy in order to balance the deficit resulting from milk production and decreased dry matter intake (**DMI**) is important for health (Ospina et al., 2010b) and milk production. Lipid reserves are mobilized as non-esterified fatty acids (**NEFA**) and are used for energy in both the mammary gland and non-mammary tissues (Bell, 1995). NEFA are also oxidized by the liver to ketone bodies, including

β-hydroxybutyrate (**BHB**), which can also be used as energy sources (Drackley et al., 2001). Plasma NEFA and BHB above various thresholds is strongly associated with detrimental effects on health and production (Ospina et al., 2010b, a, Chapinal et al., 2012). The increased mobilization of NEFA and BHB production have been associated with changes in bodyweight (**BW**) and consequently body condition score (**BCS**), especially for high yield milking animals (Weber et al., 2013).

The evaluation of the change in BW over time as well as BCS can be used as a proxy for negative energy balance in transition cows; however, concurrent disease and a mineral imbalance may exacerbate BW loss during this period. Recently Weber et al. (2013) showed that, high liver fat and high concentrations of NEFA post-partum were correlated to BW loss.

Dairy herds with automatic milking systems (**AMS**) may find that the evaluation of BW change over time is a useful metric for energy balance in transition cows because both daily milk production and BW are recorded by the units. Understanding the relationship between calcium, negative energy balance indicators, milk production, and BW dynamics around parturition is important for better decision making and management of cows in the transition period.

The objectives of the present study were to characterize calcium serum concentration variability on days 1–3 post-partum and to evaluate the association between SHPC and change in BW over the first 30 DIM in Holstein dairy cows, while controlling for concurrent disease and negative energy balance.

MATERIALS AND METHODS

Study population and study design

A prospective cohort study was conducted in three commercial dairy farms in central New York State. To be included in the study a herd had (1) to have more than100 milking cows; (2) be in free-stall housing; (3) be fed a partial mixed ration (**PMR**), and (4) to use a Lely AMS (Astronaut A3 or A4, Lely Industries N.V.).

Within herds, all cows and heifers calving from 11 June until 8 August 2012 were enrolled in the study. These animals were followed up until 30 DIM. The goal was to enroll a minimum of 85 animals in the study in order to find a 1 kg difference in daily BW change between groups at the individual animal level, with a 1.5 kg standard deviation, 95% confidence interval and power of 85%.

The PMR consisted of 80% forage and 20% concentrate during the dry period, and 55% forage and 45% concentrate for milking cows. The diets were formulated to meet or exceed the NRC nutrient requirements for lactating Holsteins and complementary pelleted grain was offered in the robots during milking. In the North Eastern United States, anionic salts are not commonly used as dairy herds manage the potassium content of dry cow diets through manipulation of manure application fields and forage choice, e.g. limiting alfalfa feeding to dry cows. None of the herds in this study were feeding a diet where DCAD was managed by the addition of anionic salts during the study period.

Data collection

Enrollment for each cow in the study occurred weekly between 3 and 10 days prior to the expected calving date. Blood was collected from the coccygeal vessels; within 30 min of collection the blood samples were spun for 15 min at 2000g. Serum was harvested and stored at

-20 °C following the recommendations of Stokol and Nydam (2005). Five blood samples were collected from each cow over several days, at similar times each day, namely, pre-partum (7 ± 3 days before expected calving), within 24 h of calving, and on days 2, 3, and 5 post-partum. Total calcium, NEFA, and BHB concentrations were measured depending on the day (Figure 3.1).

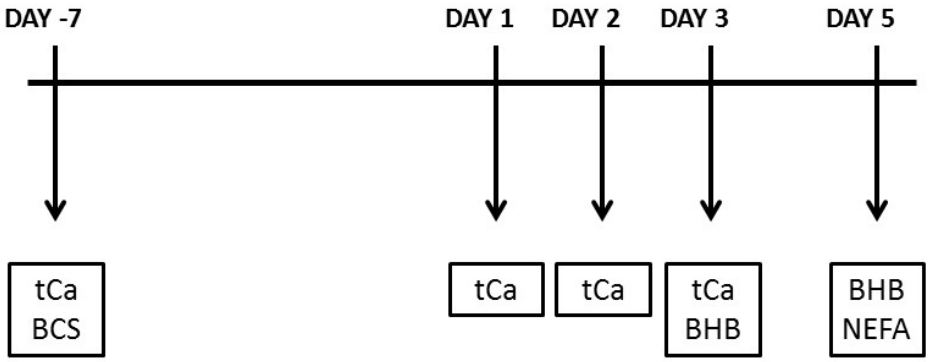

Figure 3.1. Sampling timeline for the animals enrolled in the study.
Calving was used as reference (day = 0). tCa = total calcium; NEFA = non-esterified fatty acid; BHB = β-hydroxybutyrate.

Serum samples were sent to Cornell University Animal Health Diagnostic Center for total calcium concentration (Roche Diagnostic reagents) and serum NEFA concentration analysis (NEFA-C, Wako Chemicals). The Precision Xtra meter (Abbott Laboratories) was used to evaluate BHB concentrations at the cow side (Iwersen et al., 2009).

Information on daily BW and daily milk production, from day 0 up to 30 DIM were downloaded from reports on the AMS system. Information as to whether animals developed any disease (e.g., displaced abomasum, clinical ketosis, metritis, milk fever, and retained placenta) was documented in Dairy Comp 305 (Valley Agricultural Software) from two of the three herds.

Standard disease definitions were discussed with the farms at the start of the study although each farm implemented their own standardized care. The standard disease definitions were as follows: (1) displaced abomasum – movement of the abomasum to a location on the left side of the cow, which was detected by auscultating a 'ping' sound with finger percussion; (2) clinical ketosis– a cow that was off feed, positive ketone test, and having decreased milk production, with no other detectable signs of disease; cases were treated with propylene glycol (Duffield et al., 1999); (3) metritis – sick cow (dull, decreased milk yield) that had a temperature > 39.5 °C with a fetid (purulent or red to brown color or both) vulval discharge and was < 21 DIM (Sheldon et al., 2006); and (4) retained fetal membranes – failure to expel membranes within 24 h after calving.

Statistical analysis

Daily BW information was imported from the Lely T4C management system (Lely Industries) into Excel (Microsoft). The daily BW was plotted against time to evaluate its change over time and a simple linear regression line of best fit was estimated. The slope from the line of best fit was used as the estimate of change in BW for each animal during the first 30 DIM. Because the slope was used in the statistical analysis, the effect of individual missing points was negligible. The change in BW over the first 30 DIM was used as the dependent variable in the analysis.

Total milk production over the first 30 DIM was also evaluated using similar methods. The data were imported into Excel from the T4C management system and a line of best fit was used to estimate any missing values. The sum of all values over the first 30 DIM was used to estimate total milk production over the first 30 DIM. Total milk production over the first 30 DIM was used as an explanatory variable in the analysis.

Descriptive statistics were generated with the FREQ, MEANS, and BOX PLOT procedures of SAS version 9.3 (SAS Institute). The *t* test was used to evaluate the mean difference in change in BW within first 30 DIM between parity groups (parity = 1, parity = 2, and parity ≥ 3). Statistical analyses to determine meaningful predictors of change in BW were conducted in SAS using the MIXED procedure. The LSMEANS with the DIFF option was used to evaluate the interaction terms. The development of any disease (displaced abomasum, metritis, clinical ketosis, or retained placenta) was dichotomized if it occurred within 10 DIM. The following were dichotomized at a concentration below or above which animals were more likely to develop disease: pre-partum NEFA was dichotomized at ≥ 0.3 mEq/L (Ospina et al., 2010b); post-partum NEFA ≥ 0.7 mEq/L (Ospina et al., 2010b), and BHB at ≥ 1.2 mmol/L (McArt et al., 2012). Subclinical hypocalcemia was determined by total calcium concentrations between 6 and 8.0 mg/dL (Reinhardt et al., 2011). The NEFA and BHB predictors were included in the analysis as one dichotomous variable (**NEB**) and considered positive if any measured concentration was above predetermined cut-points. The cows were considered positive for SHPC if any calcium measured post-partum was between 6 and 8 mg/dL.

The evaluation of change in BW was stratified into three parity groups (parity = 1, parity = 2, parity ≥ 3) and within these groups the following model was evaluated: the fixed effects of SHPC, NEB, the development of any disease, total milk production within the first 30 DIM, and two sets of interactions: SHPC and NEB; and SHPC and disease, with herd as a random effect. Interactions and potential explanatory variables were removed in a manual backward stepwise fashion if $P > 0.1$, the interaction with the largest *P*-value was removed first, then the explanatory variable if necessary.

RESULTS

Descriptive results

The herds enrolled in this study had 200 (Herd A), 400 (Herd C), and 700 (Herd B) milking cows, and 4, 7, and 14 AMS, respectively. In total, 114 animals were enrolled during the study period. Nine animals were excluded: three animals died, two were culled by farm personnel before 30 DIM and four were excluded because of incomplete data collection due to problems in the AMS. Herd A was excluded from the statistical model because this herd did not use Dairy Comp 305 for health record keeping, which could lead to missing information. Ninety-two animals were available for the multivariable analysis (Table 3.1).

Table 3.1. Distribution of the animals enrolled in the study divided into different parity groups by herd.

Parity group	Herd A	Herd B	Herd C	Total
Parity = 1	5	17	13	35 (33%)
Parity = 2	2	9	18	29 (28%)
Parity ≥ 3	6	13	22	41(39%)
Total	13	39	53	105

During the study period, the average disease incidence at the herd level was 18% for clinical ketosis (range: 0–21%), 1% for milk fever (range: 0–2.5%), 5% for displaced abomasum (range: 0–8%), 8% for retained fetal membranes (range: 0–9.5%), and 10% for metritis (range: 0–18%) (Figure 3.2).

Blood calcium concentration, by lactation and DIM is presented in a box and whisker plot (Figure 3.3). Most first lactation animals were normocalcemic, and only 6% of cows with parity ≥ 3 were clinically hypocalcemic; however, the majority (60%) of the animals in this parity

group experienced SHPC at 1 DIM. The prevalence of SHPC on day 1, 2, or 3 was 17% for first parity animals, 55% for second parity animals, and 73% of animals with parity ≥ 3.

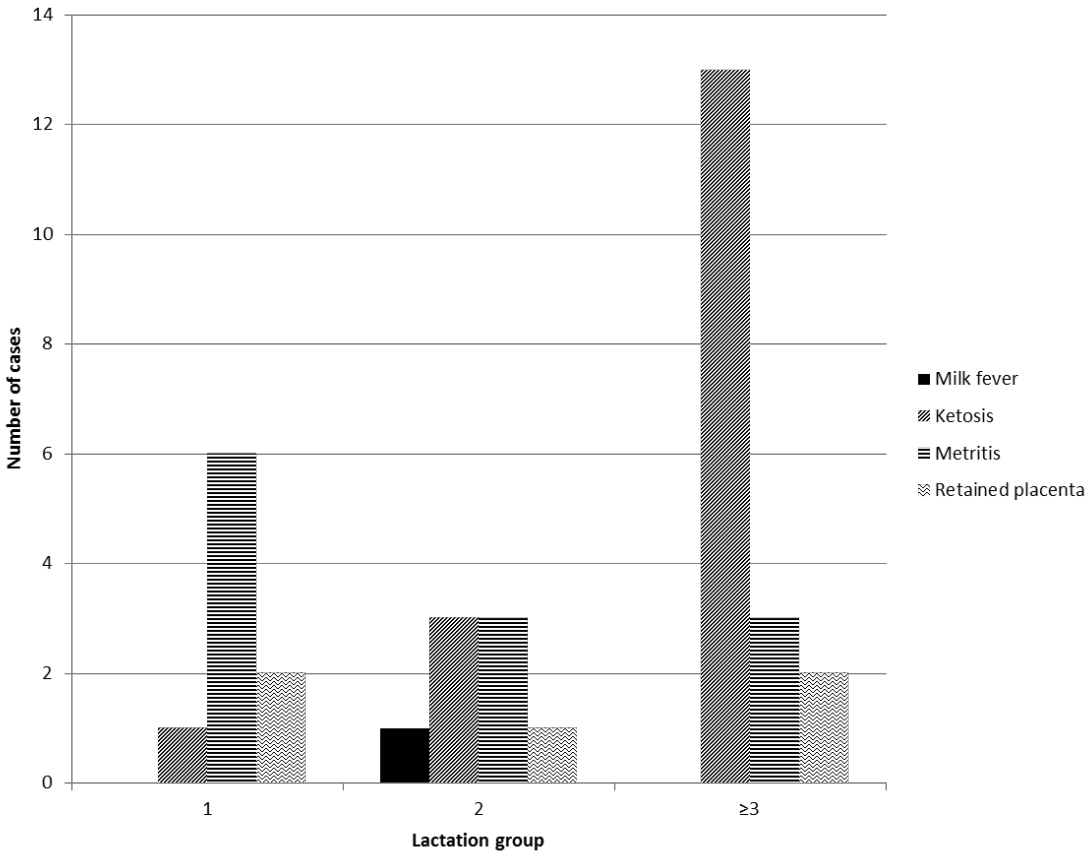

Figure 3.2. Incidence of disease in the first 10 DIM in the study population stratified by lactation group.
The diseases of interest were: milk fever, ketosis, metritis and retained placenta. Lactation group was defined as: 1 = first parity animals, 2 = second parity animals, ≥3 = third and greater parity animals.

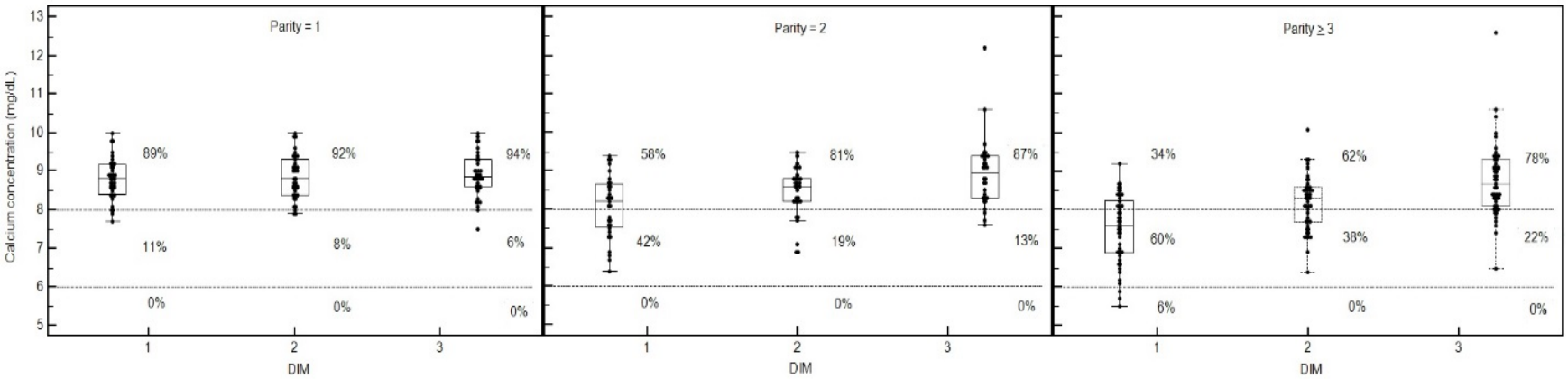

Figure 3.3. Serum calcium concentrations (Ca) for all animals enrolled in the study.
Samples were collected in the first 24 h after calving (1 DIM) and on days 2 and 3 after calving (2 and 3 DIM). Calcium concentration results are stratified by lactation group. The two horizontal lines define the hypocalcemia levels: subclinical hypocalcemia as those with calcium concentrations between 6 and 8 mg/dL, and clinical hypocalcemia as those with calcium concentrations lower than 6 mg/dL. The percentage of animals presenting normocalcemia, subclinical hypocalcemia, and clinical hypocalcemia in the first 3 DIM is shown.

Inferential results

There was no meaningful difference in milk production between the herds; daily average milk production was 32.6 kg/day for herd A and the average milk production for the first 30 days was 980 kg, for herd B it was 36.2 kg/day and the 30 day average milk production was 1085 kg; for herd C it was 35 kg/day and 1050 kg, respectively ($P = 0.7$). All animals lost weight in the first 30 DIM; however, on average, parity ≥ 3 animals lost the most weight (33.6 kg), while parity 2 lost 32.7 kg and parity 1 animals lost 21.3 kg.

The mean change in BW was different between parities ($P < 0.001$), with one exception: there was no difference between change in BW in normocalcemic animals in parity 2 and normocalcaemic animals in parity ≥ 3 ($P = 0.07$). There was a difference in BW change when comparing across calcium status ($P < 0.001$) within parity, i.e. animals with SHPC lost weight faster than those with normocalcemia (Figure 3.4). The BW loss was 19 and 39 kg, for parity 1; 29 and 38 kg for parity 2, and 36 and 53 kg for parity ≥ 3 for normocalcemic and SHPC animals, respectively.

The multivariable results are found in Table 3.2 where final models were stratified by parity. The results of the interactions from these models and the effect on BW are found in Figures 3.5–3.7.

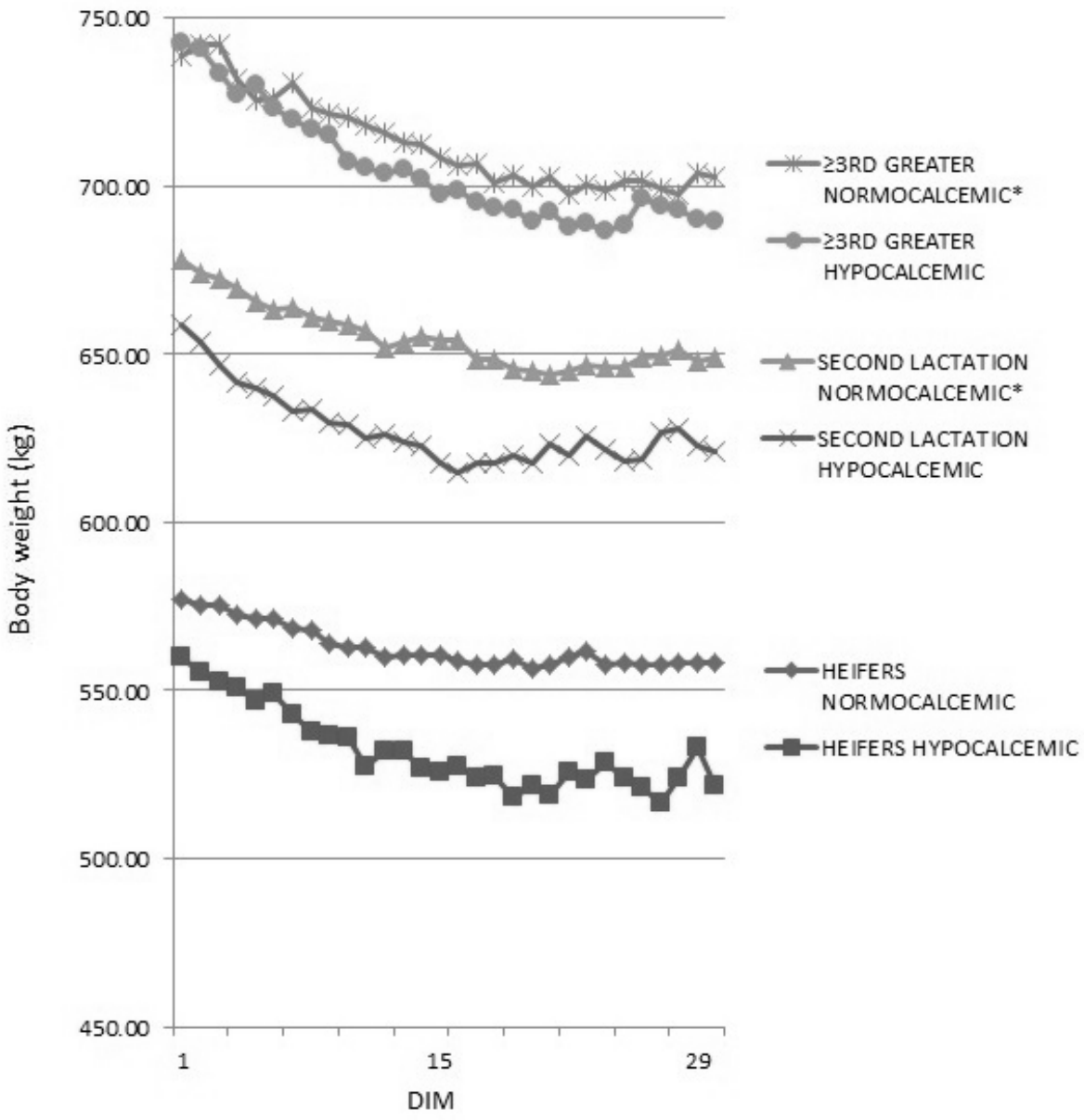

Figure 3.4. Bodyweight change over the first 30 DIM was stratified by parity and within parity by SHPC status.
Animals with SHPC were defined as having at least one calcium concentration between 6 and 8 mg/dL on days 1, 2, or 3, and normocalcemia was defined as having no measured calcium concentration below 8 mg/dL. All animals lost weight and weight loss was different between parity groups ($P < 0.001$), with one exception. Normocalcemic second lactation animals (*) and normocalcemic $\geq$ 3 lactation animals (*) did not have different bodyqeight slopes over the first 30 DIM ($P = 0.07$). Within all parity groups, animals with SHPC lost more weight than those with normocalcemia ($P < 0.001$).

Parity 1

Subclinical hypocalcemia, disease, milk production and the interaction between SHPC and disease were associated with change in BW in first lactation animals ($P < 0.001$). Negative energy balance ($P = 0.2$) and the interaction between SHPC and NEB ($P = 0.3$) were not associated with BW change and thus were removed from the final model. With every additional 45 kg of milk produced, heifers lost 0.3 kg/day. The interaction between SHPC and disease showed that normocalcemic animals with disease lost the most weight (2.6 kg/day), while those with neither hypocalcemia nor disease lost the least weight (0.8 kg/day) (Figure 3.5).

Parity 2

Milk production was not associated with the change in BW ($P = 0.3$) and was removed from the final model. Although there was a 10% chance of committing a type I error with the interaction between SHPC and NEB, it was retained in the model so that the differences in the combinations could be analyzed. The interaction between SHPC and disease and SHPC and NEB, demonstrated that animals with normocalcemia, but with either disease or NEB lost the more weight (5.1 and 5.2 kg/day, respectively) than the other combinations within their respective interactions (Figure 3.6).

Parity ≥ 3

All explanatory variables were associated with change in BW. The change in BW based on milk production was statistically significant, but numerically unimportant; for every additional 45 kg of milk, the animal gained 0.1 kg/day (Table 3.2). Animals with SHPC regardless of disease or NEB status lost weight faster (≥ 3.7 kg/day) than those without SHPC (< 3.3 kg/day) (Figure 3.7). In addition, animals with both SHPC and disease or SHPC and NEB lost weight faster than those with just disease or NEB combined with normocalcemia.

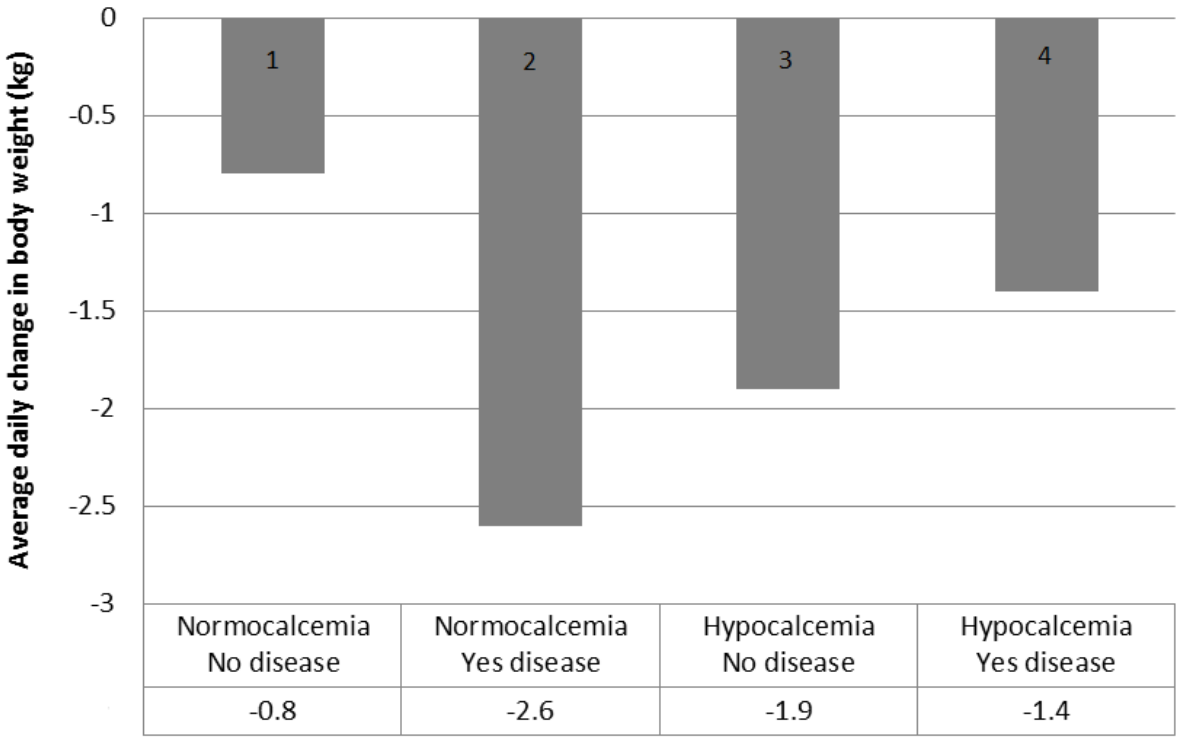

Figure 3.5. Change in daily bodyweight based on the interaction between subclinical hypocalcemia and disease in parity = 1 animals.
Subclinical hypocalcemia was defined as at least one calcium concentration between 6 and 8 mg/dL on days 1, 2, or 3, and disease was defined as any disease within 10 DIM (displaced abomasum, retained placenta, metritis, milk fever, or ketosis). Normocalcemia defined as having no measured calcium reading < 8 mg/dL. Results based on the interaction term in the model. All contrast terms are significantly different from 0 and all are significantly different from each other ($P < 0.01$). The number of animals in each combination group is: 20, 5, 3, and 2 in columns 1, 2, 3, and 4, respectively.

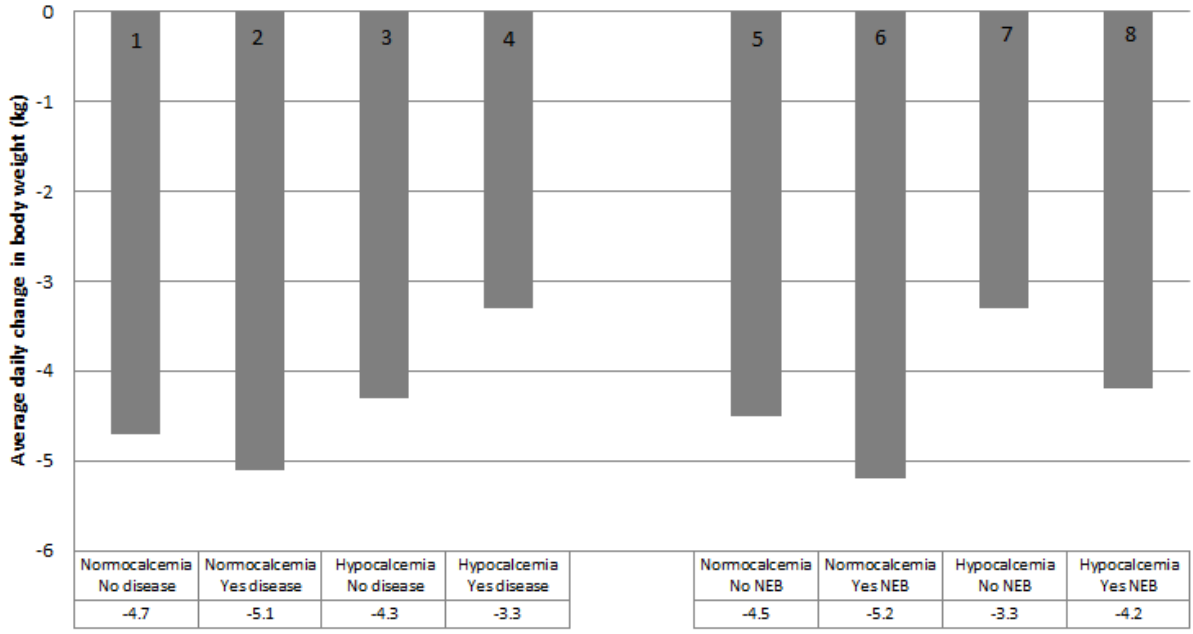

Figure 3.6. Change in daily bodyweight based on the interaction between subclinical hypocalcemia and disease, and subclinical hypocalcemia and negative energy balance in parity = 2 animals.

Subclinical hypocalcemia was defined as at least one calcium concentration between 6 and 8 mg/dL on days 1, 2, or 3. Disease was defined as the occurrence of any disease (displaced abomasum, retained placenta, metritis, milk fever, or ketosis) within 10 DIM. Negative energy balance was defined as at least one β-hydroxybutyrate concentration ≥ 1.2 mmol/L on days 1, 2, or 3; or non-esterified fatty acid concentration ≥ 0.7 mEq/L at 5 DIM. Normocalcemia defined as having no measured calcium reading < 8 mg/dL. Results based on the interaction term in the model. All contrast terms are significantly different than 0 ($P < 0.01$) and all are significantly different from each other ($P < 0.05$). The number of animals in the disease combination group is: 12, 1, 10, 4 for columns 1–4, respectively; and 7, 6, 5, 9 for NEB columns 5–8, respectively.

Table 3.2. Multivariable analysis stratified by parity of the effect of subclinical hypocalcemia (SHPC)[a] on change in bodyweight (kg/day) while controlling for disease[b], negative energy balance (NEB)[c], milk[d], and the interaction between SHPC and disease and SHPC and NEB; herd treated as a random effect.

Effect	Estimate (kg/day)	Standard error	*P*-value
Parity = 1			
Intercept	2	0.4	<0.0001
SHPC[a]	-2.6	0.2	<0.0001
Disease[b]	-1	0.2	<0.0001
SHPC x disease	4.9	0.2	<0.0001
Milk[d]	-0.3	0.01	<0.0001
Parity = 2			
Intercept	-8.2	0.5	<0.001
SHPC[a]	-3.8	0.5	<0.001
Disease[b]	-2.2	0.3	<0.002
NEB[c]	1.9	0.3	<0.003
SHPC x disease	3.1	0.5	<0.004
SHPCP x NEB	-0.5	0.3	0.1
Parity $\geq$ 3			
Intercept	-11.6	0.7	<0.001
SHPC[a]	2.6	0.3	<0.001
Disease[b]	0.8	0.2	<0.001
NEB[c]	0.5	0.2	0.01
SHPCP x disease	-2.2	0.4	<0.001
SHPC x NEB	0.8	0.4	0.06
Milk[d]	0.1	0.02	<0.001

[a]SHPC defined as positive if any measured total calcium concentration was between 6 and 8mg/dL on day 1, 2, or3.
[b]Disease defined as positive if animal developed any of the following diseases within 10 days in milk: displaced abomasum, retained placenta, metritis or clinical ketosis.
[c]NEB defined as positive if any of the following concentrations were above the cut-point: pre-partum non-esterified fatty acids (NEFA) $\geq$ 0.3mEq/L, β-hydroxybutyrate (BHB) $\geq$ 1.2 mmol/L, post-partum NEFA $\geq$ 0.7 mEq/L.
[d]One unit change in milk is equivalent to an increase in 45.5kg of total milk in the first 30 DIM.

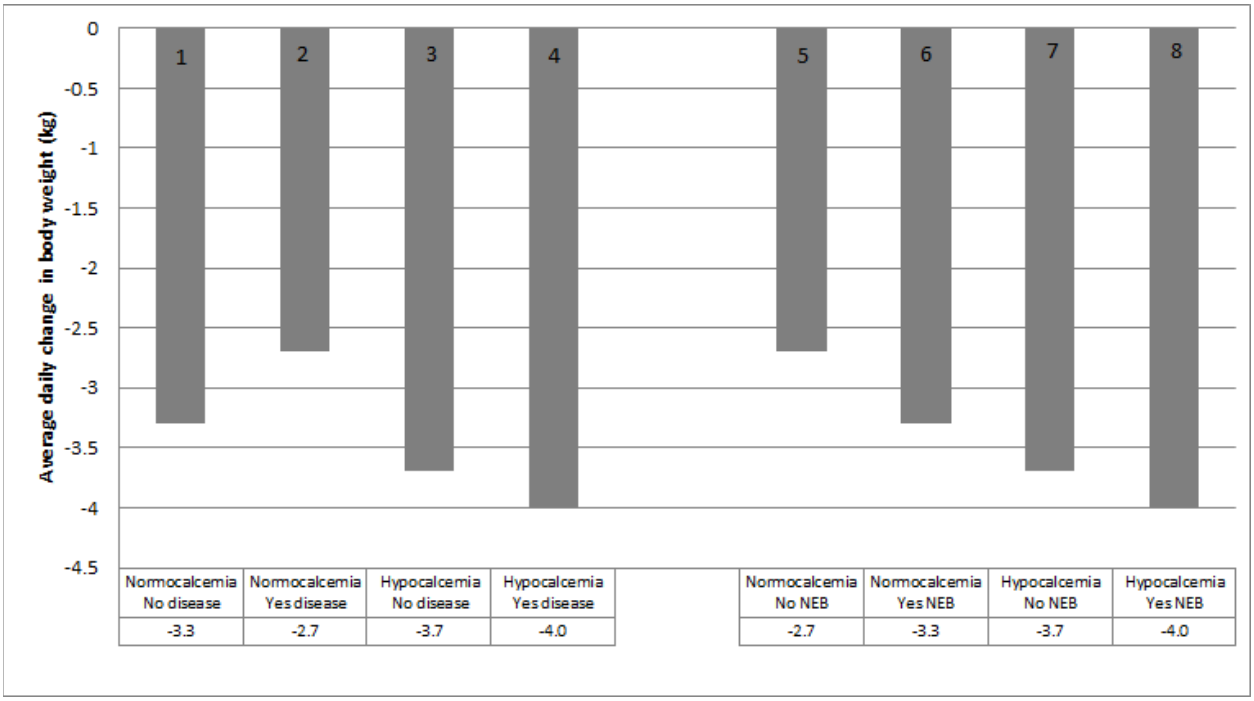

Figure 3.7. Change in daily bodyweight based on the interaction between subclinical hypocalcemia and disease, and subclinical hypocalcemia and negative energy balance in parity = 3 animals.
Subclinical hypocalcemia was defined as at least one calcium concentration between 6 and 8 mg/dL on days 1, 2, or 3. Disease was defined as the presence of any of the following diseases within 10 DIM; displaced abomasum, retained placenta, metritis, milk fever, or ketosis. The variable negative energy balance was defined as at least one β-hydroxybutyrate concentration ≥ 1.2 mmol/L on days 1, 2, or 3; or non-esterified fatty acid concentration ≥ 0.7 mEq/L at 5 DIM. Normocalcemia defined as having no measured calcium reading < 8 mg/dL. Results based on the interaction term in the model. All contrast terms are significantly different from 0 ($P < 0.01$) and all are significantly different from each other ($P < 0.05$). The number of animals in each disease combination is: 6, 5, 14, 10 for columns 1–4, respectively; and 8, 3, 8 16 for NEB columns 5–9, respectively.

DISCUSSION

The objectives of this study were to determine the influence of SHPC on changes in BW during the first 30 DIM while controlling for NEB, disease, and biologically relevant interactions in primiparous and multiparous cows in commercial herds that used AMS. Few reports have evaluated BW, mostly due to the limitations of weighing cows on commercial farms; however, herds using AMS technology have access to this information daily.

It is important to note that our study evaluated these effects in only three herds in New York State; due to the small sample size results may not be representative of all herds. The criteria used for selection were proximity to the laboratory and willingness to participate in the study; the herds were not selected due to on-going or identified health or production problems. Additionally, the cows in the study were also selected based on whether they calved during our study window. Sampling all cows should not bias the results. Nevertheless, because sampling only took place over the summer there might be some seasonal differences that were not evaluated in this study.

Subclinical hypocalcemia is encountered in most of the herds in the US, and its prevalence increases with parity (Reinhardt et al., 2011). Several cut-points have been used to define SHPC, ranging from 8.0 to 8.8 mg/dL (Goff, 2008, Chapinal et al., 2011, Martinez et al., 2012). The current study used 6–8 mg/dL to define SHPC similar to the range used by Reinhardt et al. (2011), but not as wide as the 5.5–8 mg/dL used by Kamgarpour et al. (1999) and (Horst et al., 2003). In the current study, the incidence of SHPC was in agreement with previous data for parity 2 and parity $\geq$ 3 animals (Goff, 2008, Reinhardt et al., 2011, Martinez et al., 2012); however, first lactation animals showed a lower incidence of SHPC. Despite some number differences, the dynamics were similar between our study and previous reports; calcium

concentrations increased in the early post-partum period after a drop in the first 2 days post-partum (Kamgarpour et al., 1999, Goff, 2008, Reinhardt et al., 2011, Martinez et al., 2012).

Although NEFA can be used as an alternative source of energy (Herdt, 2000), excessive concentrations of NEFA and BHB are disadvantageous to the animal's production and health (Ospina et al., 2010a, b, Martinez et al., 2012). The NEFA and BHB cut-points used in this study were based on Ospina et al. (2010b) in which these markers of negative energy balance were associated with subsequent increase risk of disease early in lactation.

The use of the AMS system made it possible to obtain daily BW and daily milk weights; however, there were some missing data points due to identification failures or improper data storage at the AMS. These usually involved just one missing value per day. As the slope of the regression line for BW was used in the analysis, missing values did not affect the outcome. To estimate total milk production missing values were averaged over the two nearest data points.

In this analysis many interaction terms were significant and thus included in the final model. When interaction terms are included in the model, the independent effect of each factor should no longer be evaluated independently from the second factor. For example, if the interaction between NEB and disease is in the model, then the change in NEB is based on disease status (i.e., disease or no disease) and the levels of both factors need to be taken into consideration.

To the authors' knowledge no other peer-reviewed publication has discussed the differences between BW dynamics for the different parities. In our study, first lactation animals lost the least amount of weight in the first 30 DIM; however those with disease (regardless of calcium status) lost the most weight. The fact that those animals are still growing, not producing as much milk as other groups, and there were few animals with NEB may explain the decreased role played by SHPC and NEB with the BW change. This finding also indicates that additional

research with a larger number of cows is necessary to accurately evaluate the calcium threshold, below which detrimental effects are likely in first parity animals.

Although the prevalence of SHPC was similar between parity 2 and parity ≥ 3 animals, parity 2 animals were evaluated separately due to due differences in BW dynamics. SHPC was significantly related to BW loss; however, disease and NEB had a stronger association with BW loss. This may be because parity 2 animals are still growing and calcium dynamics may be different when compared with more mature animals, even though they are producing similar quantities of milk.

Parity ≥ 3 animals had both the largest change in BW due to SHPC and the highest prevalence of SHPC. This is not surprising because mature animals are making the most milk and not mobilizing calcium for growth, thus the calcium dynamics seen in first and second lactation animals may not be in effect. In addition, this group of animals also had higher prevalence of disease and NEB than younger animals. Similar dynamics have been described in other studies in commercial herds, where greater loss in BCS score was encountered in hypocalcemic cows (Martinez et al., 2012). Multiparous cows lost the most weight in the first 30 DIM, in accordance with the study by Kamgarpour et al. (1999). The pattern of BW loss in these older animals showed that animals with SHPC and an additional insult (e.g., disease or NEB) lost more BW than animals with either disease or NEB but normocalcemia.

CONCLUSION

Bodyweight measurements can be easily assessed using the milking system technologies based on AMS, therefore this has great potential to be used as an accurate source of information for dairy research and management. Blood calcium concentrations for all parity groups were

lower in the first DIM but regained normal levels by day 3 of lactation. Incidence of SHPC increased concomitantly with parity reaching 73% for cows in parity ≥ 3. All animals lost weight in the first 30 DIM; however, some animals lost weight faster depending on SHPC, disease and NEB status. Within first parity animals those with disease (and normocalcemia) lost the most weight. Those cows within parity 2 with both disease and NEB (and normocalcemia) lost the most weight, and, conversely, those in parity ≥ 3 with SHPC and either disease or NEB lost the most weight.

REFERENCES

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63(9):1514-1529.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 73(9):2804-2819.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. Journal of dairy science 94(10):4897-4903.

Chapinal, N., S. J. Leblanc, M. E. Carson, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. Journal of dairy science 95(10):5676-5682.

DeGaris, P. J. and I. J. Lean. 2008. Milk fever in dairy cows: a review of pathophysiology and control principles. Veterinary journal (London, England : 1997) 176(1):58-69.

Drackley, J. K., T. R. Overton, and G. N. Douglas. 2001. Adaptations of glucose and long-chain fatty acid metabolism in liver of dairy cows during the periparturient period. Journal of dairy science 84:E100-E112.

Duffield, T. F., K. E. Leslie, D. Sandals, K. Lissemore, B. W. McBride, J. H. Lumsden, P. Dick, and R. Bagg. 1999. Effect of prepartum administration of monensin in a controlled-release capsule on milk production and milk components in early lactation. Journal of dairy science 82(2):272-279.

Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Veterinary journal (London, England : 1997) 176(1):50-57.

Horst, R. L., J. P. Goff, and T. A. Reinhardt. 2003. Role of vitamin D in calcium homeostasis and its use in prevention of bovine periparturient paresis. Acta veterinaria Scandinavica. Supplementum 97:35-50.

Iwersen, M., U. Falkenberg, R. Voigtsberger, D. Forderung, and W. Heuwieser. 2009. Evaluation of an electronic cowside test to detect subclinical ketosis in dairy cows. Journal of dairy science 92(6):2618-2624.

Kamgarpour, R., R. C. Daniel, D. C. Fenwick, K. McGuigan, and G. Murphy. 1999. Post partum subclinical hypocalcaemia and effects on ovarian function and uterine involution in a dairy herd. Veterinary journal (London, England : 1997) 158(1):59-67.

LeBlanc, S. J., K. E. Leslie, and T. F. Duffield. 2005. Metabolic predictors of displaced abomasum in dairy cattle. Journal of dairy science 88(1):159-170.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao, and J. E. Santos. 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.

McArt, J. A., D. V. Nydam, and G. R. Oetzel. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. Journal of dairy science 95(9):5056-5066.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010a. Association between the proportion of sampled transition cows with increased nonesterified fatty acids and beta-hydroxybutyrate and disease incidence, pregnancy rate, and milk production at the herd level. Journal of dairy science 93(8):3595-3601.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010b. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. Journal of dairy science 93(2):546-554.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Veterinary journal (London, England : 1997) 188(1):122-124.

Sheldon, I. M., G. S. Lewis, S. LeBlanc, and R. O. Gilbert. 2006. Defining postpartum uterine disease in cattle. Theriogenology 65(8):1516-1530.

Stokol, T. and D. V. Nydam. 2005. Effect of anticoagulant and storage conditions on bovine nonesterified fatty acid and beta-hydroxybutyrate concentrations in blood. Journal of dairy science 88(9):3139-3144.

Weber, C., C. Hametner, A. Tuchscherer, B. Losand, E. Kanitz, W. Otten, S. P. Singh, R. M. Bruckmaier, F. Becker, W. Kanitz, and H. M. Hammon. 2013. Variation in fat mobilization during early lactation differently affects feed intake, body condition, and lipid and glucose metabolism in high-yielding dairy cows. Journal of dairy science 96(1):165-180.

CHAPTER 4: Association between subclinical hypocalcemia in the first 3 days of lactation and reproductive performance of dairy cows*

* Caixeta LS, Ospina PA, Capel MB, Nydam DV. Association between subclinical hypocalcemia in the first 3 days of lactation and reproductive performance of dairy cows. Theriogenology – *In press.*

.

ABSTRACT

The objective of this study was to determine the effects of subclinical hypocalcemia on reproductive performance in dairy cows. In a prospective cohort study, 97 cows on 2 dairy farms with automatic milking systems were monitored for subclinical hypocalcemia. Animals were enrolled 7 ± 3 days prior to estimated calving date and three parity groups were defined based on the lactation that the animals were going to start: lactation = 1, lactation = 2, and lactation ≥ 3. Serum calcium concentration (**Ca**) was measured in all animals in the first 3 DIM and subclinical hypocalcemia (**SHPC**) was defined as Ca ≤ 8.6 mg/dL; animals that presented a low Ca level during all 3 days were classified as chronic SHPC (**cSHPC**). Return to cyclicity (progesterone ≥ 1 ng/mL between 21 ± 3 DIM and 49 ± 3 DIM) during the voluntary waiting period was analyzed based on weekly progesterone concentrations measured in serum. Information on reproductive outcomes (i.e., number of breedings, pregnancy status, days open, etc.), were collected from on-farm software after all study cows had completed their study period. Chronic SHPC was present in all parity groups with higher incidence in multiparous animals (20% of parity = 1, 32% of parity = 2; and 46% of parity ≥ 3 animals). The cSHPC animals took longer to return to cyclicity when compared to eucalcemic and SHPC animals. In a multivariable Cox's Proportional Hazard model animals with normal Ca were 1.8 times more likely to return to cyclicity by the end of the voluntary waiting period when compared to cSHPC animals. Animals with cSHPC also had 0.27 odds of being pregnant at first service compared to eucalcemic cows when analyzed by multivariable logistic regression. Subclinical hypocalcemia had a negative effect on return of ovarian function during the voluntary waiting period and decreased the odds of pregnancy at first service. Cows with cSHPC had an even more pronounced impaired reproductive function than those with one subclinical measurement.

INTRODUCTION

High producing dairy cows face a challenging period when transitioning from late pregnancy to early lactation. Energy demand increases by 2.5-fold (Bell, 1995, Reynolds et al., 2003) and mineral requirements, especially calcium, are increased by over 65% to support lactogenesis in early lactation (DeGaris and Lean, 2008). As a result, homeorhetic adaptations take place to adjust for such increased demands (Bauman and Currie, 1980). Unsuccessful adaptation to transition period challenges has been associated with increased occurrence of diseases (LeBlanc et al., 2005, Ospina et al., 2010a, Chapinal et al., 2011), decreased milk production (Ospina et al., 2010b, Chapinal et al., 2011) and impaired reproductive performance (Ospina et al., 2010a, Chapinal et al., 2011).

Hypocalcemia has been reported as a problem in the dairy industry for over two centuries, especially clinical cases also known as milk fever (Murray et al., 2008). Nutritional management of cations and anions during dry period and early lactation, along with an increased understanding of transition period physiology have been the key to decreasing the incidence of milk fever to rates as low as 1% (Reinhardt et al., 2011, Oetzel and Miller, 2012). However, despite the low incidence of clinical cases in modern dairy cattle, reports have shown that prevalence of subclinical hypocalcemia is high in the US (Reinhardt et al., 2011) with as many as 73% of animals of parity $\geq$ 3 experiencing subclinical hypocalcemia during the first 3 DIM (Caixeta et al., 2015). Subclinical hypocalcemia is defined as a low calcium concentration without the development of clinical signs (e.g. recumbency, lethargy, hypothermia, and rumen atony). Several thresholds have been used to define subclinical hypocalcemia and they range from 8.0 mg/dL to 8.8 mg/dL (DeGaris and Lean, 2008, Goff, 2008, Chapinal et al., 2011, Reinhardt et al., 2011, Martinez et al., 2012).

Even though, hypocalcemic animals may not develop clinical signs, further metabolic and health consequences have been associated with the occurrence of this mineral imbalance during early lactation and it has a great economic impact in modern dairy enterprises (Oetzel and Miller, 2012). Traditionally, hypocalcemia has been associated with occurrence of dystocia, uterine prolapse, retained placenta, mastitis and decreased rumen and abomasum motility (Curtis et al., 1983, Risco et al., 1984, Goff, 2008), as well as impaired immune cell functions (Kimura et al., 2006). More recently, research has shown that subclinical hypocalcemia is associated with an increased risk of metritis (Martinez et al., 2012) and displaced abomasum (Chapinal et al., 2011) as well as an increase in culling rates (Duffield et al., 1999, Seifi et al., 2011) in dairy cows. Additionally, it has been reported that grazing animals that have low calcium concentrations within the first week post-partum have increased chances of developing multiple clinical disorders during lactation (Sepulveda-Varas et al., 2015). Although hypocalcemia has been associated with impaired reproductive performance by delaying resumption of ovarian cyclicity (Jonsson et al., 1999) and impaired response to estrus synchronization protocols (McNally et al., 2014), the effect of prolonged low blood calcium concentration in early lactation on reproductive performance have not been described. The objective of the present study is to evaluate the association between subclinical hypocalcemia in the first 3 days of lactation with reproductive performance during the first 120 DIM.

MATERIALS AND METHODS

Study Population, Study Design, and Sample Size Calculation

A prospective cohort study was conducted from a convenience sample of 2 commercial farms in Central New York. The 2 herds were selected as part of another study (Caixeta et al., 2015) because of the use of automatic milking systems (**AMS** - Astronaut A3 and A4, Lely Industries N.V, Rotterdam, The Netherlands). Herd A milked over 700 cows using 14 AMS while herd B used 7 AMS to milk 400 cows. Within herd, all cows and heifers calving between June 11th of 2012 and August 8th of 2012 were enrolled in the study. These animals were followed forward for the first 120 days of lactation.

In both farms cows were housed in free-stall barns; concrete stalls were covered with mattresses. Herd A bedded with waste paper-pulp while herd B used sand bedding. In both farms, animals from different parity groups were co-mingled in the fresh cow pen where only one milking unit was available; after leaving the fresh cow pen cows were separated according to their parity group into pens with at least 2 milking units. A ratio of 60 animals/AMS was observed regardless of the lactation period of the cows. Animals were fed partial mixed ration (**PMR**) consisted of 80% forage and 20% concentrate during the dry period, and 55% forage and 45% concentrate for the lactation groups. The diet was formulated to meet or exceed the NRC nutrient requirements for lactating Holsteins according to farm conditions. A part of the total diet was offered to the animals as a grain mixture in the form of pellets in the AMS, the amount of pellets fed depended on both DIM and milk being produced at the cow level. In the North Eastern United States, dairy herds, like the study herds, routinely manage the potassium content of dry cow diets through manipulation of manure application fields and forage choice, e.g. limiting alfalfa feeding to dry cows.

The sample size was calculated in order to detect a minimum of 1.3 kg difference in milk production between eucalcemic and hypocalcemic animals with a standard deviation of 3.5 kg, power of 80% and a 95% confidence interval (Caixeta et al., 2015). A post-hoc evaluation determined that the 97 animals enrolled in this study would achieve 80% power and 95% confidence interval, given the 15% difference between the proportions of animals returning to cyclicity at the end of the voluntary waiting period (**VWP**) between the two calcium status groups.

Data Collection and Case Definition

Animals were enrolled 7 ± 3 days prior to expected calving date and body condition score (**BCS**) was determined using a 5-point scale (Ferguson et al., 1994). A blood sample was collected from coccygeal vessels using a Vacutainer tube without anticoagulant and a 20-gauge x 2.54 cm Vacutainer needle (Becton, Dickson and Company, Franklin Lakes, NJ) at the following time points: pre-partum, at 1, 2, 3, 5, and 7 days in milk (**DIM**); and weekly thereafter during the VWP (between second (14 ± 3 DIM) and seventh week (49 ± 3 DIM) post-partum).

Total calcium concentration was determined pre-partum and at d 1, 2, and 3 after calving to assess calcium status around parturition. Negative energy balance was determined by the concentrations of serum non-esterified fatty-acid (**NEFA**) concentration measured pre-partum and 5 days postpartum; and serum β-hydroxybutyrate (**BHB**) concentration measured on d 3 and 5 post-partum. Finally, serum progesterone (**P4**) concentration was used to determine the presence of an active corpus luteum, and consequently return to cyclicity, at d 7 and weekly starting at the second week of lactation (14 ± 3 DIM) until the seventh week post-partum (49 ± 3 DIM).

All blood samples were spun for 15 minutes at 2,000x *g* at the farm in a portable centrifuge within 30 minutes of collection and immediately placed in ice to be transported back to the laboratory where samples were kept at -20°C until analysis were performed according to recommendations (Stokol and Nydam, 2005). Serum samples were sent to Cornell University Animal Health Diagnostic Center (Ithaca, NY) for determination of calcium and NEFA concentration. Serum β-hydroxybutyrate (**BHB**) concentration was evaluated cow side using the Precision Xtra meter (Abbott Laboratories, Abbott Park, IL) (Iwersen et al., 2009). And progesterone concentrations were determined using radioimmunoassay as previous described by Beam and Butler (1997).

Negative energy balance (**NEB**) was defined using pre- and post-partum measurements of NEFA and BHB as described by previous reports (Ospina et al., 2010c, McArt et al., 2012). Briefly, metabolites concentrations were dichotomized as follows: pre-partum NEFA $\geq$ 0.3 mEq/L, post-partum NEFA $\geq$ 0.7 mEq/L, and post-partum BHB $\geq$ 1.2 mmol/L. Return to cyclicity during the VWP was determined by measurement of P4 during the VWP. Cows having P4 concentration $\geq$ 1 ng/mL were considered to have a functional corpus luteum, thus cyclic (Canfield and Butler, 1991). Subclinical hypocalcemia was defined based on serum total calcium concentration (**Ca**). A cut-off point of Ca $\leq$ 8.6 mg/dL was used to dichotomize each individual sample into hypocalcemic or eucalcemic (Martinez et al., 2012).

Occurrence of diseases of interest (e.g., displaced abomasum, clinical ketosis, metritis, and retained placenta) during the first 10 DIM and reproductive performance data was recorded in Dairy Comp 305 (Valley Ag. Software) and retrieved at the end of the study period. Standard disease definitions as described elsewhere (Caixeta et al., 2015) were discussed with the farms at the start of the study; however, each farm implemented their own standardized care.

Statistical Analysis

The sequential Ca concentrations were used to divide the animals into three groups based on total serum calcium concentration: eucalcemia, subclinical hypocalcemia (**SHPC**), and chronic subclinical hypocalcemia (**cSHPC**). Eucalcemic animals did not have an abnormal Ca (Ca > 8.6 mg/dL); SHPC animals had at least one low Ca measurement during the first 3 DIM; while cSHPC animals had low Ca concentration for all three days measured.

Descriptive statistics were generated using FREQ and MEANS procedures of SAS version 9.3 (SAS Institute Inc., Cary, NC). Comparison between the proportions of SHPC animals among parity groups and within DIM, as well as cSHPC between parity groups, were obtained with procedure GENMOD of SAS.

Dynamics of serum calcium concentration during the first 3 DIM for the different groups, according to calcium status, were analyzed by the MIXED procedure in SAS, with repeated measurements and Bonferroni adjustment for multiple comparisons. A Kenward-Roger degrees of freedom approximation was used to calculate the denominator degrees of freedom.

A general linear mixed model carried out using the GLIMMIX procedure of SAS was used to determine the association between early lactation calcium status and the reproductive variable: days open during the first 120 DIM. The *t* test statement was used to evaluate the mean difference, between groups with different calcium status. Statistical analysis for pregnancy at first service was carried out using multivariable logistic regression with the LOGISTIC procedure of SAS.

The effect of subclinical hypocalcemia on return to cyclicity during the VWP and time to pregnancy before 120 DIM were determined by multivariable Cox's Proportional Hazard models

of MedCalc version 14.12.0 for Windows (MedCalc Software, Mariakerke, Belgium). Cows that were not pregnant and were sold or died before 120 DIM were right censored.

Throughout statistical analysis herd was included in the models as random effect while other potential explanatory variables (i.e. calcium status group, parity, disease during the first 10 DIM, NEB, and total milk production during the first 60 DIM) were included as fixed effects. All interactions between calcium status group and the other explanatory variables were analyzed. Calcium status was forced into all models and other explanatory variables and interactions were removed in a manual backwards stepwise fashion if $P > 0.10$.

RESULTS

Descriptive Statistics

In total, 101 animals were enrolled during the study period, but only 97 were used for statistical analysis. Four animals were excluded from the study for the following reasons: one animal died, one was culled by farm personnel during the first week of lactation, and two were excluded because of incomplete data collection due to software problems between AMS system and farm management software.

Nineteen animals (20%) did not have low Ca concentration during the first 3 DIM, while 78 were hypocalcemic; of those 45 were classified as SHPC (46%) and 33 as cSHPC (34%). The percentage of animals presenting abnormal Ca decreased with time, independent of parity group. Despite the elevated number of animals presenting low calcium concentrations in early lactation, no clinical hypocalcemia, i.e. milk fever, cases were diagnosed during the study period. Chronic SHPC increased with parity; parity ≥ 3 (46%) when compared to parity = 1 (20%) ($P = 0.03$;

Table 4.1). Serum calcium concentrations decreased to levels below cut-off values for both SHPC groups at 1 DIM while eucalcemic animals continued to have normal levels, despite reaching Ca nadir (8.8 ± 0.21 mg/dL) at 1 DIM. Calcium levels had an upwards trend during the following days and after 3 DIM SHPC animals had normal Ca concentrations (9.0 ± 0.14 mg/dL) while cSHPC (7.9 ± 0.16 mg/dL) still presented low calcium concentrations (Figure 4.1).

Table 4.1. Proportion of animals with low blood calcium concentration (Ca[1]) during the first 3 DIM[2] by parity. Data: number of animals with low blood calcium concentration divided by the total number of animals in a given parity (percent).

	Parity = 1	Parity = 2	Parity ≥ 3	*P*-value
DIM[2]				
1	11/30 (37)[a]	21/28 (75)[b]	38/39 (97)[c]	< 0.001
2	12/30 (40)[a]	17/28 (61)[a]	32/39 (82)[b]	0.001
3	10/30 (33)[a]	12/28 (43)[a]	20/39 (51)[a]	0.3
cSHPC[3]	6/30 (20)[a]	9/28 (32)[a,b]	18/39 (46)[b]	0.07

[1] Ca = low blood calcium concentration was defined as Ca ≤ 8.6 mg/dL.
[2] DIM = days in milk.
[3] cSHPC = Chronic subclinical hypocalcemia was defined as total blood calcium concentration ≤ 8.6 mg/dL for all the first 3DIM.
[a,b,c] Different letters in the same row indicate P<0.05 between percentage of animals presenting low blood calcium concentration for the different parity groups.

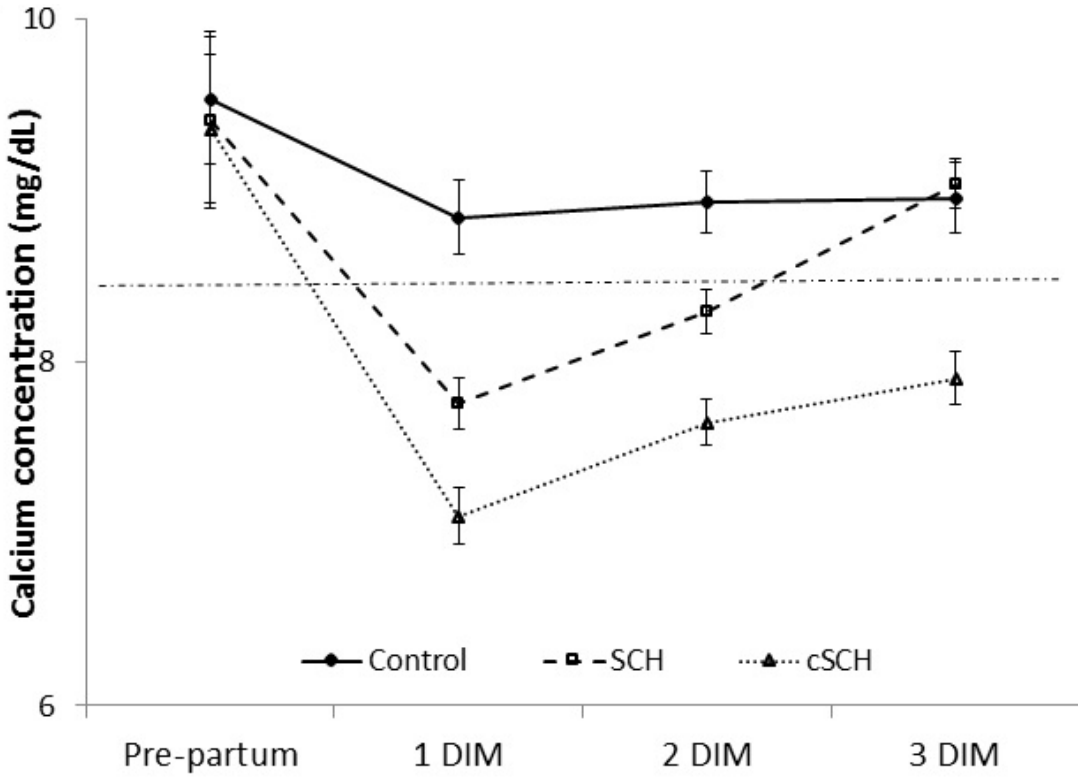

Figure 4.1. Dynamics of serum calcium concentration (mg/dL) in the first 3 DIM according to calcium status group.
Cows were classified as having eucalcemia (Ca > 8.6 mg/dL) or subclinical hypocalcemia (Ca ≤ 8.6 mg/dL). Subclinical hypocalcemia was categorized according to persistence of low serum calcium concentration: subclinical hypocalcemia (SHPC) animals had 1 or 2 low measurements while chronic subclinical hypocalcemia (cSHPC) animals presented low calcium concentration during all first 3 DIM. Horizontal dashed line represents serum calcium concentration cut-off point (8.6 mg/dL). Data are presented in LSM ± SEM and effects of the interaction between calcium status and DIM was analyzed ($P < 0.01$).

When controlling for parity group and occurrence of disease in the first 10 DIM, no statistical differences in the total milk production during the first 60 DIM were observed when comparing the groups with different calcium status (2006.7 ± 101.7 kg vs. 2169.0 ± 68.3 kg vs. 2259.9 ± 80.7 kg for eucalcemia, SHPC, and cSHPC respectively; $P = 0.18$). No statistical differences between the different calcium status groups were observed when comparing the concentration of circulating NEFA pre-partum ($P = 0.77$) and at 5 DIM ($P = 0.14$); as well as BHB at 3 DIM ($P =$

0.61) and 5 DIM ($P = 0.60$). Nonetheless, when these metabolites were analyzed together, i.e. NEB variable, the proportion of animals considered to be in NEB was elevated in both hypocalcemic groups when compared to eucalcemia animals (eucalcemia = 21% vs. SHPC = 76% vs. cSHPC 85%; $P = 0.007$).

The frequency of disease occurrence during the first 10 DIM was different when comparing the eucalcemic animals to both hypocalcemic groups ($P = 0.006$). When comparing the different calcium status groups separately, it was observed that fewer eucalcemic animals developed any disease when compared to cSHPC animals (16% vs. 57%; $P = 0.009$) but not when compared to SHPC (16% vs. 42%; $P = 0.14$). Additionally the proportion of animals developing any disease in early post-partum was not different when comparing both hypocalcemia groups to each other (42% vs. 57% for SHPC and cSHPC respectively; $P = 0.49$).

Reproductive performance

Out of the 97 animals enrolled in the study, nine (9.3%) were right censored being culled not pregnant before reaching 120 DIM; three animals died in the first 30 days of the trial (1 presumptive listeriosis case, 1 back injury and 1 non-defined), two animals were culled due to mastitis, two animals were culled due to lameness, and two animals were culled due to low milk production possible as a consequence of clinical ketosis during early lactation. From the rest of the study population 29 animals (~ 30%) did not get pregnant until after 120 DIM, therefore they were considered open for the purpose of this study.

Days open during the first 120 of lactation was similar between the three groups, however days to return to cyclicity was longer for cSHPC. Only a numerical difference on days open was observed when comparing the three calcium groups during the first 120 days of lactation: 85 ± 9 days for eucalcemia; 87 ± 8 days for SHPC; and 89 ± 8 days for cSHPC animals ($P = 0.89$).

Days to return to cyclicity during the VWP was similar when comparing eucalcemia (28 ± 3 DIM) and SHPC (29 ± 2 DIM), but cSHPC (36 ± 2 DIM) tended to take longer to return to cyclicity ($P = 0.07$). No interaction between calcium status and other explanatory variables were observed for these reproductive outcomes ($P > 0.20$ and $P > 0.30$ for days open during the first 120 DIM and days to return to cyclicity, respectively).

The odds of pregnancy at first service for the different calcium status groups was determined by multivariable logistic regression and including parity, NEB, milk production during the VWP, and development of any disease during the first 10 DIM as covariates; however, none of these variables nor the interaction terms between these variables and calcium status were significantly associated with the outcome ($P > 0.16$), and were removed. Chronic SHPC cows had lower odds of pregnancy at first service (OR = 0.27; 95% CI = 0.080 – 0.876; $P = 0.03$) when compared to eucalcemic animals (Table 4.2).

Cox Proportional-Hazard models were calculated to show the risk of return to ovarian function during the VWP and time to pregnancy based on calcium groups (Figures. 4.2 and 4.3). During the voluntary waiting period SHPC animals had a similar hazard to return of cyclicity as eucalcemic animals (HR = 0.86; 95% CI = 0.48 – 1.52; $P = 0.6$), while the cSHPC group tended to have lower hazard for return to cyclicity (HR = 0.55; 95% CI = 0.30 – 1.02; $P = 0.06$). There was no difference between the eucalcemic and subclinical hypocalcemia animals ($P = 0.2$) and no difference between the eucalcemia and cSHPC ($P = 0.3$) when evaluating pregnancy by 120 DIM as the outcome.

Table 4.2. Logistic regression of the association of subclinical hypocalcemia, measured within 3 DIM, with pregnancy at first service.

Variable	n[1]	Pregnant at 1st Service (%)	Odds ratio	95% CI	*P*-value
Pregnant at first service					
Calcium Status[2]					
Eucalcemia[3]	19	63[a]	*Ref.*	-	-
SHPC[4]	45	44[a,b]	0.46	0.152 – 1.401	0.2
cSHPC[5]	33	31[b]	0.27	0.080 – 0.876	0.03

[1] Total number of animals in each group according to total blood calcium concentration.
[2] Calcium status was defined according to the number of days the animals were classified as subclinical hypocalcemic.
[3] Eucalcemia = eucalcemic animals had all measurement of total blood calcium concentrations > 8.6 mg/dL.
[4] SHPC = Subclinical hypocalcemia. Subclinical hypocalcemia was defined as at least one measurement of total blood calcium concentration ≤ 8.6 mg/dL.
[5] cSHPC = Chronic subclinical hypocalcemia. Chronic subclinical hypocalcemia was defined as total blood calcium concentration ≤ 8.6 mg/dL for all the first 3DIM.
[a,b,c] Different letters in the same column indicate P <0.05.

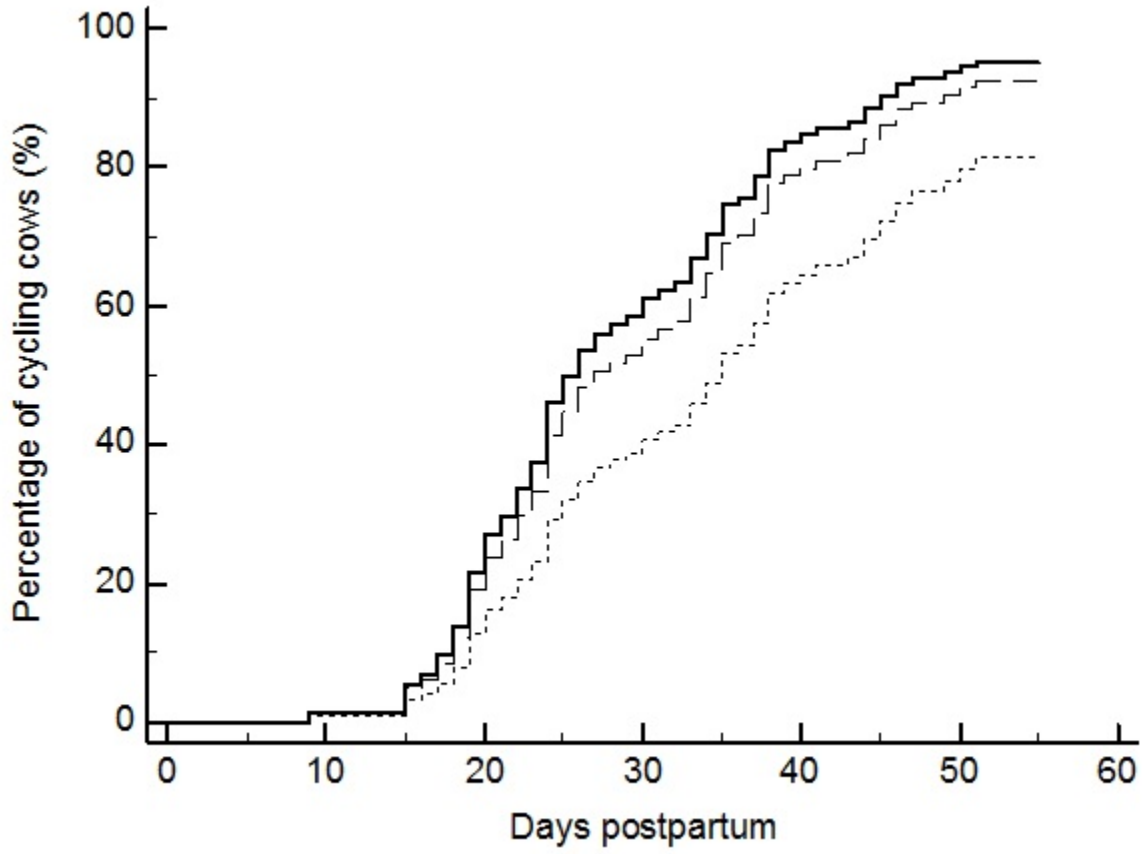

Figure 4.2. Cox Proportional-Hazard curves for time to detection of an active corpus luteum during the voluntary waiting period.
Subclinical hypocalcemia (SHPC - dashed line) animals had similar hazard to return to cyclicity when compared to eucalcemic (solid line) animals (HR = 0.86; 95% CI = 0.48 – 1.52; *P* = 0.6). Chronic SHPC (dotted lines) animals tended to take longer to return to cyclicity when compared to eucalcemia and SHPC animals (HR = 0.55; 95% CI = 0.30 – 1.02; *P* = 0.06). The median days to active corpus luteum were, respectively, 25, 27, and 35 for eucalcemic, SHPC, and cSHPC animals. Active corpus luteum was defined as progesterone > 1.0 ng/mL. The total animals per each cohort were: 19, 45, and 33 for eucalcemic animals, SHPC, and cSHPC respectively.

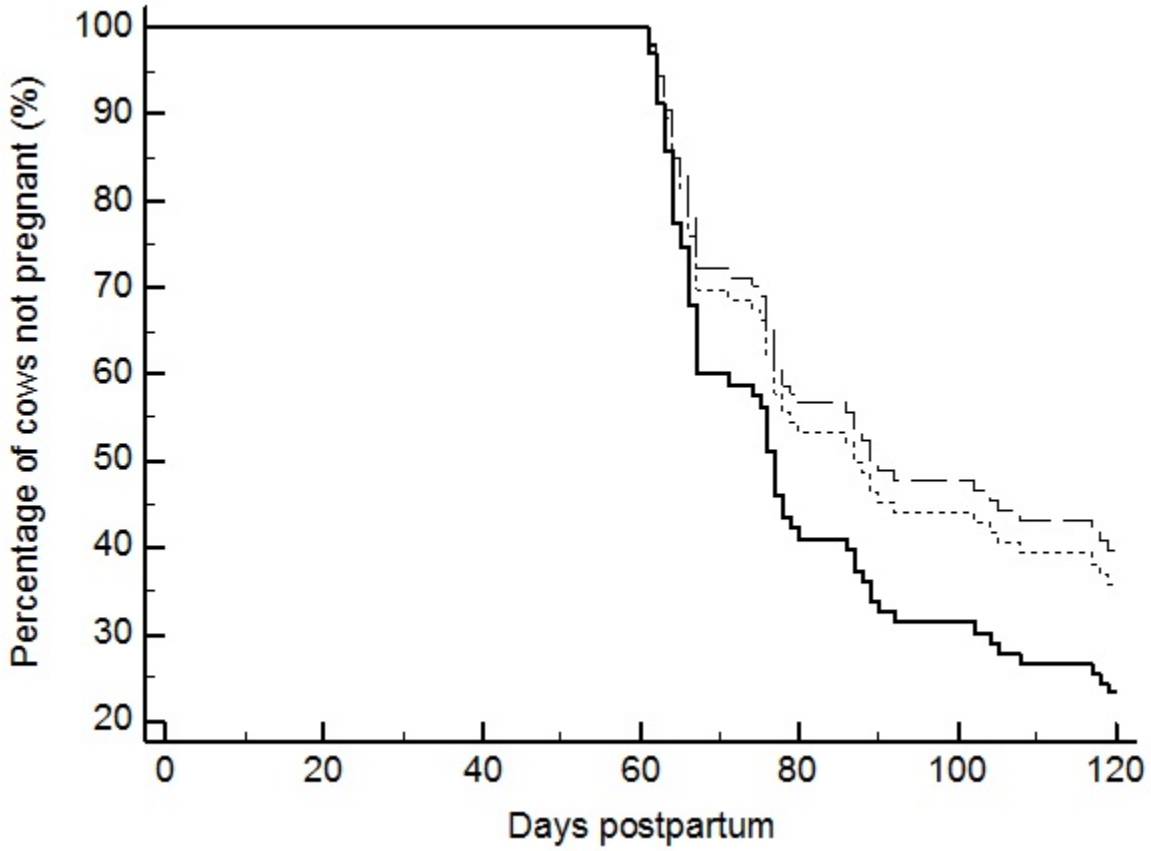

Figure 4.3. Time to pregnancy during the first 120 DIM for the different cohorts.
Cox Proportional-Hazard curves for time to pregnancy in the first 120 DIM for eucalcemic animals (eucalcemia; n = 19; solid line), subclinical hypocalcemia (SHPC; n = 45; dashed line) or chronic subclinical hypocalcemia (cSHPC; n = 33; dotted line). Compared to the eucalcemic group the hazard ratio for subclinical hypocalcemic cows was 0.63(95% CI = 0.33 – 1.23; *P* = 0.2) and 0.71 for chronic SHPC (95% CI = 0.36 – 1.39; *P* = 0.3).

DISCUSSION

The objective of this study was to evaluate the effects of subclinical hypocalcemia during the first 3 DIM on the reproductive performance of dairy cows. It is important to note that although there are several factors that may be associated with observed calcium concentration of a cow in the current lactation (e.g., previous lactation milk production, dry cow diet formulation, breed, and the use of anionic salts (Erb and Martin, 1978, Horst et al., 1997, Fleischer et al., 2001)) the objective was to evaluate the effect of subclinical hypocalcemia, irrespective of the cause. Several cut-points ranging from 8.0 to 8.8 mg/dL have been reported (Goff, 2008, Chapinal et al., 2011) for defining subclinical hypocalcemia; the cut-off used in this study was within the mentioned range, 8.6 mg/dL. Martinez et al. (2012) identified 8.59 mg/dL as the value with the highest sensitivity (88.5%) and specificity (55.2%) to predict cows that would develop metritis post-partum; therefore this cut-off was chosen to dichotomize blood calcium concentration and analyze reproductive performance of dairy cows in this study.

During this study only total calcium was measured. It is important to highlight that only part of the blood Ca pool is free and readily available for biological activities, also referred to as ionized calcium (**iCa**), the rest is transported in blood bonded to albumin (Sava et al., 2005). Additionally, increased blood pH can influence iCa concentration in blood, since alkaline environment determine a stronger binding of this ions to albumin (Wang et al., 2002). Therefore the determination of the iCa:Ca ratio has been studied under different conditions. In humans, iCa has been shown to correspond to half of Ca circulating calcium under normal conditions (Forman and Lorenzo, 1991, Kragh-Hansen and Vorum, 1993), but during periods of abnormal calcium states such association is not maintained and measurement of iCa is necessary to improve calcium status diagnostics accuracy (Ong et al., 2012). Similarly, a slight change in the iCa-Ca

ratio, high iCaA:Ca due to increased percentage of Ca being ionized, was observed on dairy cows immediately after parturition depending on calcium status (Sweeney et al., 2014). Despite this discrepancy, measurements of Ca were considered adequate when predicting neutrophil function, and therefore acceptable as am index of calcium status in periparturient dairy cow (Sweeney et al., 2014). Ionized Ca represents the bioactive calcium in blood, but its determination is complicated and costly. Moreover iCa is believed to not predict functional outcomes significantly better than Ca. Accordingly, total calcium is commonly measured in dairy cattle research.

The sample size was estimated to compare two groups: hypocalcemic animals, defined as low calcium concentration within the first 3 DIM, and a eucalcemic group, defined as no low calcium concentrations within the first 3 DIM. However, the incidence of cSHPC animals was alarming and this led to an additional level of analysis. The consequence being that the inclusion of the third group in the analysis, decreased the statistical power. It was not an objective of this study to detect differences in the reproductive performance of the different hypocalcemia groups, but the prevalence of cSHPC led to the division into two hypocalcemic groups. A 45% power was determined by post-hoc power analysis when accounting for three calcium status groups instead of two groups (hypocalcemic vs. eucalcemic). The reduced number of individuals per group and lower power of the statistical model may explain the higher p-values. There is a chance that type II errors, i.e. indicating no difference when there truly is one, could be interpreted from some of the results presented in this study where the numerical differences where intriguing. If the prevalence of cSHPC was known to be as elevated as encountered, more animals would have been enrolled decreasing the chances of a type II error.

In the current study, the 80% prevalence of subclinical hypocalcemia during the first 3 DIM was higher than results obtained in a survey of dairy farms in the United States (Reinhardt et al., 2011). However, the cut-off point to determine subclinical hypocalcemia in the survey and previous studies were lower than the cut-off used in the current study (8.0 mg/dL vs 8.6 mg/dL). Differently from previous reports (Chapinal et al., 2011, Reinhardt et al., 2011, Jawor et al., 2012, Martinez et al., 2012), the current study also defined subclinical hypocalcemia based on the duration of low calcium concentration during the first 3 DIM and not only based on a single low blood calcium concentration. Nonetheless, when SHPC was defined as low calcium concentrations throughout the first 3 DIM the numbers of affected animals were surprisingly high indicating that calcium homeostatic mechanisms were not sufficient to overcome the challenge imposed by the increased milk production for more than 30% of animals in this study (Martin-Tereso and Verstegen, 2011, Goff, 2014).

Some degree of hypocalcemia is expected in early lactation with a nadir between 12 and 24 hours after calving (Goff, 2014). Nonetheless, several metabolic adaptations are triggered to overcome this challenge including enhanced absorption of dietary calcium, increased mobilization of calcium from bones, and enhanced renal re-absorption of calcium (DeGaris and Lean, 2008, Goff, 2008, Martin-Tereso and Martens, 2014), as a consequence serum calcium concentration should rise to normal values within 2 to 3 DIM (Kamgarpour et al., 1999, Martinez et al., 2012, Chamberlin et al., 2013, Sato et al., 2013). However, when these mechanisms fail and cSHPC is the result we have an indication of a decreased capacity to adapt to the new physiological state in early lactation. The persistence of abnormal metabolite levels may substantiate the exacerbated negative outcomes in cSHPC animals. The increased prevalence of any disease during the first 10 DIM in the cSHPC animals observed in this study agrees with

results from previous reports (Kimura et al., 2006, Martinez et al., 2014) that described an association between hypocalcemia and immune suppression due to impaired neutrophil function in periparturient animals.

The dynamics of total serum calcium concentration presented in this study are in agreement with previous reports in which Ca nadir is reached in the first day of lactation and normal values are regained by 3 DIM in the subclinical hypocalcemia groups (Kamgarpour et al., 1999, Martinez et al., 2012, Chamberlin et al., 2013). The analysis of cSHPC is a novel concept used by our group showing that some of animals do not adapt as expected to the mineral imbalances caused by the beginning of lactation. The length of time that dairy cows remain in a SHPC state in early lactation might be more detrimental to health, milk production, and reproductive performance than the actual calcium concentration nadir in circulation.

Various factors throughout lactation have been associated with impaired reproductive performance in dairy cows. Among those factors, subclinical hypocalcemia happening during the very early stages of lactation have been associated with poor reproduction (Chapinal et al., 2012, Martinez et al., 2012). In an attempt to minimize the effect of other confounding factors and isolate the association between subclinical hypocalcemia and the reproductive performance of dairy cows the analysis of the reproductive performance during the current study was restricted to the first 120 DIM.

The association between occurrence of subclinical hypocalcemia in early lactation and days open have been previously reported with inconsistent results. Martinez et al. (2012) reported that hypocalcemic animals tended to stay open 15 days longer than eucalcemic animals. On the other hand, Chamberlin et al. (2013) reported no difference in the mean days open when comparing eucalcemic and subclinical hypocalcemic animals. Even though the results of the current study

are in agreement with the latter, a distinction between these results must be made because time to pregnancy was only analyzed up to 120 DIM and not for the whole lactation period. It is possible that other confounders, related or not to calcium metabolism, play a role in reproductive performance later than 120 of lactation leading to the variable results reported to date.

In the present study return to cyclicity tended to be different ($P = 0.07$) when comparing the calcium status groups; with animals having abnormal Ca levels for longer being negatively affected. Similar results were reported in hypocalcemic animals (Ribeiro et al., 2013) while no difference has been reported in hypocalcemic cows with high risk of developing uterine disease (Martinez et al., 2012). Abnormal blood calcium concentrations during the peri-parturient period has also been associated to reproductive impairment decreasing response to synchronization protocols (McNally et al., 2014) and decreased odds of pregnancy for cows (Chapinal et al., 2012). Similar results were observed in this study, with animals presenting abnormal concentration of Ca during the first 3 DIM; especially cSHPC animals which were less likely to have active ovaries by the time ovulation synchronization protocols were started. This might have influenced the efficiency of protocols and contributed to the lower odds of pregnancy at first service (Santos et al., 2009, Bisinotto et al., 2010).

Impaired reproductive performance in hypocalcemic animals is, in part, explained by the association between mineral and metabolic adaptations during the transition period. Hypocalcemia can be detrimental to reproductive performance through two different interconnected pathways. First, subclinical hypocalcemia reduces calcium availability to immune cells (Kimura et al., 2006) impairing neutrophil function (Martinez et al., 2012) leading to an increased risk of infectious uterine diseases; aggravated by the incapacity of the uterus to expel uterine content due to suppressed smooth muscle contraction caused by the calcium

deficiency (Hansen et al., 2003). Secondly, subclinical hypocalcemia has been reported to exacerbate negative energy balance (Reinhardt et al., 2011, Ribeiro et al., 2013) and impair lipid metabolism (Chamberlin et al., 2013). The decreased energy availability further intensifies immune cell dysfunction and increased occurrence of uterine diseases (Galvao et al., 2010). Clinical and subclinical diseases are associated with reduced pregnancy per artificial insemination rates and delayed return to cyclicity (Ribeiro et al., 2013). Even though, the mechanisms by which lower calcium levels delays ovarian activity has not been described, the negative impacts of the early lactation metabolic challenges in high producing dairy cows has been reported to have effects that are carried over on fertility months later (Wathes et al., 2007).

The successful transition by eucalcemic cows is confirmed by improved early lactation reproductive performance, increased odds of getting pregnant at first service and increased hazard of getting pregnant before 120 DIM of these animals when compared to their chronic subclinically hypocalcemic counterparts. These results are confirmed by previous reports showing that increased odds of pregnancy (Chapinal et al., 2012, Martinez et al., 2012) and decreased reproductive disorders (Martinez et al., 2012, Ribeiro et al., 2013) are observed in animals that maintain normal serum blood concentration during the periparturient period.

The incidence and persistency of SHPC during the first week of lactation is associated with impaired reproductive performance. Identification and appropriate management of these animals is important to overcome the metabolic challenge the animal is facing during early lactation.

CONCLUSION

Approximately 1/3 of animals of all parities experienced cSHPC; with the incidence of low blood calcium concentration increasing directly associated with parity. Subclinical hypocalcemia had a negative effect on return of ovarian function during the voluntary waiting period and decreased the odds of pregnancy at first service. Cows with chronic subclinical hypocalcemia tended to have an even more pronounced impaired reproductive function than eucalcemic animals.

REFERENCES

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63(9):1514-1529.

Beam, S. W. and W. R. Butler. 1997. Energy balance and ovarian follicle development prior to the first ovulation postpartum in dairy cows receiving three levels of dietary fat. Biology of reproduction 56(1):133-142.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 73(9):2804-2819.

Bisinotto, R. S., R. C. Chebel, and J. E. Santos. 2010. Follicular wave of the ovulatory follicle and not cyclic status influences fertility of dairy cows. Journal of dairy science 93(8):3578-3587.

Caixeta, L. S., P. A. Ospina, M. B. Capel, and D. V. Nydam. 2015. The association of subclinical hypocalcemia, negative energy balance and disease with bodyweight change during the first 30 days post-partum in dairy cows milked with automatic milking systems. Veterinary journal (London, England : 1997) 204(2):150-156.

Canfield, R. W. and W. R. Butler. 1991. Energy balance, first ovulation and the effects of naloxone on LH secretion in early postpartum dairy cows. Journal of animal science 69(2):740-746.

Chamberlin, W. G., J. R. Middleton, J. N. Spain, G. C. Johnson, M. R. Ellersieck, and P. Pithua. 2013. Subclinical hypocalcemia, plasma biochemical parameters, lipid metabolism, postpartum disease, and fertility in postparturient dairy cows. Journal of dairy science 96(11):7001-7013.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. Journal of dairy science 94(10):4897-4903.

Chapinal, N., S. J. Leblanc, M. E. Carson, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. Journal of dairy science 95(10):5676-5682.

Curtis, C. R., H. N. Erb, C. J. Sniffen, R. D. Smith, P. A. Powers, M. C. Smith, M. E. White, R. B. Hillman, and E. J. Pearson. 1983. Association of parturient hypocalcemia with eight periparturient disorders in Holstein cows. Journal of the American Veterinary Medical Association 183(5):559-561.

DeGaris, P. J. and I. J. Lean. 2008. Milk fever in dairy cows: a review of pathophysiology and control principles. Veterinary journal (London, England : 1997) 176(1):58-69.

Duffield, T. F., K. E. Leslie, D. Sandals, K. Lissemore, B. W. McBride, J. H. Lumsden, P. Dick, and R. Bagg. 1999. Effect of prepartum administration of monensin in a controlled-release capsule on milk production and milk components in early lactation. Journal of dairy science 82(2):272-279.

Erb, H. N. and S. W. Martin. 1978. Age, breed and seasonal patterns in the occurrence of ten dairy cow diseases: a case control study. Canadian journal of comparative medicine : Revue canadienne de medecine comparee 42(1):1-9.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. Journal of dairy science 77(9):2695-2703.

Fleischer, P., M. Metzner, M. Beyerbach, M. Hoedemaker, and W. Klee. 2001. The relationship between milk yield and the incidence of some diseases in dairy cows. Journal of dairy science 84(9):2025-2035.

Forman, D. and L. Lorenzo. 1991. Ionized calcium: its significance and clinical usefulness. Annals of Clinical & Laboratory Science 21(5):297-304.

Galvao, K. N., M. Frajblat, W. R. Butler, S. B. Brittin, C. L. Guard, and R. O. Gilbert. 2010. Effect of early postpartum ovulation on fertility in dairy cows. Reproduction in domestic animals = Zuchthygiene 45(5):e207-211.

Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Veterinary journal (London, England : 1997) 176(1):50-57.

Goff, J. P. 2014. Calcium and magnesium disorders. The Veterinary clinics of North America. Food animal practice 30(2):359-381, vi.

Hansen, S. S., J. Y. Blom, A. Ersboll, and R. J. Jorgensen. 2003. Milk fever control in Danish dairy herds. Acta veterinaria Scandinavica. Supplementum 97:137-139.

Horst, R. L., J. P. Goff, T. A. Reinhardt, and D. R. Buxton. 1997. Strategies for preventing milk fever in dairy cattle. Journal of dairy science 80(7):1269-1280.

Iwersen, M., U. Falkenberg, R. Voigtsberger, D. Forderung, and W. Heuwieser. 2009. Evaluation of an electronic cowside test to detect subclinical ketosis in dairy cows. Journal of dairy science 92(6):2618-2624.

Jawor, P. E., J. M. Huzzey, S. J. LeBlanc, and M. A. von Keyserlingk. 2012. Associations of subclinical hypocalcemia at calving with milk yield, and feeding, drinking, and standing behaviors around parturition in Holstein cows. Journal of dairy science 95(3):1240-1248.

Jonsson, N. N., W. J. Fulkerson, P. M. Pepper, and M. R. McGowan. 1999. Effect of genetic merit and concentrate feeding on reproduction of grazing dairy cows in a subtropical environment. Journal of dairy science 82(12):2756-2765.

Kamgarpour, R., R. C. Daniel, D. C. Fenwick, K. McGuigan, and G. Murphy. 1999. Post partum subclinical hypocalcaemia and effects on ovarian function and uterine involution in a dairy herd. Veterinary journal (London, England : 1997) 158(1):59-67.

Kimura, K., T. A. Reinhardt, and J. P. Goff. 2006. Parturition and hypocalcemia blunts calcium signals in immune cells of dairy cattle. Journal of dairy science 89(7):2588-2595.

Kragh-Hansen, U. and H. Vorum. 1993. Quantitative analyses of the interaction between calcium ions and human serum albumin. Clinical chemistry 39(2):202-208.

LeBlanc, S. J., K. E. Leslie, and T. F. Duffield. 2005. Metabolic predictors of displaced abomasum in dairy cattle. Journal of dairy science 88(1):159-170.

Martin-Tereso, J. and H. Martens. 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.

Martin-Tereso, J. and M. W. Verstegen. 2011. A novel model to explain dietary factors affecting hypocalcaemia in dairy cattle. Nutrition research reviews 24(2):228-243.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao, and J. E. Santos. 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.

Martinez, N., L. D. Sinedino, R. S. Bisinotto, E. S. Ribeiro, G. C. Gomes, F. S. Lima, L. F. Greco, C. A. Risco, K. N. Galvao, D. Taylor-Rodriguez, J. P. Driver, W. W. Thatcher, and J. E. Santos. 2014. Effect of induced subclinical hypocalcemia on physiological responses and neutrophil function in dairy cows. Journal of dairy science 97(2):874-887.

McArt, J. A., D. V. Nydam, and G. R. Oetzel. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. Journal of dairy science 95(9):5056-5066.

McNally, J. C., M. A. Crowe, J. F. Roche, and M. E. Beltman. 2014. Effects of physiological and/or disease status on the response of postpartum dairy cows to synchronization of estrus using an intravaginal progesterone device. Theriogenology 82(9):1263-1272.

Murray, R. D., J. E. Horsfield, W. D. McCormick, H. J. Williams, and D. Ward. 2008. Historical and current perspectives on the treatment, control and pathogenesis of milk fever in dairy cattle. The Veterinary record 163(19):561-565.

Oetzel, G. R. and B. E. Miller. 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.

Ong, G. S., J. P. Walsh, B. G. Stuckey, S. J. Brown, E. Rossi, J. L. Ng, H. H. Nguyen, G. N. Kent, and E. M. Lim. 2012. The importance of measuring ionized calcium in characterizing calcium status and diagnosing primary hyperparathyroidism. The Journal of clinical endocrinology and metabolism 97(9):3138-3145.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010a. Association between the proportion of sampled transition cows with increased nonesterified fatty acids and beta-hydroxybutyrate and disease incidence, pregnancy rate, and milk production at the herd level. Journal of dairy science 93(8):3595-3601.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010b. Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. Journal of dairy science 93(4):1596-1603.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010c. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. Journal of dairy science 93(2):546-554.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Veterinary journal (London, England : 1997) 188(1):122-124.

Reynolds, C. K., P. C. Aikman, B. Lupoli, D. J. Humphries, and D. E. Beever. 2003. Splanchnic metabolism of dairy cows during the transition from late gestation through early lactation. Journal of dairy science 86(4):1201-1217.

Ribeiro, E. S., F. S. Lima, L. F. Greco, R. S. Bisinotto, A. P. Monteiro, M. Favoreto, H. Ayres, R. S. Marsola, N. Martinez, W. W. Thatcher, and J. E. Santos. 2013. Prevalence of periparturient diseases and effects on fertility of seasonally calving grazing dairy cows supplemented with concentrates. Journal of dairy science 96(9):5682-5697.

Risco, C. A., J. P. Reynolds, and D. Hird. 1984. Uterine prolapse and hypocalcemia in dairy cows. Journal of the American Veterinary Medical Association 185(12):1517-1519.

Santos, J. E., H. M. Rutigliano, and M. F. Sa Filho. 2009. Risk factors for resumption of postpartum estrous cycles and embryonic survival in lactating dairy cows. Animal reproduction science 110(3-4):207-221.

Sato, R., K. Onda, H. Kato, H. Ochiai, K. Kawai, T. Iriki, K. Kaneko, Y. Yamazaki, and Y. Wada. 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 (London, England : 1997) 197(2):358-362.

Sava, L., S. Pillai, U. More, and A. Sontakke. 2005. Serum calcium measurement: Total versus free (ionized) calcium. Indian journal of clinical biochemistry : IJCB 20(2):158-161.

Seifi, H. A., S. J. Leblanc, K. E. Leslie, and T. F. Duffield. 2011. Metabolic predictors of post-partum disease and culling risk in dairy cattle. Veterinary journal (London, England : 1997) 188(2):216-220.

Sepulveda-Varas, P., D. M. Weary, M. Noro, and M. A. von Keyserlingk. 2015. Transition diseases in grazing dairy cows are related to serum cholesterol and other analytes. PloS one 10(3):e0122317.

Stokol, T. and D. V. Nydam. 2005. Effect of anticoagulant and storage conditions on bovine nonesterified fatty acid and beta-hydroxybutyrate concentrations in blood. Journal of dairy science 88(9):3139-3144.

Sweeney, B., E. Martens, M. Felippe, and T. Overton. 2014. Impacts and Evaluation of Subclinical Hypocalcemia in Dairy Cattle. in Proc. Cornell Nutrition Conference.

Wang, S., E. H. McDonnell, F. A. Sedor, and J. G. Toffaletti. 2002. pH effects on measurements of ionized calcium and ionized magnesium in blood. Archives of pathology & laboratory medicine 126(8):947-950.

Wathes, D. C., M. Fenwick, Z. Cheng, N. Bourne, S. Llewellyn, D. G. Morris, D. Kenny, J. Murphy, and R. Fitzpatrick. 2007. Influence of negative energy balance on cyclicity and fertility in the high producing dairy cow. Theriogenology 68 Suppl 1:S232-241.

CHAPTER 5: Finding an objective measurement to body condition scoring dairy cows: dynamics of body condition score, ultrasound measured back fat thickness, and body weight in early lactation and its association with milk production in herds using automatic milking systems*

*Chapter formatted according to Journal of Dairy Science author's guideline.

ABSTRACT

Dairy cows undergo a state of negative energy balance after parturition and use their body fat reserves as an energy source. Body condition score (**BCS**) is the most common subjective method to determine body fat reserves and has been shown to be correlated to fat reserves in dairy cows. Alternatively, back-fat thickness (**BFT**) and body weight (**BW**) are potential objective methods to determine body energy reserves and change in these measures can indicate mobilization of body reserves. The objectives of this study were to describe the dynamic of BCS, BFT, and BW in the first 60 DIM, and to evaluate the association between these variables and production outcomes in herds using automatic milking systems. In a longitudinal prospective study, data was collected from 105 cows. Body condition scores and BFT were measured weekly and BW, daily. A repeated measures model was used for the descriptive analysis of the parameters and a mixed procedure with repeated measures was used to determine the potential explanatory variables related to milk production. Animals from both parity groups reached BW, BCS, and BFT nadir within 60 DIM and an upwards trend was observed by the end of the voluntary waiting period. Daily BW change and BW change during the first 30 DIM better explains milk production when compared to BCS and BFT. Daily BW data might be a better tool to monitor dairy cow during lactation when compared to BCS.

Key words: automatic milking systems, body condition score, body weight, back-fat thickness, dairy cows.

INTRODUCTION

In early lactation, cows are not able to fulfill the requirements for milk production by feed intake alone therefore they undergo a period of negative energy balance (Bauman and Currie, 1980, Herdt, 2000). In order to overcome this challenge animals will mobilize their body reserves, mostly adipose tissue, releasing metabolites (non-esterified fatty acids – **NEFA**) that can be used by various tissues as an energy source (Bauman and Currie, 1980, Bell, 1995, Drackley, 1999). However, excessive levels of either of these metabolites has been associated with negative health, reproductive and productive outcomes (Duffield et al., 2009, Ospina et al., 2010c, b, Chapinal et al., 2011, McArt et al., 2012). Various attempts to decrease lipid mobilization in the early lactation and re-establish energy balance have been made with limited success due to the genetic predisposition for milk production (Grummer et al., 1995, Andersen et al., 2004, Delaby et al., 2009). Although, lipid mobilization and negative energy balance may not be eliminated from the early post-partum period, managing the use of fat reserves to avoid excessive mobilization can limit possible negative effects during lactation. Currently, several options for monitoring and measuring changes in the use of energy reserves in early lactation are used in animal agriculture: body condition score, back-fat measurement, and monitoring body weight.

Various body condition score (**BCS**) systems have been described in different parts of the world using different scales (Ferguson et al., 1994, Roche et al., 2004); regardless of scale, lower scores reflect thinner animals and higher score reflect over-conditioned cows. Even though BCS is an effective and simple way to determine the cow's energy stores (Fox et al., 1999), it is a subjective measurement with undesirable inter and intra-observer variation (Ferguson et al., 1994, Kristensen et al., 2006). Nonetheless, BCS has been widely used as a management tool to

determine body fat. In fact, optimum BCS and BCS dynamics during lactation have been determined for dairy cows: the optimum calving BCS for milk production has been reported to be around 3.5, in a 5-point scale; and a moderate BCS loss in early lactation, specifically less than 0.75 points on a 5-point scale, has been associated with increased milk production (Roche et al., 2007a). Additionally, it has been determined that dairy cows calving with BCS $\geq$ 3.75 are less likely to get pregnant and BCS loss of over 1 unit (~ 430 Mcal/BCS unit) in early lactation is associated with impaired reproductive performance (Fox et al., 1999, Lopez-Gatius et al., 2003, Roche et al., 2007b, Hoedemaker et al., 2009, Pires et al., 2013). Previous reports have demonstrated that cows with higher BCS at calving lost more BCS during early lactation because of decreased dry matter intake and higher incidence of ketosis (Treacher et al., 1986, Gillund et al., 2001). Animals at either extremity of the BCS scale have an increased risk of compromised animal welfare (Roche et al., 2009, Matthews et al., 2012), including increased prevalence of claw-horn disruption in animals with low BCS (Bicalho et al., 2009).

The measurement of the layer of subcutaneous fat (back-fat-thickness – **BFT**) has been commonly used to assess energy stores and carcass quality in beef cattle (Bullock et al., 1991, Greiner et al., 2003, Emenheiser et al., 2014). Yet, only recently this approach was used to determine dairy cows BFT (Schroder and Staufenbiel, 2006). Ultrasonography of BFT can reasonably predict, with an acceptable degree of accuracy, the subcutaneous fat (Brethour, 1992, Bruckmaier et al., 1998); therefore, BFT has the potential to be used as an objective measurement of body reserves. Similar to BCS, BFT has been associated with milk production and reproductive performance (Mosenfechtel et al., 2002).

Another objective measurement that can be used as an alternative to BCS is body weight (**BW**). However, single measurements of BW may not be a good indicator of body reserves

because parity, stage of lactation, gestation, breed, gastrointestinal fill, and udder weight can lead to variations in this measurement. On the other hand, the use of repeated measurements of BW allows an assessment of BW variation throughout lactation enabling its use as an accurate measure, even though gut fill might influence individual measurements (Thorup et al., 2013). Some major challenges for the sequential BW measures in commercial dairies are: the initial investment to install the scales, as well as maintenance costs, and the costs related to the excessive handling of the animals and time budget. Nonetheless, the utilization of automatic milking systems (**AMS**) with built-in scales makes the daily weighing of cows in commercial farms feasible. Additionally, gut and udder fill can change BW measurements of dairy cows. The association between BW and production outcomes has been determined previously with extremely heavy animals that present a rapid weight loss after calving having decreased milk production and impaired reproductive performance due to decrease dry matter intake and a more severe negative energy balance status (Berry et al., 2003, Berry et al., 2007, Roche et al., 2007a, Roche et al., 2007b, Sakaguchi, 2009).

Body condition score has been reported to be correlated to BFT and BW measurements with all three measurement being used to determine dairy cows' energy stores (Domecq et al., 1995, Hussein et al., 2013). Thus, understanding the normal dynamics of the BCS, BFT, and BW is essential to identify animals that are not complying with expected patterns during early lactation allowing implementation of preventive interventions. A rigorous analysis of variation of these 3 parameters in early lactation, with detection of target values can be beneficial to assessment of management programs, preventing metabolic problems and avoid decreased production (Roche et al., 2013). The extent of the energy deficit, indirectly determined by assessment of variation in body condition measurement, and the use of tools and strategies to overcome this challenge

are important to achieve greater production. Therefore, the objectives of this study were to describe the dynamics of BCS, BFT, and BW in the first 60 DIM, evaluate the correlation between the three different strategies to measure body reserves and body reserves utilization, and to determine the association between these potential explanatory variables and milk production.

MATERIALS AND METHODS

Study population and herds characteristics

The study was conducted in 2 commercial herds in central New York. These herds met the following criteria: 1) greater than 100 milking cows, 2) free-stall housing, 3) fed a partial mixed ration (**PMR**), and 4) use of AMS. Herd A milked over 700 cows using 14 AMS while herd B used 7 AMS to milk 400 cows.

The cows were housed in free-stall barns with concrete stalls and sand bedded in herd A, and concrete stalls covered with mattresses and bedded with waste paper-pulp in herd B. In both herds, the alleys had grooved-concrete flooring and were cleaned by automatic scrapers. The dry cow total mixed ration consisted of 80% forage and 20% concentrate during the dry period, and lactating cows received a PMR consisting of 55% forage and 45%. The diet was formulated to meet or exceed the NRC (2001) nutrient requirements for dry and lactating Holsteins. After parturition part of the ration was offered to lactating animals as a grain mixture in the form of pellets at the milking units to stimulate visits to AMS, the amount received by each animal varied according to estimated milk production and stage of lactation.

Study design and data collection

A longitudinal prospective observational study was conducted during the summer of 2012. Animals were enrolled in the study once a week, between 3 and 10 days prior to the expected calving date reported by the management software present at the farm; once enrolled animals were closely monitored until 60 DIM. At enrollment, animals were restrained to allow determination of BCS (Ferguson et al., 1994) by two trained veterinarians and scores were averaged to minimize inter-observer bias. Body condition score was determined prior to measurement of BFT ultrasound to avoid bias.

The BFT was determined by ultrasound measurements of the subcutaneous fat in the middle point between the hook and the pin bones in the thurl area according to Schroder and Staufenbiel (2006) by the same trained veterinarian throughout the experiment to avoid measurement discrepancies. The depth of the subcutaneous fat was assessed using a portable ultrasound machine and a linear probe (IBEX pro, E.I. Medical Imaging, Colorado, USA) set at a frequency of 5 MHz. Prior to scanning the area was cleaned and 70% alcohol was applied to improve image quality and a single measurement was taken at each sampling day. Upon start of lactation BW was measured every time the cows were milked by the AMS built-in scale. Even though most of the cows had multiple BW measurements in a day, only a daily average was exported from the Lely T4C management system (Lely Industries N.V/, Rotterdam, The Netherlands) into Excel (Microsoft, v. 97-2003). Unlike BW which was measured daily after calving, the measurements of BCS and BFT to determine their dynamics in early lactation were assessed one week prior to expected calving date and weekly after parturition, until 60 DIM.

Information on whether animals developed any diseases (i.e., displaced abomasum, clinical ketosis, metritis, milk fever, and retained placenta) during the first 10 DIM was documented on

Dairy Comp 305 (Valley Ag. Software, 2009). Standard disease definitions were discussed with farm employees and veterinary responsible for the herd at the start of the study. Nevertheless, disease diagnosis and treatment was performed by farm personnel according to herd protocols defined by the herd veterinarian.

Blood samples were collected 7 ± 3 days pre-partum and post-partum on days 3 and 5. Serum samples were stored at -20°C and laboratory assays to determine metabolites concentration were performed upon end of collection period. Serum NEFA concentration was determined by a commercial kit (NEFA-C, Wako Chemicals); and BHB was evaluated at cow side using the Precision Xtra meter (Abbott Laboratories) (Iwersen et al., 2009). Blood metabolites were dichotomized using the following cut- offs points: pre-partum NEFA was dichotomized at $\geq$ 0.3 mEq; post-partum NEFA $\geq$ 0.7 mEq/L, and post-partum BHB $\geq$ 1.2 mmol/L. Previous reports have shown that individuals presenting plasma metabolite concentrations exceeding these thresholds were more likely to develop diseases during early lactation, and have impaired milk production and reproductive performance (Ospina et al., 2010c, McArt et al., 2012). Therefore, a dichotomous variable, elevated energy balance metabolites (**EEBM**), was defined as positive if any of those measurements exceeded the thresholds established.

Milk production throughout lactation was used as outcome for the current study. Daily milk production data was extracted from the AMS software at the end of the 60 day period and total lactation milk production, adjusted for 305 DIM (**305M**), was collected from the management software used by the farm.

Statistical analysis

The variables BCS, BFT, and BW were checked for normality using the UNIVARIATE procedure of SAS. Descriptive statistics and comparison between parity groups for average

BCS, BFT, and BW at calving and 30 DIM was generated using ANOVA procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). The analysis of the dynamics of BCS, BFT, and BW in the first 60 DIM was generated using a MIXED procedure with repeated measures and Tukey's adjustments for multiple comparisons in SAS (version 9.3 SAS Inst. Inc., Cary, NC). Animals were stratified into 2 parity groups (primiparous, multiparous).

The change in BW (**BWΔ**), BCS (**BCSΔ**), and BFT (**BFTΔ**) at 30 DIM was calculated subtracting the value at 30 DIM from that at the week prior to calving for BCS and BFT, and calving day for BW. Daily body weight change (**DBWC**) during the first 30 DIM was determined using the daily BW measurements taken by the AMS. The daily BW data was used to in a simple linear regression with the coefficient of the regression line corresponding to the DBWC (JMP statistical package, version 9 SAS Inst. Inc., Cary, NC, 2011). The distribution of BWΔ, BCSΔ, BFTΔ, and DBWC within parity groups were determined using UNIVARIATE procedure. To facilitate data analysis and results interpretation, the variables DBWC, BWΔ, BCSΔ, and BFTΔ were categorized into 3 levels according to their distribution quantile within parity group. Values below the 25^{th} percentile were identified as low variation (**LOW**), values between the 25^{th} and 75^{th} percentile were identified as medium variation (**MEDIUM**), and values above the 75^{th} percentile were identified as high variation (**HIGH**). Upper and lower levels for each quantile of all the explanatory variables are presented in Table 5.1. Other explanatory variables used in the statistical models were: development of any of the aforementioned diseases within 10 DIM and dichotomous variable created based on plasma metabolite concentration, EEBM. Herd was used as random effect in the models and cows were nested within herd.

Table 5.1. Limits for quantiles of each body condition score measurement according to parity group.

	Primiparous (n = 35)		Multiparous (n = 70)	
Variable quantile	25th Percentile	75th Percentile	25th Percentile	75th Percentile
DBWC[1] (kg/day)	0.18	-1.31	-0.47	-2.17
BWΔ[2] (kg)	-6.36	-38.18	-21.8	-64.1
BCSΔ[3]	-0.25	-0.75	-0.25	-0.75
BFTΔ[4] (mm)	-3	-11	-3	-10

[1] Daily body weight change
[2] Body weight variation in the first 30 DIM
[3] Body condition score variation in the first 30 DIM
[4] Back-fat thickness variation in the first 30 DIM

The correlation between the three different methods of measuring body condition was determined using CORR procedure of SAS. Milk production was evaluated on a daily basis during the first 60 DIM; total milk produced within 60 DIM and total milk produced during the entire lactation were later used to determine possible associations with study variables. For statistical analysis the continuous variable, milk production, was checked for normality and repeated measures over time were analyzed using MIXED procedure of SAS. The covariance structure used for each model was chosen based on the smallest Akaike information criterion. A Kenward-Roger degrees of freedom approximation was used to calculate the denominator degrees of freedom. The evaluation of the influence of DBWC, BWΔ, BCSΔ, and BFTΔ in the total milk produced over 60 days and throughout whole lactation was determined by MIXED procedure in SAS using herd as random effect with animals nested within. Development of any disease and EEBM were added as dichotomous explanatory variables. Potential explanatory variables were removed from the statistical models in a manual backward stepwise fashion if $P > 0.15$.

RESULTS

In total, 114 animals were enrolled during the study period; however 9 were excluded due to removal from herd that resulted in less than 30 days of milk production and body weight information. Of these 9, three animals died in the first 30 days of the trial (1 presumptive listeriosis case, 1 back injury and 1 non-defined), 2 animals were culled by farm personnel and 4 cows were excluded from the analysis because of incomplete data collection due to problems in the AMS. The remaining 105 animals were comprised of 35 primiparous animals and 70 animals from multiparous.

Body weight, body condition score, and back-fat thickness

Descriptive statistics of BCS, BFT, and BW for the different parity groups at calving and at 30 DIM is presented in Table 5.2. Body condition measurements were normally distributed within parity groups and quantiles calculations were performed. Daily body weight change during the first 30 DIM ranged from -4.0 to 0.94 kg/day, with a mean of -0.7 kg/day for primiparous; older animals had a greater range of DBWC varying from -4.42 to 1.79 kg/day (mean of -1.48kg/day). Following the same pattern BWΔ varied differently depending parity group: -94.1 to 8.2 kg for primiparous and -123.2 to 20.9 kg for multiparous over the first 30 DIM. Change in BFT and BCS during the first 30 DIM were similar for both groups with lowest BFTΔ around -20 mm and highest at 3 mm; both groups had their most extensive BCS loss as -1.25 units and the least change in BCS as an increase of 0.25 units.

Table 5.2. Descriptive statistics of BW[1], BCS[2], and BFT[3] for the different parity groups at calving and at 30 DIM.

	Primiparous (n = 35) Mean (±SE)	Multiparous (n = 70) Mean (±SE)	*P*-value
Average BW at calving, kg	577 ± 13.1	693 ± 8.9	< 0.001
Average BW at 30 DIM, kg	554 ± 11.8	669 ± 8.4	< 0.001
Average BCS at calving	3.9 ± 0.05	4.2 ± 0.04	0.08
Average BCS at 30 DIM	3.4 ± 0.05	3.5 ± 0.04	0.003
Average BFT at calving, mm	28.4 ± 1.1	27.1 ± 0.7	0.3
Average BFT at 30 DIM, mm	21.6 ± 0.7	20.5 ± 0.5	0.2

[1] Body weight (kg)
[2] Body condition score
[3] Back-fat thickness (mm)

On average cows from both parity groups had similar dynamics of BCS, BFT and BW; most animals, regardless of parity group, lost BW, BCS, and BFT in early lactation, especially within 30 DIM. A downwards trend on all three body condition measurements was observed during early lactation but it was reversed around the 4th week of lactation for primiparous animals (Figure 5.1). Regardless of the measurement used, multiparous animals took longer to reach nadir when compared to primiparous. Primiparous animals reached BCS nadir at 4 weeks post-partum while multiparous at about 5 weeks; similarly 1 week difference was observed when comparing the time to lowest BFT during early lactation (5 weeks vs. 6 weeks, for primiparous and multiparous respectively). A greater difference, two weeks, was observed when analyzing BW change in early lactation; primiparous animals reached their lightest weight at 20 DIM while multiparous around 33 DIM. Overall all types of measurement techniques were correlated ($P <$ 0.001) to each other regardless of parity group (Table 5.3).

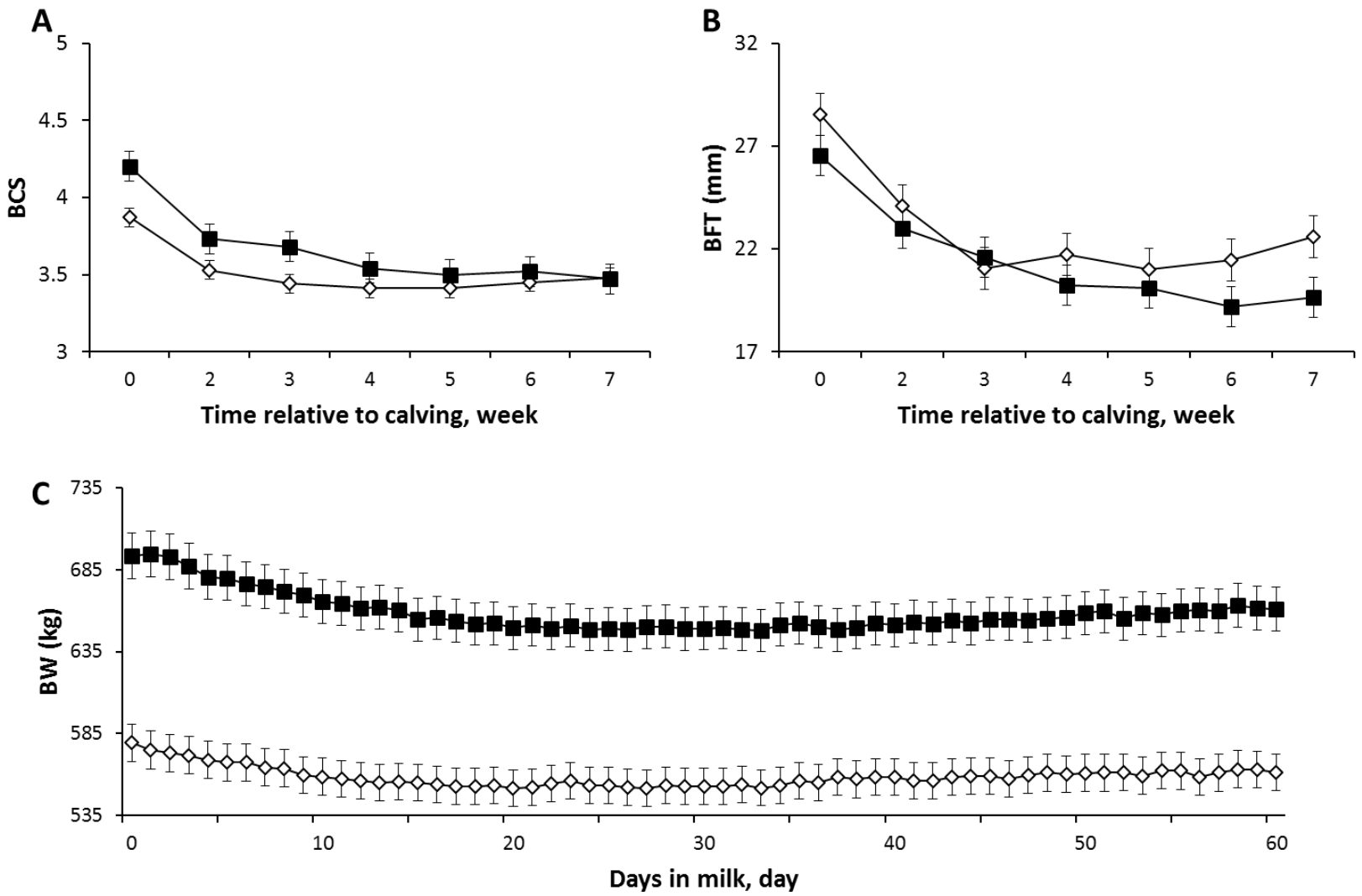

Figure 5.1. Dynamics of BCS (A), BFT (B), and bodyweight (C) during the voluntary waiting period for primiparous (◊) and multiparous (■).

Table 5.3. Correlation among the different methods of measuring body condition in the study.

		BCS[1]		BFT[2]	
		Correlation coefficient	*P*-value	Correlation coefficient	*P*-value
BFT	Primiparous	0.60	<0.0001		
	Multiparous	0.69	<0.0001		
BW[3]	Primiparous	0.41	<0.0001	0.38	<0.0001
	Multiparous	0.44	<0.0001	0.47	<0.0001

[1]Body condition score
[2]Backfat thickness (mm)
[3]Body weight (kg)

Milk production

Daily milk production increment up to 30 DIM did not differ between body measurements quantiles irrespective of parity groups. On average all animals increased their milk production during the first 30 DIM by about 250 g daily, regardless of health and energy balance status. Daily milk production during the first 60 DIM for both parity groups according to DBWC quantiles is presented in Figure 5.2. All animals presented a greater increase in milk production during the first 20 DIM, independently of quantile groups.

Primiparous animals that had the least variation in weight daily ($DBWC_{LOW}$) produced less milk when compared to the other quantile groups (quantile x day interaction; $P = 0.04$), with these animals producing on average sixe fewer kilos of milk daily between 2 DIM and 48 DIM. On the other hand DBWC did not significantly influence daily milk production of multiparous which is shown by the similar milk curves of the three quantiles (quantile x day interaction; $P = 0.2$)

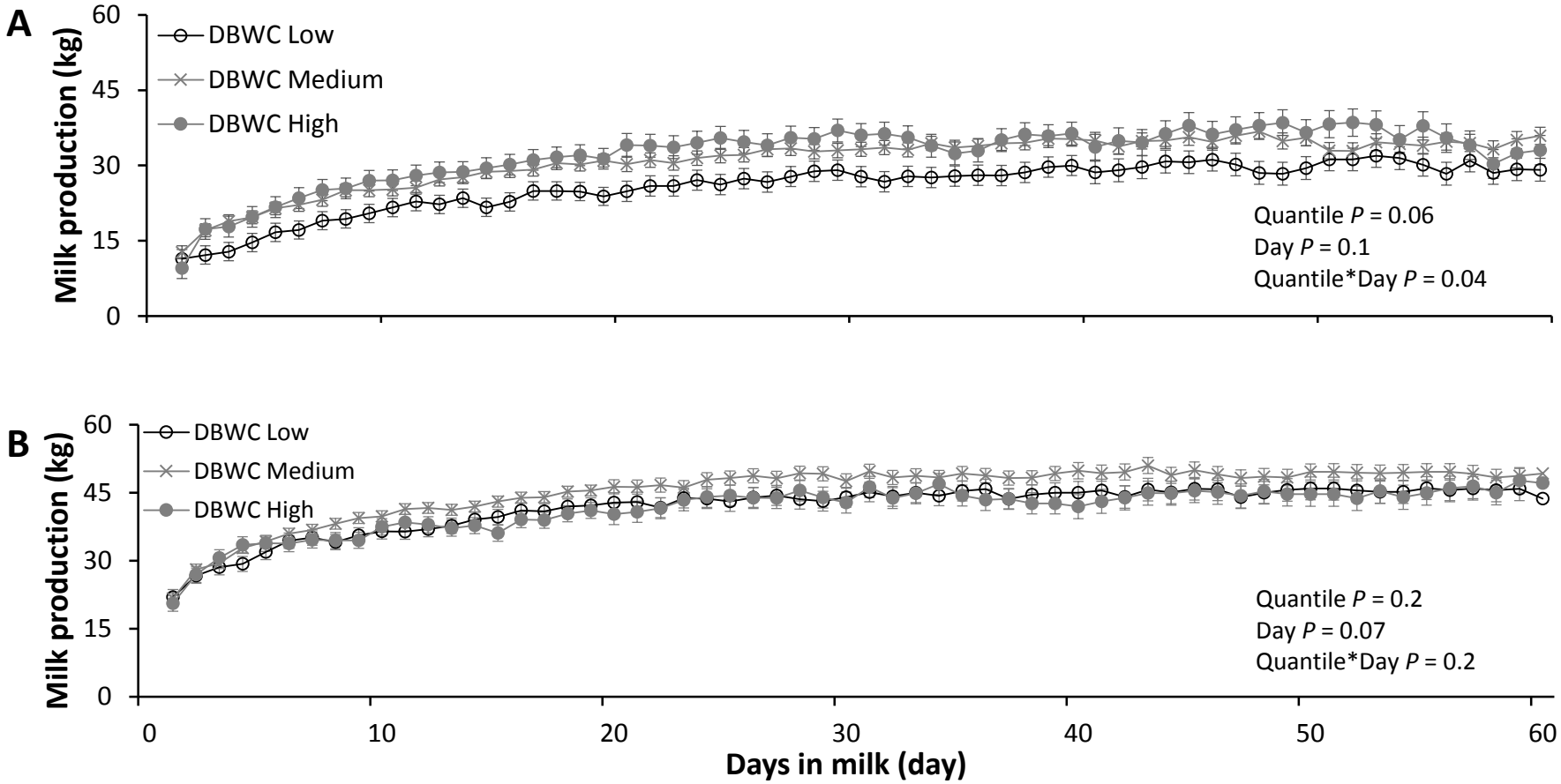

Figure 5.2. Daily milk production during the first 60 DIM according to daily body weight change in different parity groups. A total of 35 primiparous animals (A) are represented in the figure ($DBWC_{LOW}$ n = 9; $DBWC_{MEDIUM}$, n = 17, $DBWC_{HIGH}$, n = 9). First lactation individuals that presented the least daily bodyweight change ($DBWC_{LOW}$) produced significantly less milk when compared to the groups. Seventy multiparous animals (B) are represented ($DBWC_{LOW}$ n = 18; $DBWC_{MEDIUM}$, n = 34, $DBWC_{HIGH}$, n = 18). Amongst the multiparous animals milk production was similar for the 3 different groups with animals losing moderate body weight producing numerically more milk throughout the first 60 days of lactation. For each individual time point results represents the least square mean ± SEM of all cows in each group.

The association between DBWC, BWΔ, BCSΔ, and BFTΔ and total milk production during the first 60 DIM and throughout the whole lactation (305 DIM) was analyzed for the different parity groups accounting for early lactation disease and EEBM. Independently of the period analyzed (30DIM, 60 DIM, 305DIM) BCSΔ and BFTΔ were not associated ($P > 0.1$) with milk production. Similarly, EEBM did not affect milk production of animals enrolled in this study; hence no data are presented. Results for the effect of DBWC and BWΔ, as well as disease occurring during the first 10 DIM is presented in Table 5.4 and 5.5.

For primiparous animals, DBWC in the first 30 DIM influenced milk production ($P < 0.01$) in the first 60 DIM while BWΔ did not change milk production. The effect of DBWC was opposite when comparing early lactation and whole lactation milk production: in our experiment it was calculated that for each extra kg of daily body weight lost in the first 30 DIM animals increased their total milk produced in the first 60 DIM by approximately 274 kg ($P < 0.001$) while the total milk production during the first lactation was decreased by 432 kg ($P = 0.06$). Interestingly, the occurrence of any disease in the first 10 DIM was associated with an increased total milk produced during the first 60 DIM (approximately 490 kg; $P = 0.05$) with no effects on total milk produced throughout 305 DIM.

Even though whole lactation milk production for multiparous tended to be affected by DBWC ($P = 0.1$) no other association between body weight changes and milk production was observed for this parity group in the study animals. On the other hand, the occurrence of disease during the first 10 DIM was associated with decreased milk production during the first 60 DIM (-256 kg ± 115, $P = 0.03$ when analyzing DBWC; and -276 kg ± 125, $P = 0.03$ when analyzing BWΔ) and tended to decreased milk production during 305 DIM (-1127 kg ± 597, $P = 0.06$ when analyzing DBWC; and -1088 ± 631, $P = 0.09$ when analyzing BWΔ) for this parity group.

Table 5.4. Estimated effects of body weight change and disease in early lactation on milk production (kg) during the first 60 DIM according to parity.

Effect	Estimate	Standard Error	*P*-value
Primiparous			
DBWC[1]			
Intercept[2]	2427	117.6	< 0.001
DBWC	273.9	97.7	< 0.01
Disease[3]	489.8	238.8	0.05
Multiparous			
DBWC			
Intercept[2]	2400	95.8	< 0.001
DBWC	82.8	44.3	0.6
Disease[3]	-255.8	115.2	0.03
BWΔ			
Intercept[2]	2362	108.4	< 0.001
BWΔ[4]	1.8	1.9	0.4
Disease[3]	-275.8	124.9	0.03

[1] DBWC was defined as the daily bodyweight change during the first 30DIM.
[2] Total milk production for animals that did not have daily bodyweight change, and did not develop any disease during the first 10 DIM.
[3] Disease defined as positive if animal developed any of the following diseases within 10 days in milk: retained placenta, metritis, clinical ketosis, or displaced abomasum.
[4] BWΔ was defined as the difference in bodyweight between 30 DIM and 1DIM.

Table 5.5. Estimated effects of body weight change and disease in early lactation on milk production (kg) during the whole lactation (305 DIM) according to parity.

Effect	Estimate	Standard Error	*P*-value
Primiparous			
DBWC[1]			
Intercept[2]	9433	290.4	< 0.001
DBWC	-432.9	225.9	0.06
Multiparous			
DBWC			
Intercept[2]	13608	485.8	< 0.001
DBWC	347.5	222.2	0.1
Disease[3]	-1127.3	596.9	0.06
BWΔ			
Intercept[2]	12672	711.55	< 0.001
BWΔ[4]	13.7	9.8	0.2
Disease[3]	2003.5	1322.16	0.3
BWΔ x Disease	-8160.96	1662.82	0.03

[1] DBWC was defined as the daily bodyweight change during the first 30DIM.
[2] Total milk production for animals that did not have daily bodyweight change, and did not develop any disease during the first 10 DIM.
[3] Disease defined as positive if animal developed any of the following diseases within 10 days in milk: retained placenta, metritis, clinical ketosis, or displaced abomasum.
[4] BWΔ was defined as the difference in bodyweight between 30 DIM and 1DIM.

After dividing body weight changes into quantiles to facilitate statistical interpretation, the interaction between diseases and DBWC was not significant for both parity groups ($P = 0.2$ for primiparous; and $P = 0.13$ for multiparous) even though the number of sick animals was not equally distributed among the different groups. Similarly, the BWΔ and disease interaction was not important for primiparous animals ($P = 0.6$). On the contrary, the interaction of BWΔ and disease in the first 10 DIM significantly influence whole lactation milk production of multiparous animals ($P = 0.03$). The results of the interaction between BWΔ quantiles and disease occurrence in the first 10 DIM for multiparous animals are presented in Figure 5.3. Briefly, animals that did not have a disease event in early lactation produced similar amounts of milk throughout lactation even though a numerical difference was observed ($BW\Delta_{LOW}$ = 12,672kg; $BW\Delta_{MEDIUM}$ = 13,369 kg; $BW\Delta_{HIGH}$ = 13,150 kg; $P \geq 0.1$). On the other hand, whole lactation milk production of animals that were sick in early lactation was significantly different when comparing the different amount of BW lost during the first 30DIM: in fact, animals that $BW\Delta_{LOW}$ produced significantly more than animals that developed disease in the first 10 DIM and lost more BW during the first 30 DIM ($BW\Delta_{MEDIUM}$ = 11,467 kg, $P = 0.01$; $BW\Delta_{HIGH}$ = 10,898 kg, $P < 0.01$).

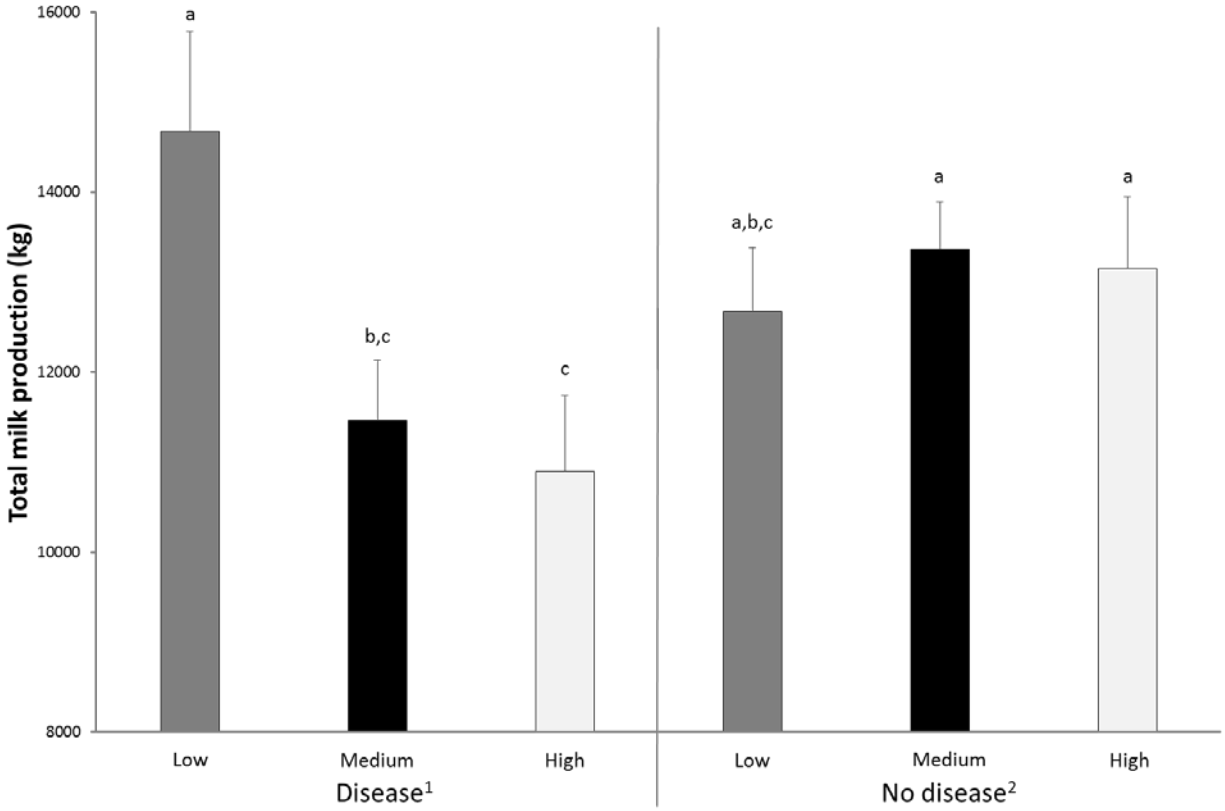

Figure 5.3. Total milk produced during the whole lactation based on the interaction between difference in bodyweight during the first 30 DIM and occurrence of disease in multiparous cows.

Animals that lost the most weight in the first 30 DIM and got sick produced statistically significant less milk that their healthy counterparts (10,898kg ± 839 vs. 13,150kg ± 798; P = 0.05). Similarly, these animals produced less milk than animals that were sick in the first 10 DIM but lost the least amount of weight in the first 30 DIM (10,898kg ± 839 vs. 14,675kg ± 1,114; P < 0.001). Whole lactation milk production was adjusted for 305 DIM. Difference in body weight during the first 30 DIM was calculated subtracting the value at 30 DIM from that at calving (BWΔ). Disease was defined as development of displaced abomasum, clinical ketosis, metritis, milk fever, and retained placenta during the first 10 DIM; while no disease was defined as the absence of any disease during the same period.). Data presented in LSM ± SE. [a,b,c] Different letters indicates that milk production differs between quantile and health status (P < 0.05).

DISCUSSION

The objectives of the current study were to characterize the dynamics of BW, BCS, and BFT in the early lactation period, the correlation between the different measurements, and to determine the association of these possible explanatory variables with milk production. Few reports have evaluated BFT and BW, mostly due to the limitations of weighing cows in conventional farms and lack of user friendly ultrasound technology; however, herds using AMS technology have access to the BW information daily and the development of better portable ultrasound machines have facilitated the assessment of these measurements.

In the current study pre-partum BCS and BFT measurement were used as proxy to define calving BCS and BFT in order to decrease the handling time of this animals in the first day after calving. Conversely, BW was only recorded when animals entered the lactating herd. Study animals lost BW, BFT, and BCS during early lactation similarly to previous reports by many authors (Roche et al., 2006, Sumner and McNamara, 2007, Hussein et al., 2013). A reason for the use of energy reserves that lead to loss of BW, BCS, and BFT is the state of negative energy balance that dairy cows experience in early lactation (Bell, 1995, LeBlanc et al., 2005, Ospina et al., 2010a, Chapinal et al., 2011, McArt et al., 2012).

Similar to our results, BCS, BFT, and BW have been shown to be correlated to each other and to this day this is the first report to measure all three parameters concomitantly (Domecq et al., 1995, Hussein et al., 2013). The correlation between BCS and BFT was higher ($r^2 \geq 0.6$) than the correlation between BW with either one ($r^2 < 0.45$ and $r^2 < 0.48$ for BCS and BFT, respectively) for both parity groups. This discrepancy was expected because gut fill influences BW measurement while it is not considered for the other two methods.

Not surprisingly, the BW of primiparous and multiparous animals was different ($P < 0.01$) during early lactation (Blottner et al., 2011). The enrollment of animals in herds using AMS allowed the determination of the exact day of the nadir because BW was measured on a daily basis, while the BW data from other studies was determined in a weekly basis. Therefore, the BW difference and variation in the early lactation in the present study is robust and accurate. The measurements of BFT were higher than previous reports (Blottner et al., 2011, Hussein et al., 2013). Previous studies have grouped all parities while analyzing BFT change and animals were managed within different feeding systems leading to the differences encountered between results of this studies and previous published data.

When analyzing the comparison between BW, BCS, and BFT for the different parity groups it is important to point out that primiparous animals have a less accentuated change in BW and BCS than their multiparous counterparts, while BFT change is not different. The fact that primiparous animals do not produce as much milk as multiparous animals might explain the difference in BCS at 30 DIM and a less accentuated BW loss. Even though adipose tissue reserves are being used to support milk production the growth of other tissues is likely to influence the subjective measurement of BCS while the objective measurements BW and BFT are not influenced as much.

In our study, DBWC better predicted milk production when compared to the other two parameters. Primiparous animals that presented the lowest daily change in BW had lower milk production throughout the whole lactation. On the other hand, multiparous animals that had the fastest weight loss rate had similar milk production to their counterparts, with disease being more influential than weight loss. Interestingly, an interaction between BWΔ and disease was very important to determine whole lactation milk production of multiparous animals. Decreased milk

production due to clinical and subclinical diseases and increased BW loss associated with subclinical hypocalcemia and other diseases have been previously reported and support this finding (Ospina et al., 2010b, Chapinal et al., 2011, McArt et al., 2012, Caixeta et al., 2015). As expected the associations between BCS and BFT change with milk production followed a similar pattern (Domecq et al., 1995, Roche et al., 2007a).

Even though occurrence of any disease event has been determined to be more influential for milk production in older animals (Caixeta et al., 2015), the same association could not be observed in the younger animals probably because the number of disease episodes for this group was very low. Based on the results of this study the rate of BW loss should be monitored since animals that lose weight rapidly tend to produce less total milk. This finding is supported by previous reports showing that cattle which have higher dairy genetic merit take longer to reach their BW and BCS nadir in comparison to their lower dairy genetic counterparts (Gallo et al., 1996, Berry et al., 2007).

The intensity of the BW, BCS, and BFT change as well as how rapidly these changes are happening are important to determine animals' capacity to adapt to the beginning of the lactation period. The possibility of the use of these different parameters to produce a more accurate report can be extremely important to decision making and earlier responses to abnormalities in order to prevent future losses.

CONCLUSION

The dynamics of BW, BCS, and BFT are similar for the different parity groups; animals lost BW, BCS, and BFT during early lactation but started to recuperate body measurements by the end of the of the 60 days period. Daily change in BW and BW change over the first 30 DIM were better predictors of milk production than BCS and BFT for both parity groups, while occurrence of disease in association with increased BW change in early lactation significantly affect total milk production throughout whole lactation of multiparous animals. The increased use of technology by progressive dairy farms and the pursue for accurate measurement capable of helping in the decision making of modern dairy farms may set the stage for the use of sequential BW as an objective measurement with the potential to replace body condition scoring.

REFERENCES

Andersen, J. B., N. C. Friggens, T. Larsen, M. Vestergaard, and K. L. Ingvartsen. 2004. Effect of energy density in the diet and milking frequency on plasma metabolites and hormones in early lactation dairy cows. Journal of veterinary medicine. A, Physiology, pathology, clinical medicine 51(2):52-57.

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63(9):1514-1529.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 73(9):2804-2819.

Berry, D. P., F. Buckley, P. Dillon, R. D. Evans, M. Rath, and R. F. Veerkamp. 2003. Genetic relationships among body condition score, body weight, milk yield, and fertility in dairy cows. Journal of dairy science 86(6):2193-2204.

Berry, D. P., J. M. Lee, K. A. Macdonald, and J. R. Roche. 2007. Body condition score and body weight effects on dystocia and stillbirths and consequent effects on postcalving performance. Journal of dairy science 90(9):4201-4211.

Bicalho, R. C., V. S. Machado, and L. S. Caixeta. 2009. Lameness in dairy cattle: A debilitating disease or a disease of debilitated cattle? A cross-sectional study of lameness prevalence and thickness of the digital cushion. Journal of dairy science 92(7):3175-3184.

Blottner, S., B. J. Heins, M. Wensch-Dorendorf, L. B. Hansen, and H. H. Swalve. 2011. Brown Swiss x Holstein crossbreds compared with pure Holsteins for calving traits, body weight, backfat thickness, fertility, and body measurements. Journal of dairy science 94(2):1058-1068.

Brethour, J. R. 1992. The repeatability and accuracy of ultrasound in measuring backfat of cattle. Journal of animal science 70(4):1039-1044.

Bruckmaier, R. M., L. Gregoretti, F. Jans, D. Faissler, and J. W. Blum. 1998. Longissimus dorsi muscle diameter, backfat thickness, body condition scores and skinfold values related to metabolic and endocrine traits in lactating dairy cows fed crystalline fat or free fatty acids. Zentralblatt fur Veterinarmedizin. Reihe A 45(6-7):397-410.

Bullock, K. D., J. K. Bertrand, L. L. Benyshek, S. E. Williams, and D. G. Lust. 1991. Comparison of real-time ultrasound and other live measures to carcass measures as predictors of beef cow energy stores. Journal of animal science 69(10):3908-3916.

Caixeta, L. S., P. A. Ospina, M. B. Capel, and D. V. Nydam. 2015. The association of subclinical hypocalcemia, negative energy balance and disease with bodyweight change during the first 30 days post-partum in dairy cows milked with automatic milking systems. Veterinary journal (London, England : 1997) 204(2):150-156.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. Journal of dairy science 94(10):4897-4903.

Delaby, L., P. Faverdin, G. Michel, C. Disenhaus, and J. L. Peyraud. 2009. Effect of different feeding strategies on lactation performance of Holstein and Normande dairy cows. Animal : an international journal of animal bioscience 3(6):891-905.

Domecq, J. J., A. L. Skidmore, J. W. Lloyd, and J. B. Kaneene. 1995. Validation of body condition scores with ultrasound measurements of subcutaneous fat of dairy cows. Journal of dairy science 78(10):2308-2313.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? Journal of dairy science 82(11):2259-2273.

Duffield, T. F., K. D. Lissemore, B. W. McBride, and K. E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. Journal of dairy science 92(2):571-580.

Emenheiser, J. C., R. G. Tait, Jr., S. D. Shackelford, L. A. Kuehn, T. L. Wheeler, D. R. Notter, and R. M. Lewis. 2014. Use of ultrasound scanning and body condition score to evaluate composition traits in mature beef cows. Journal of animal science 92(9):3868-3877.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. Journal of dairy science 77(9):2695-2703.

Fox, D. G., M. E. Van Amburgh, and T. P. Tylutki. 1999. Predicting requirements for growth, maturity, and body reserves in dairy cattle. Journal of dairy science 82(9):1968-1977.

Gallo, L., P. Carnier, M. Cassandro, R. Mantovani, L. Bailoni, B. Contiero, and G. Bittante. 1996. Change in body condition score of Holstein cows as affected by parity and mature equivalent milk yield. Journal of dairy science 79(6):1009-1015.

Gillund, P., O. Reksen, Y. T. Grohn, and K. Karlberg. 2001. Body condition related to ketosis and reproductive performance in Norwegian dairy cows. Journal of dairy science 84(6):1390-1396.

Greiner, S. P., G. H. Rouse, D. E. Wilson, L. V. Cundiff, and T. L. Wheeler. 2003. The relationship between ultrasound measurements and carcass fat thickness and lonizissimus muscle area in beef cattle. Journal of animal science 81(3):676-682.

Grummer, R. R., P. C. Hoffman, M. L. Luck, and S. J. Bertics. 1995. Effect of prepartum and postpartum dietary energy on growth and lactation of primiparous cows. Journal of dairy science 78(1):172-180.

Herdt, T. H. 2000. Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. The Veterinary clinics of North America. Food animal practice 16(2):215-230, v.

Hoedemaker, M., D. Prange, and Y. Gundelach. 2009. Body condition change ante- and postpartum, health and reproductive performance in German Holstein cows. Reproduction in domestic animals = Zuchthygiene 44(2):167-173.

Hussein, H. A., A. Westphal, and R. Staufenbiel. 2013. Relationship between body condition score and ultrasound measurement of backfat thickness in multiparous Holstein dairy cows at different production phases. Australian veterinary journal 91(5):185-189.

Iwersen, M., U. Falkenberg, R. Voigtsberger, D. Forderung, and W. Heuwieser. 2009. Evaluation of an electronic cowside test to detect subclinical ketosis in dairy cows. Journal of dairy science 92(6):2618-2624.

Kristensen, E., L. Dueholm, D. Vink, J. E. Andersen, E. B. Jakobsen, S. Illum-Nielsen, F. A. Petersen, and C. Enevoldsen. 2006. Within- and across-person uniformity of body condition scoring in Danish Holstein cattle. Journal of dairy science 89(9):3721-3728.

LeBlanc, S. J., K. E. Leslie, and T. F. Duffield. 2005. Metabolic predictors of displaced abomasum in dairy cattle. Journal of dairy science 88(1):159-170.

Lopez-Gatius, F., J. Yaniz, and D. Madriles-Helm. 2003. Effects of body condition score and score change on the reproductive performance of dairy cows: a meta-analysis. Theriogenology 59(3-4):801-812.

Matthews, L. R., C. Cameron, A. J. Sheahan, E. S. Kolver, and J. R. Roche. 2012. Associations among dairy cow body condition and welfare-associated behavioral traits. Journal of dairy science 95(5):2595-2601.

McArt, J. A., D. V. Nydam, and G. R. Oetzel. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. Journal of dairy science 95(9):5056-5066.

Mosenfechtel, S., M. Hoedemaker, U. J. Eigenmann, and P. Rusch. 2002. Influence of back fat thickness on the reproductive performance of dairy cows. The Veterinary record 151(13):387-388.

NRC. 2001. Nutrient requirements of dairy cattle: 2001. National Academies Press.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010a. Association between the proportion of sampled transition cows with increased nonesterified fatty acids and beta-hydroxybutyrate and disease incidence, pregnancy rate, and milk production at the herd level. Journal of dairy science 93(8):3595-3601.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010b. Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. Journal of dairy science 93(4):1596-1603.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010c. Evaluation of nonesterified fatty acids and beta-hydroxybutyrate in transition dairy cattle in the northeastern United States: Critical thresholds for prediction of clinical diseases. Journal of dairy science 93(2):546-554.

Pires, J. A., C. Delavaud, Y. Faulconnier, D. Pomies, and Y. Chilliard. 2013. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. Journal of dairy science 96(10):6423-6439.

Roche, J. R., D. P. Berry, and E. S. Kolver. 2006. Holstein-Friesian strain and feed effects on milk production, body weight, and body condition score profiles in grazing dairy cows. Journal of dairy science 89(9):3532-3543.

Roche, J. R., P. G. Dillon, C. R. Stockdale, L. H. Baumgard, and M. J. VanBaale. 2004. Relationships among international body condition scoring systems. Journal of dairy science 87(9):3076-3079.

Roche, J. R., N. C. Friggens, J. K. Kay, M. W. Fisher, K. J. Stafford, and D. P. Berry. 2009. Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. Journal of dairy science 92(12):5769-5801.

Roche, J. R., J. K. Kay, N. C. Friggens, J. J. Loor, and D. P. Berry. 2013. Assessing and managing body condition score for the prevention of metabolic disease in dairy cows. The Veterinary clinics of North America. Food animal practice 29(2):323-336.

Roche, J. R., J. M. Lee, K. A. Macdonald, and D. P. Berry. 2007a. Relationships among body condition score, body weight, and milk production variables in pasture-based dairy cows. Journal of dairy science 90(8):3802-3815.

Roche, J. R., K. A. Macdonald, C. R. Burke, J. M. Lee, and D. P. Berry. 2007b. Associations among body condition score, body weight, and reproductive performance in seasonal-calving dairy cattle. Journal of dairy science 90(1):376-391.

Sakaguchi, M. 2009. Differences between body condition scores and body weight changes in postpartum dairy cows in relation to parity and reproductive indices. The Canadian veterinary journal. La revue veterinaire canadienne 50(6):649-656.

Schroder, U. J. and R. Staufenbiel. 2006. Invited review: Methods to determine body fat reserves in the dairy cow with special regard to ultrasonographic measurement of backfat thickness. Journal of dairy science 89(1):1-14.

Sumner, J. M. and J. P. McNamara. 2007. Expression of lipolytic genes in the adipose tissue of pregnant and lactating Holstein dairy cattle. Journal of dairy science 90(11):5237-5246.

Thorup, V. M., S. Hojsgaard, M. R. Weisbjerg, and N. C. Friggens. 2013. Energy balance of individual cows can be estimated in real-time on farm using frequent liveweight measures even in the absence of body condition score. Animal : an international journal of animal bioscience 7(10):1631-1639.

Treacher, R., I. Reid, and C. Roberts. 1986. Effect of body condition at calving on the health and performance of dairy cows. Animal Production 43(01):1-6.

CHAPTER 6: The effect of fatty acids and glucagon administration on liver expression of fibroblast growth factor 21 in dairy cows*

*Chapter formatted according to Journal of Dairy Science author's guideline.

ABSTRACT

Dairy cows typically experience a period of negative energy balance when transitioning from late gestation to early lactation. Higher plasma concentrations of glucagon and non-esterified fatty acids (**NEFA**) are metabolic hallmarks of this period. Dairy cows also experience a sudden increase in plasma concentration of the novel hormone fibroblast growth factor 21 (**FGF21**) in early lactation. In other species, both glucagon and NEFA have implicated in the induction of FGF21 in the liver. To assess the relative contribution of these factors in regulating FGF21, two experiments were performed in energy sufficient, non-pregnant, non-lactating dairy cows. In the first study, six cows were injected with saline or glucagon (5 mg) every 8 h over 72 h. Glucagon treatment caused a 3-fold increase in FGF21 mRNA expression in liver but did not impact plasma FGF21. In the second study, six cows received an IV infusion and SC injections of saline (Control group), an IV infusion of intralipid and SC injections of saline (Lipid group) or an IV infusion of intralipid and SC injections of glucagon (Lipid + Glcg group). Intravenous infusions lasted 16 consecutive hours and SC injections were performed every 8 h. Lipid infusion elevated circulating fatty acid concentration and successfully induced storage of triglycerides in liver. Intralipid infusion caused a 34-fold increase in FGF21 mRNA expression in liver and an 8-fold increase in plasma FGF21. Presence of glucagon during the intralipid infusion did not have any additional effects on plasma NEFA, liver triglyceride, liver FGF21 mRNA or plasma FGF21. These data indicate that increased plasma NEFA is a major factor triggering hepatic FGF21 expression in the early lactating dairy cow.

Key Words: fatty acids, glucagon, FGF21, dairy cows.

INTRODUCTION

The transition from late pregnancy to early lactation is a challenging period for the modern dairy cow. The increased nutrient demands associated with initiation of milk production occurs in the absence of adequate compensatory feed intake, and as a consequence early lactating dairy cows experience a period of negative energy balance (**NEB**) (Bell, 1995, Drackley, 1999). Excessive NEB is associated with increased risk of development of several diseases (i.e. retained fetal membranes, metritis, displaced abomasum, and clinical ketosis) and decreased milk production and reproductive performance (Duffield et al., 2009, Chapinal et al., 2011, McArt et al., 2012, Ospina et al., 2013). An efficient transition period is essential to the success of the modern dairy cow in current production systems (Drackley, 1999); therefore it is important to understand the adaptations happening in the periparturient period to develop strategies that can improve animal performance.

Dairy cows cope with NEB by calling on several metabolic adaptations triggered by changes in key metabolic hormones. Among those adaptations, elevated concentrations of circulating glucagon is important to increase gluconeogenesis, and to enhance fatty acid oxidation and ketone production (Bobe et al., 2003a, Hanigan et al., 2004, Bobe et al., 2009). Also, a lower concentration of circulating insulin, associated with increased plasma growth hormone concentrations lead to increased availability of glucose to the mammary gland and increased supply of non-esterified fatty acids (**NEFA**) as on alternative fuel source (Vernon and Finley, 1988, Bell, 1995, Drackley, 1999; Rhoads et al., 2007). Additionally, plasma leptin is reduced in early lactation leading to a reduction in thyroid hormone levels which in turn improves metabolic efficiency during this period (Block et al., 2001; Boisclair et al., 2006; Ehrhardt et al., 2016).

Fibroblast growth factor 21 (**FGF21**) is a novel protein hormone reduced by various nutritional stresses in rodents (Kharitonenkov et al., 2005, Badman et al., 2007, Inagaki et al., 2007, Lundasen et al., 2007). FGF21 has been shown to improve fatty acid oxidation capacity in liver and to coordinate liver and adipose tissue functions in various species (Inagaki et al., 2007, Schoenberg et al., 2011). In rodents and humans increased plasma concentrations of fatty acids and glucagon have been reported to enhance FGF21 gene expression in liver (Badman et al., 2007, Inagaki et al., 2007, Cyphert et al., 2012, Arafat et al., 2013, Kinoshita et al., 2014). Interestingly, Schoenberg et al. (2011) demonstrated that FGF21 plasma concentration peaks on the day of parturition when both glucagon and NEFA are elevated. Accordingly, the major objective of the present work was to assess the possibility that glucagon and NEFA are positive regulators of FGF21 production in dairy cattle.

MATERIALS AND METHODS

Animal and Experimental design

Two experiments were performed with non-pregnant, non-lactating Holstein dairy cows with approval of the Cornell University Institutional Animal Care and Use Committee. Procedures common to both experiments were as follow. Cows were held in individual tie-stalls and fed non-limiting amounts of a total mixed ration (**TMR**). The TMR consisted of grass hay, wheat straw, dried distiller's grains and mineral supplement in the ratio of 58:23:14:5 and containing 1.55 Mcal of metabolizable energy (**ME**) and 143 g crude protein per kg DM (Table 6.1). The TMR was offered as either 2 (experiment 1) or 12 daily meals (experiment 2) and the daily feed intake was calculated for each animal as the difference between feed offered and feed refusal.

Energy intake and energy balance was calculated based on feed composition analysis and estimated maintenance energy requirements. Liver and adipose tissue biopsies were obtained after surgical preparation and local anesthesia of the biopsy site. Liver tissue was harvested via percutaneous puncture with a biopsy trocar and adipose tissue via dissection of the tail head region (Block et al., 2001, Schoenberg et al., 2011). Liver and adipose tissue samples were divided into aliquots, snap-frozen in liquid N and stored at -80°C until further analysis. Indwelling jugular catheters were fitted the day before each experimental period. After collection, jugular blood samples were mixed immediately with heparin (15 IU/ml) and spun at 3,000x *g* for 15 min at 4°C. Resulting plasma was stored at -20°C until analyzed for metabolites and hormones.

Table 6.1. Chemical composition of the experimental diet used in chapter 6.

Nutrient	Content[1]
Metabolizable energy (Mcal/kg)	1.55
Crude protein (%)	14.30
ADF (%)	32.20
NDF (%)	46.10
Calcium (%)	0.78
Phosphorus (%)	0.36
Potassium (%)	1.21
Magnesium (%)	0.27
Sodium (%)	0.16
Sulfur (%)	0.26

[1]Values given on a dry matter basis.

Experiment 1: effect of glucagon

The objective of this study was to determine whether glucagon alone triggers FGF21 production. Six multiparous Holstein cows were selected on the basis of uniform age (4.75 ± 0.75 year), body condition score (3.6 ± 0.2 on a scale of 1 to 5) and weight (698.4 ± 36.8 kg).

They were randomly allocated to a single reversal design with experimental periods of 72 h separated by a 3-day intervening period. Treatments were initiated at 0 h and consisted of subcutaneous (**SC**) injection of either saline solution (0.9% Sodium Chloride USP; Abbott Laboratories, North Chicago, IL) or bovine glucagon (5 mg dissolved in saline, Eli Lilly and Co., Indianapolis, IN). Each treatment was administered as a 60 ml solution every 8 h as we previously described (Bobe et al., 2003b, Osman et al., 2008). Blood samples were collected at -2, -1, 0, 1, 2, 3, 4, and 8 h relative to first SC injection and immediately before SC injections at 16, 24, 48 and 72 h. Liver biopsies were obtained immediately before SC injections at 16 and 72 h (Fig. 6.1A).

Experiment 2: effect of increased plasma NEFA with and without glucagon

This study involved a second group of 6 multiparous dairy cows with average age, body condition score and weight of 4.3 ± 0.3 year, 3.25 ± 0.2 and 682.5 ± 21.2 kg, respectively. They were randomly assigned to two – 3 x 3 Latin squares with experimental periods of 17 h separated by 3-day intervals. Each 17 h experimental period consisted of 1h of basal blood sampling followed by a 16 h period of treatment. Treatments consisted of various combinations of SC injections of saline or bovine glucagon (Eli Lilly; Indianapolis, IN) and intravenous (**IV**) infusion of saline or 20% intralipid solution (Frasenius, Kabi; Deeriled, IL). These treatments were: 1) IV infusion and SC injections of saline (Control); 2) IV infusion of intralipid and SC injections of saline (Lipid); 3) IV infusion of intralipid and (SC) injections of glucagon (Lipid + Glcg). Saline and intralipid solutions were infused at the rate of 100 mL/hr for the entire treatment period using a controlled infusion pump (Abbot Plum XL Infusion Pump; Abbott Laboratories, North Chicago, IL). Subcutaneous injections of saline and glucagon (5 mg dissolved in saline) were given in a 60 ml volume at the 0 and 8 h time point of treatment period. Blood samples were

collected at -1, -0.5 and 0 h relative to the start of treatment, hourly during the first 4 hours of treatment and every 3 hours thereafter. Liver and adipose tissue biopsies were obtained at the end of each experimental period (Fig. 6.1B).

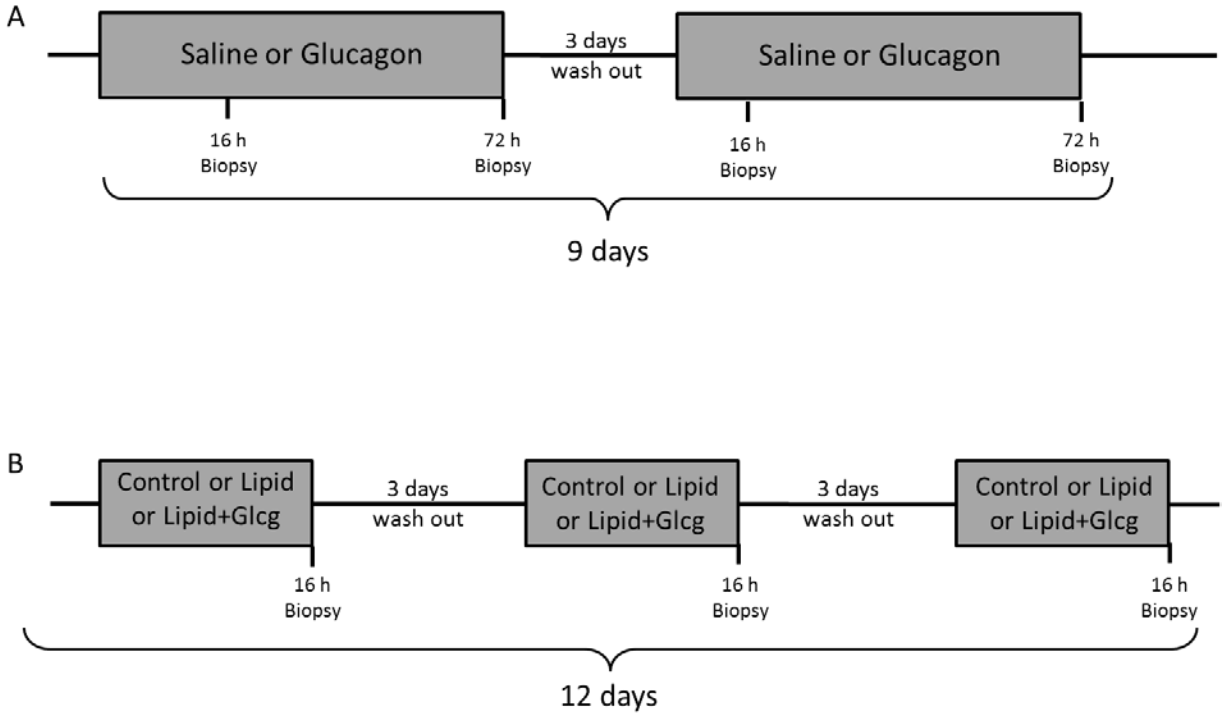

Figure 6.1. Sampling timeline for the animals enrolled in experiment 1 (A) and experiment 2 (B).
In experiment 1, dairy cows received subcutaneous injection of saline or glucagon every 8 h for a 72 h period. Liver triglyceride and glycogen contents were measured on biopsies obtained after 16 or 72 hours of treatment. During experiment 2, dairy cows were treated for 16 h with intravenous (IV) infusion and subcutaneous (SC) injections of saline (control), IV infusion of intralipid and SC injections of saline (Lipid), or IV infusion of intralipid and SC injections of glucagon (Lipid + Glcg).

Analysis of metabolites and hormones

Plasma glucose, NEFA and β-hydroxybutyrate (**BHBA**) were analyzed by spectrophotometric methods based respectively on the enzymes glucose oxidase, acyl-CoA/oxidase, and β-hydroxybutyrate dehydrogenase (Block et al., 2001). Liver triglycerides (**TG**) and glycogen content were measured using procedures validated in cattle (Folch et al., 1957, Bernal-Santos et al., 2003). In brief, total lipids were extracted using the Folch procedure followed by determination of TG by the colorimetric Hantzsch condensation method. Glycogen was extracted with KOH, precipitated with ETOH and converted into glucose with amyloglucosidase; the spectrophotometric glucose oxidase was then used to measure released glucose. The plasma concentrations of insulin and FGF21 were determined using double-antibody assays (Porcine insulin RIA, Millipore Corp; Human FGF21 ELISA, Eli Lilly Corp) previously validated with bovine plasma (Pires et al., 2007, Osman et al., 2008, Schoenberg et al., 2011), The standard curve range was 50 – 3200 pg/mL for FGF21 and from 2 – 200 ng/dL for insulin. The FGF21 assay had a sensitivity of 36pg/mL while the insulin assay had a sensitivity of 12 ng/dL and a cross-reactivity with bovine insulin of 90% according to manufacturer. Samples were analyzed in triplicates for all metabolites and hormone and inter- and intra-assay coefficients of variation were < 10% and < 6% for all metabolite assays, and < 6% for all hormone assays.

Gene Expression Analysis

Frozen liver and adipose tissue were homogenized with Qiazol (QIAGEN, Inc., Valencia, CA). Total RNA was isolated and purified using RNeasy Mini columns and on-column RNase-free DNase treatment (Qiagen). The quantity and integrity of the RNA was assessed using an RNA Nano Lab chip kit and bioanalyzer (Agilent, Palo Alto, CA). Reverse transcription reactions were performed with 2 μg of RNA in a total 20 μL volume with the high-capacity

cDNA reverse transcription kit and RNase inhibitor (Applied Biosystems). Gene expression was analyzed with quantitative real-time PCR assays using Power SYBR Green Mix (Applied Biosystems). Real-time PCR assays were performed in duplicate with a total 25 μL reaction volume containing 500 nM concentration of each primer and reverse transcribed mRNA (25 ng except 2.5 ng for the internal standard gene 18S). The sequences of all primers used are given in Table 6.2. mRNA data were analyzed using a relative standard curve based on serial 2-fold dilutions of pooled cDNA from adipose or liver tissue. Unknown sample expression levels were calculated from the standard curve and adjusted to the geometric mean of the invariant genes 18s, Rps2, and B2M unless otherwise specified.

Table 6.2. Bovine primers used in real-time PCR analysis.

Transcript[a]		Sequence [b]	Product (bp)	Accession No.
RN18S1				
	F	GATCCATTGGAGGGCAAGTCT	74	NR_036642.1
	R	GCAGCAACTTTAATATACGCTATTGG		
B2M				
	F	CATCCAGCGTCCTCCAAAGAT	131	NM_173893.3
	R	CCCCATTCTTCAGCAAATCG		
FGF21				
	F	GCCAGGCGTCATTCAGATCT	110	XM_005219486.3
	R	GAAAGCTGCAGGCTTTGGG		
FGFR1c				
	F	GCAAGGTGTACAGTGACCCGCA	134	NM_001110207.1
	R	TTTGTCGGTGGTGTTAACTCCGG		
FGFR4				
	F	GAATGGGCACGTTTACCCC	67	NM_001192584.1
	R	CAGTTTCTTCTCCATGCGCTG		
KLB				
	F	TTCCCTGTGATTTCTCCTGGG	113	NM_001205326.1
	R	GTTGCCCGTCACATTCCACA		
RPS2				
	F	GGAGCATCCCTGAAGGATGA	101	NM_001033613.2
	R	TCCCCGATAGCAACAAACG		

[a] Primers were designed to measure the abundance of the following transcripts: 18S ribosomal RNA (RN18S1), beta-2-microglobulin (B2M), fibroblast growth factor 21 (FGF21), fibroblast growth factor receptor 1c (FGFR1c), fibroblast growth factor receptor 4 (FGFR4), β-Klotho (KLB), and ribosomal protein S2 (RPS2).
[b] Primers sequences are shown in a 5' to 3' orientation.

Statistical Analysis

Data were analyzed by the mixed procedure of SAS version 9.3 (SAS Institute Inc., Cary, NC). Descriptive statistics were generated using MEANS procedures and comparison among groups was obtained using ANOVA. For the glucagon experiment, data collected at multiple times were analyzed by a model accounting for treatment (saline *vs* glucagon), time and their interactions as fixed effects and animal as the random effect. For the experiment involving glucagon and intralipid, the mixed model accounted for treatment (Control, Lipid, and Lipid + Glcg), time and their interaction as fixed effects and animal as the random effect. If significant, variation accounted by treatment was partitioned in 2 orthogonal contrasts accounting for the effect of intralipid (Control *vs* Lipid and Lipid + Glcg) and the effect of glucagon (Lipid *vs* Lipid + Glcg). Both models included measurements collected during the basal period when available (e.g., plasma concentrations of metabolites and hormones) and involved the Toeplitz covariance structure resulting in the smallest Akaike's information criterion. Statistically significance and tendency were set at $P \leq 0.5$ and $P \leq 0.10$, respectively.

RESULTS

Experiment 1: effects of glucagon alone

Non-pregnant, non-lactating dairy cows received SC injections of glucagon every 8 h over a 72 h study period. Glucagon treatment had not effects on voluntary dry matter or energy intake (Table 6.3). The calculated energy balance was equally positive across treatments and averaged 122 and 123 % of estimated maintenance energy requirements for saline and glucagon treatment, respectively.

Table 6.3. Effect of glucagon treatment on whole body energetics.

	Treatment [a]			
	Saline	Glcg	SEM	P-value [b]
Whole body energetics				
Dry matter intake (kg/d)	9.96	10.17	0.19	NS
Energy intake (Mcal ME/d)	15.12	15.45	0.29	NS
Energy balance (Mcal ME/d)	4.26	4.58	0.21	NS
Energy balance (% maintenance)	122	123	5.00	NS

[a] Dairy cows (n = 6) were treated with subcutaneous injections of saline or glucagon every 8h for 72h.
[b] Type I error probability. NS = non-significant.

To document effectiveness of glucagon treatment, blood samples were obtained over the first 8 h following the first injection and analyzed for plasma glucose, insulin and plasma NEFA. Over this period, the plasma concentration of glucose, insulin, and NEFA for the cows in the saline group remained nearly invariant at ~ 82 mg/dL, 5.49 ng/mL and 261 μM (Fig. 6.2A). In contrast, the concentrations of glucose and insulin increased by 31% and 314% within the first hour after glucagon injection followed by a return to baseline over the next 3 hours (Fig. 6.2A; Treatment x Time, $P < 0.001$). A reciprocal pattern was seen for plasma NEFA, with a 36% reduction at 1 h followed by a progressive return to baseline over the next 7 hours.

To determine whether repeated glucagon injections could lead to sustained changes overtime, additional samples were obtained at 16, 24, 48 and 72 h (Fig 6.2B). These samples were taken 8 h after SC injections but immediately before the next scheduled injection. Plasma glucose remained 13% higher across all times with glucagon treatment ($P < 0.001$) but no effects were seen on plasma insulin and NEFA. Liver biopsies were also obtained in a similar manner at 16 and 72 h of the study period and analyzed for triglyceride and glycogen content (Fig. 6.3). Liver glycogen content remained more or less stable over time whereas there was a numerical reduction in triglyceride content between 16 and 72 h. Glucagon treatment had no significant effect on either variable.

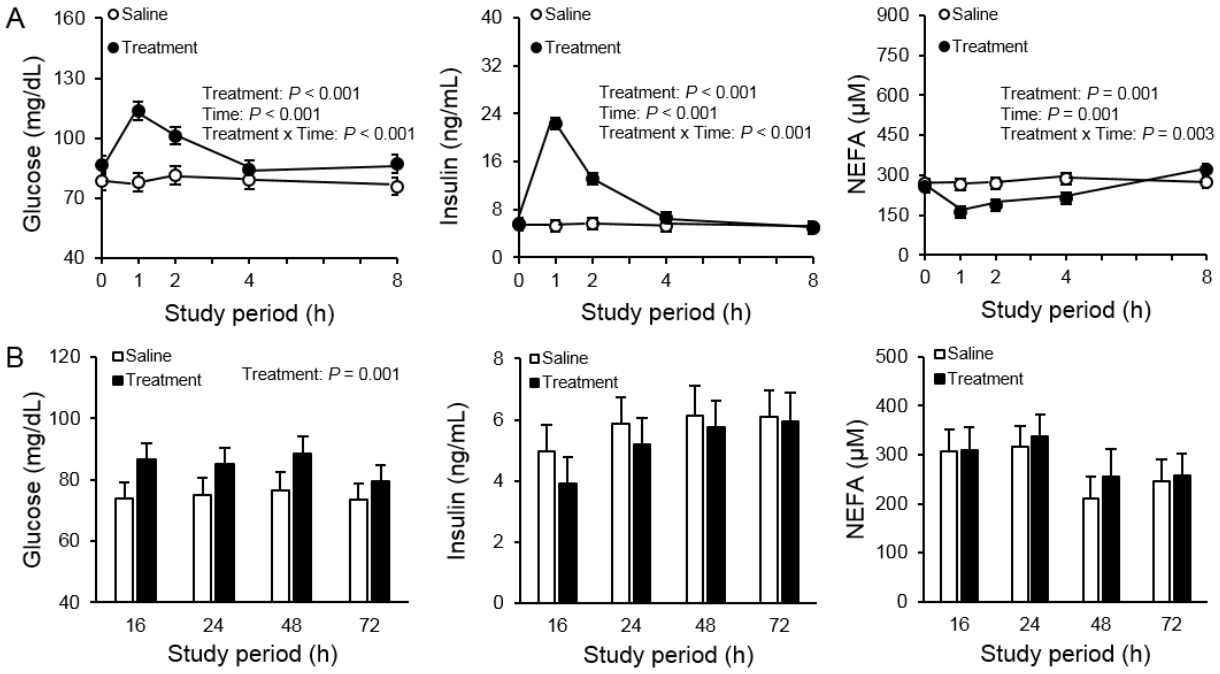

Figure 6.2. Effect of glucagon administration on the plasma concentration of glucose, non-esterified fatty acids (NEFA), and insulin.

Dairy cows received subcutaneous injections of saline or glucagon every 8 h over a 72 h period. A) Plasma concentrations of each variable were measured at the indicated times relative to first injection at 0 h. For each variable, individual time point represents the mean ± SE of 6 cows. The significant effects of treatment, time and treatment x time are reported. B) Plasma concentrations of each variable were measured at the indicated times immediately before injection of saline or glucagon. For each variable, individual bars represent the mean ± SE of 6 cows. The significant effect of treatment is reported.

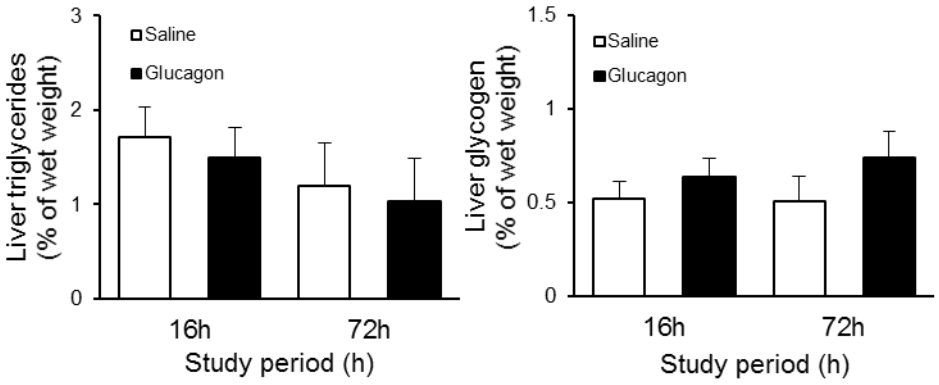

Figure 6.3. Effect of glucagon administration on liver composition.
Dairy cows received subcutaneous injection of saline or glucagon every 8 h for a 72 h period. Liver triglyceride and glycogen contents were measured on biopsies obtained after 16 or 72 hours of treatment. Each bar represents the mean ± SEM of 6 cows.

To determine effects of glucagon on FGF21 production and its signaling components, mRNA abundance was measured in liver biopsies. Glucagon caused similar increases in FGF21 mRNA expression at both 16 and 72 h (Fig 6.4A; Treatment, $P < 0.04$ and Treatment X Time, $P > 0.6$) even though FGF21 expression tended to drop over time (Time, $P = 0.093$). Glucagon reduced the abundance of both β-Klotho and FGFR1c mRNA (Fig. 6.4C; Treatment, $P < 0.05$ or less) whereas an increase in FGFR1c mRNA abundance occurred between 16 and 72 h (Time, $P < 0.03$). Neither glucagon nor time affected hepatic FGFR4 expression. Despite the positive effects of glucagon on liver FGF21 mRNA, plasma FGF21 did not differ between treatment groups at either 16 or 72 h of the study period (Fig. 6.4B).

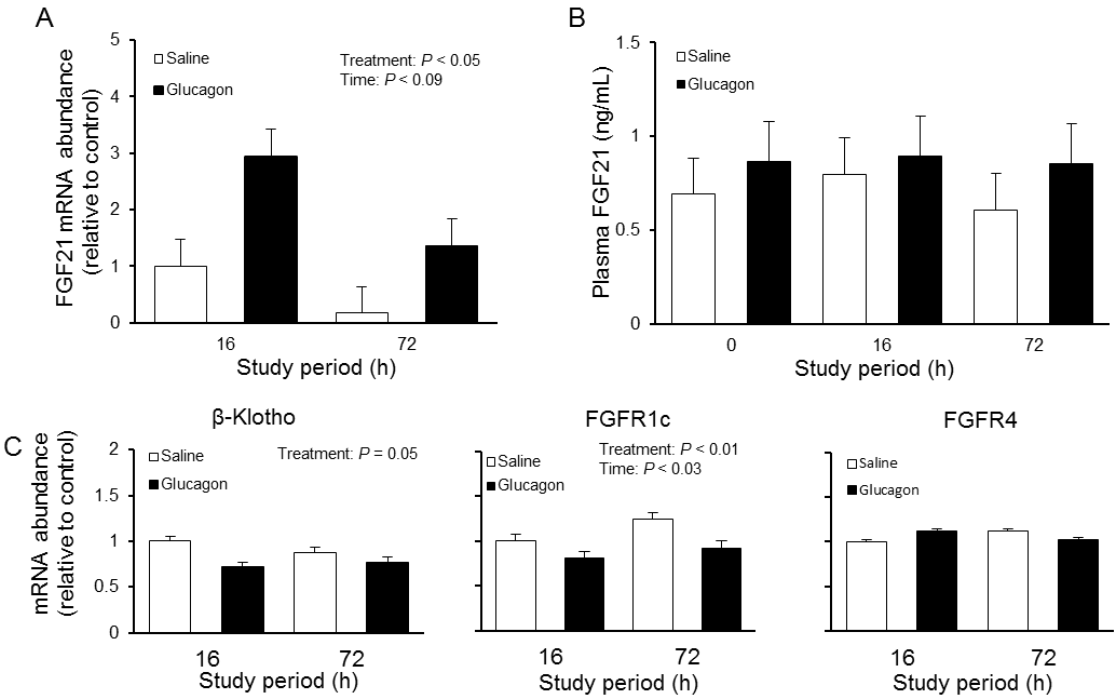

Figure 6.4. Effect of glucagon administration on FGF21 variables.
Dairy cows received subcutaneous injection of saline or glucagon every 8 h for a 72 h period. A) Total RNA was isolated from liver biopsies obtained after 16 or 72 h of treatment and analyzed for FGF21 mRNA abundance (n = 6 cows). The significant effects of treatment and time are reported. B) The plasma concentration of FGF21 was measured at the indicated times. Each bar represents the mean ± SE of 6 cows. C) Total RNA was isolated from liver biopsies obtained after 16 or 72 h of treatment and analyzed for mRNA abundance of the indicated genes. For each gene, each bar represents abundance (n = 6 cows). The significant effects of treatment and time are reported.

Experiment 2: effect of increased plasma NEFA with and without glucagon

In mice, fatty acids acting through peroxisome proliferator-activated receptor α (**PPARα**) synergizes with glucagon to increase hepatic FGF21 expression. To examine whether circulating fatty acids and glucagon interact to regulate FGF21 production in cattle, a second group of non-pregnant, non-lactating dairy cows was infused for 16 consecutive hours with intralipid solution in presence or absence of glucagon injections at 0 and 8 h. Intralipid treatments led to numerical depression in dry matter and energy intake, and as a consequence tended to reduce the extent of positive energy balance (Table 6.5; Lipid, $P < 0.10$). After correcting for the caloric value of

infused solutions, however, energy balance calculated either as an absolute value or as a % of maintenance energy requirement was higher with intralipid than saline infusion (Lipid, $P < 0.001$). Glucagon treatment during intralipid infusion had no additional effect on these energy variables (Glucagon, $P \geq 0.5$).

Consistent with positive energy balance, the concentrations of plasma glucose, insulin and NEFA immediately before infusions averaged 58 mg/dL, 8.15 ng/ml and 99 µM and did not differ between treatments (Fig. 6.5A and 6.6). Plasma NEFA averaged 92 µM throughout the 16 h study period during saline infusion but rose within 1 h to a new steady concentration of 529 - 568 µM during intralipid infusions (Fig. 6.5A; Lipid x Time, $P < 0.001$). Biopsies were also collected at the end of each infusion to determine effects on liver TG. After only 16 h, hepatic TG content increased from 1.09% of wet weight with saline infusion to an average of 4.8% with intralipid infusions (Fig 6.5B; Lipid, $P < 0.001$). No additional effects were seen on any of these variables when glucagon and intralipid were co-administered (Glucagon, $P = 0.66$).

The plasma concentrations of BHBA and insulin increased within 3 h of intralipid infusion and remained elevated for the next 13 h whereas they remained at basal levels during the saline infusion (Fig. 6.6; Lipid x Time, $P = 0.06$ or less). The elevations in the concentrations of BHBA and insulin were exacerbated when the intralipid infusion was combined with glucagon injections (Glucagon x Time, $P < 0.005$ or less). On the other hand, neither intralipid infusions nor glucagon injections impacted plasma glucose concentration or liver glycogen over the study period (Fig 6.6 and Table 6.4)

Indices of FGF21 production and signaling were measured in liver and adipose tissues collected at the end of infusions. In liver, intralipid infusions caused on average a ~ 34 fold increase in FGF21 mRNA over saline infusion (Fig. 6.7A; Lipid, $P < 0.005$), but the additional

increase seen when glucagon was present was not significant (Glucagon, $P < 0.4$). FGF21 signaling components in liver were not affected by treatments with single exception that presence of glucagon during intralipid infusion reduced β-Klotho expression. In adipose tissue, intralipid infusions had no effect on the mRNA abundance of FGF21. Intralipid infusions increased plasma FGF21 8 fold after 9 h of infusion with not further effects of glucagon; a similar increase was seen after 16 h of infusion.

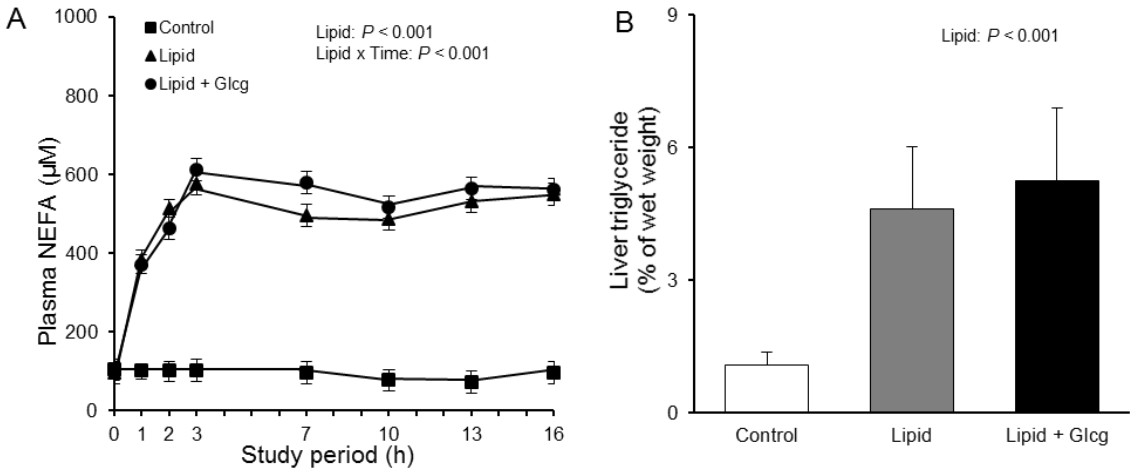

Figure 6.5. Effect of glucagon and intralipid on the plasma non-esterified fatty acids (NEFA) and liver triglyceride content.
Dairy cows were treated for 16 h with intravenous (IV) infusion and subcutaneous (SC) injections of saline (control), IV infusion of intralipid and SC injections of saline (Lipid), or IV infusion of intralipid and SC injections of glucagon (Lipid + Glcg). A) Plasma concentration of NEFA, individual time points represent the mean ± SE of 6 cows. The significant effects of the lipid contrast and its interaction with time are reported. B) Triglyceride content was measured on liver biopsies obtained at the end of treatment. Each bar represents the mean ± SE of 6 cows. The significant effect of the lipid contrast is reported.

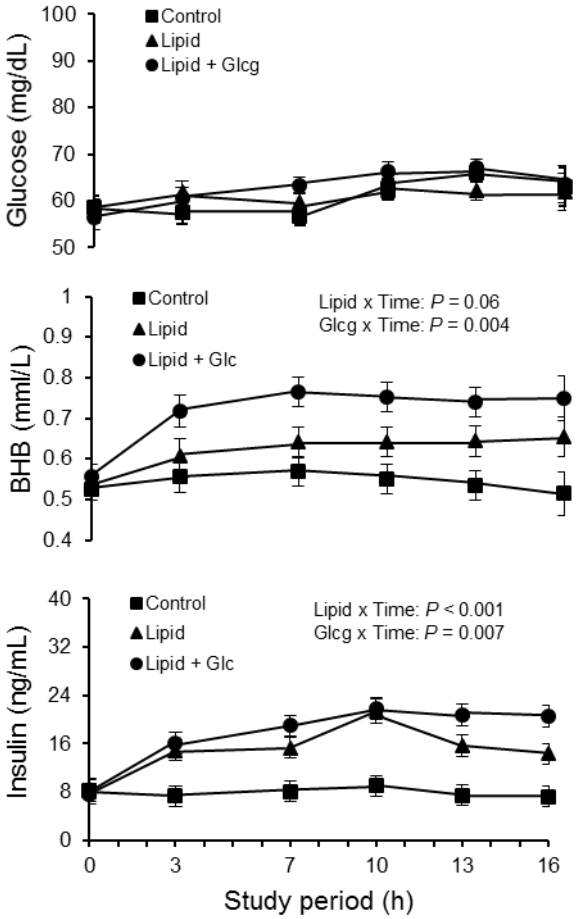

Figure 6.6. Effect of glucagon and intralipid on the plasma concentration of glucose, β-hydroxybutyrate (BHB), and insulin.
Dairy cows were treated for 16 h with intravenous (IV) infusion and subcutaneous (SC) injections of saline (control), IV infusion of intralipid and SC injections of saline (Lipid), or IV infusion of intralipid and SC injections of glucagon (Lipid + Glcg). Plasma concentrations of each variable were measured at the indicated times relative to start or infusion. For each variable, individual time point represents the mean ± SE of 6 cows. The significant effects of the lipid contrast and its interaction with time are reported.

Table 6.4. Effect of intralipid infusion in absence or presence of glucagon injection on whole body energetics and liver glycogen.

	Treatment [a]				*P* value of contrast [b]	
	Control	Lipid	Lipid + Glcg	SEM	Lipid	Glucagon
Whole animal energetics						
Dry matter intake (kg/d)	11.33	10.62	10.91	0.21	NS	NS
Energy intake (Mcal ME/d)	17.95	16.82	17.28	0.43	NS	NS
Energy balance (Mcal ME/d)	7.26	6.14	6.60	0.45	NS	NS
Corrected energy balance (Mcal ME/d) [c]	7.26	9.34	9.80	0.45	< 0.001	NS
Corrected energy balance (% maintenance)	129	137	140	4.00	0.04	NS
Liver glycogen (% wet weight)	1.16	1.03	1.00	0.09	NS	NS

[a] Dairy cows (n = 6) were treatment with intravenous (IV) infusion and subcutaneous (SC) injection of saline (Control), IV infusion of intralipid and SC injection of saline (Lipid), or IV infusion of intralipid and SC injections of glucagon every 8 hours (Lipid + Glcg) *P*-value indicate the results when comparing all treatments.

[b] Linear contrasts were Lipid (Lipid and Lipid + Glcg vs. Control) and Glucagon (Lipid + Glcg vs. lipid). Type I error probability. NS = non-significant.

[c] Estimated energy balance after including the caloric value of infused intralipid..

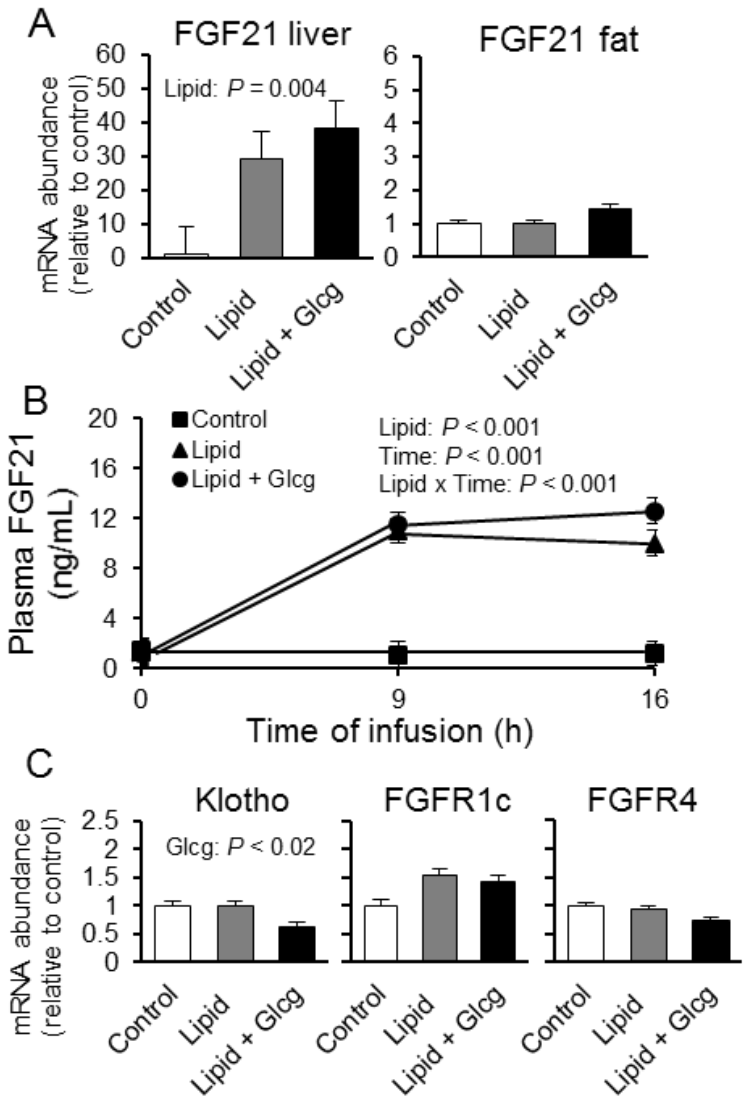

Figure 6.7. Effect of glucagon and intralipid administration on FGF21 variables.
Dairy cows were treated for 16 h with intravenous (IV) infusion and subcutaneous (SC) injections of saline (control), IV infusion of intralipid and SC injections of saline (Lipid), or IV infusion of intralipid and SC injections of glucagon (Lipid + Glcg). A) Total RNA was isolated from liver and fat biopsies obtained after 16 h of treatment and analyzed for FGF21 mRNA abundance (n = 6 cows). The significant effect of the lipid contrast is reported. B) The plasma concentration of FGF21 was measured at the indicated times. Each bar represents the mean ± SE of 6 cows. C) Total RNA was isolated from liver biopsies obtained after 16 h of treatment and analyzed for mRNA abundance of the indicated genes. For each gene, each bar represents abundance (n = 6 cows). Significant effects of the lipid contrast are reported.

DISCUSSION

Our previous work show that plasma FGF21 rises abruptly at the onset of lactation in dairy cows and remains elevated during the ensuing period of energy insufficiency (Schoenberg et al., 2011). Early lactation in dairy cows also features dynamic changes in the plasma concentration of a multitude of metabolites and hormones, many of which have been implicated in regulating plasma FGF21 in other physiological states and species (Herdt, 2000, Block et al., 2001, Ohtani et al., 2012, De Koster and Opsomer, 2013). Early lactation is therefore a challenging physiological context to identify factors capable of regulating FGF21. For this reason, we performed studies in energy-sufficient, non-pregnant and non-lactating dairy cows characterized by low plasma concentration of FGF21 and presumptive regulatory factors.

We selected glucagon as our first candidate for 2 reasons. First, plasma glucagon is induced at the onset of lactation as a homeostatic response to counter the hypoglycemia associated with copious milk secretion (Bell, 1995). The latter reflects the utilization by the mammary gland of > 80% of all available glucose in support of lactose synthesis (Bell and Bauman, 1997). Second, glucagon has been shown to stimulate FGF21 production in both rodents and humans (Berglund et al., 2010, Cyphert et al., 2012, Arafat et al., 2013). As previously showed in lactating dairy cattle using the same dose and mode of administration [i.e., 5 mg SC every 8 h] (Bobe et al., 2003b, Osman et al., 2008, Osman et al., 2010), glucagon led to peak in plasma glucose and insulin within 1 h of SC injection. These effects dissipated over the next 3 h such that they were no longer visible immediately before the next injection, with the exception of plasma glucose which remained elevated over the entire study period. Despite these data demonstrating efficacy of glucagon treatment, we did not detect any measurable changes in plasma FGF21, either measured within the initial 8 h window or throughout the experiment. It is important to note that

positive effects of glucagon on FGF21 in humans and rats were detected under insulinopenic conditions (Type I diabetes in humans and streptozotocin-diabetes in rats) (Arafat et al., 2013). This context allowed increased plasma NEFA, a necessary condition to the positive effects of glucagon and other lipolytic hormones on FGF21 in rodents and humans. In our experimental setting, however, plasma NEFA were reduced for much of the 8 h periods separating glucagon injections, likely reflecting the anti-lipolytic effects of increased insulin. Moreover, glucagon is unable of lipolytic effects in bovine adipose tissue (Etherton et al., 1977), negating the possibility an indirect effect through plasma NEFA even in physiological states where plasma insulin is low.

Profound energy insufficiency is another hallmark of early lactation in dairy cows (Bauman and Currie, 1980). This feature reflects insufficient voluntary feed intake after parturition and therefore insufficient substrates of dietary origin to fulfill the rapidly rising energy demand of the mammary gland for milk synthesis (Bell, 1995). As a consequence, plasma NEFA peaks ~ 500-800 µM around parturition and remains higher than 300 µM for weeks (Block et al., 2001, McCarthy et al., 2015, McCarthy et al., 2016), reflecting, the intensity and extent of lipid mobilization. To assess the role of plasma NEFA in inducing FGF21, we infused an intralipid solution directly in the vascular system for 16 h. Relative to saline treatment, intralipid caused a chronic increase in plasma NEFA from 92 to ~ 550 µM, in line with plasma NEFA concentration prevailing over the first 2 weeks of lactation. This single experimental manipulation was sufficient to increase plasma FGF21 from 1.3 ng/mL to 11.3 ng/ml. Our data agree with stimulation of plasma FGF21 in rodents and humans by treatments increasing circulating NEFA such as fasting, intralipid infusion and GH therapy (Badman et al., 2007, Inagaki et al., 2007, Kharitonenkov et al., 2007, Badman et al., 2009). We also tested glucagon in the presence of

intralipid on the basis of its synergistic effect in increasing FGF21 production (Berglund et al., 2010). Such glucagon actions, however, do not appear to occur in cattle as the combination of glucagon and intralipid did not lead to additional effects on plasma FGF21. Overall, these data implicate elevated plasma NEFA as a key factor driving increased plasma FGF21 in early lactating dairy cows.

We previously reported that liver expressed FGF21 at 25-fold higher levels than White adipose tissue (**WAT**) in late pregnancy when plasma FGF21 is nearly undetectable, and represents the only tissue with increased FGF21 expression in early lactation (Schoenberg et al., 2011). To verify that intralipid mimics this mechanism, we measured FGF21 expression in both liver and adipose tissue. Intralipid caused a 34-fold increase in FGF21 expression in liver. This effect is likely to involve transcriptional effects through activation of the nuclear receptor PPARα as shown in humans and rodents (Badman et al., 2007, Inagaki et al., 2007, Lundasen et al., 2007, Galman et al., 2008, Domouzoglou and Maratos-Flier, 2011, Cyphert et al., 2012). Our data also show that glucagon alone is able to increase FGF21 expression in liver in absence of increased plasma NEFA. This effect could still involve PPARα if, as demonstrated in the mouse, glucagon promotes translocation of this transcription factor to the nucleus (Longuet et al., 2008). Finally, lipids and their derivatives are able to stimulate FGF21 expression in adipose tissue. These effects were inferred to depend on PPARγ rather than PPARα, based on stimulation of FGF21 mRNA by rosiglitazone but not GW4674 (Muise et al., 2008, Dutchak et al., 2012). Our data show that increased NEFA are unable of such effects in cattle as intralipid failed to stimulate FGF21 expression in adipose tissue. Overall, these data implicate NEFA activation of FGF21 production as a major mechanism accounting for increased plasma FGF21 in transition dairy cows.

Our experiment also provided an opportunity to assess effects of glucagon and intralipid treatment on expression of FGF21 signaling components in liver. FGF21 is devoid of a proteoglycan binding domain necessary for high affinity binding to FGF receptors. Instead, FGF21 secure high affinity binding through recruitment of the single pass transmembrane protein β-Klotho as a co-receptor (Ogawa et al., 2007, Kharitonenkov et al., 2008, Adams et al., 2012). Accordingly, we measured expression of β-Klotho (**KLB**), the preferred FGF21 receptor (FGFR1c) and the FGFR receptor expressed at the highest level in liver (FGFR4). When administered alone, glucagon reduced hepatic expression of KLB mRNA whereas intralipid had no effect. Impact of these treatments were reversed for FGFR1c, with intralipid increasing expression and glucagon lacking effects. As a consequence, the combination of glucagon and intralipid reduced KLB mRNA but increased FGFR1c mRNA. The functional significance of opposite directional changes for these signaling components on FGF21 action in liver remains unknown. On the other hand, neither treatment affected hepatic expression of FGFR4. This is not surprising given that this receptor conveys predominantly FGF19 rather than FGF21 signaling (Kuro-o, 2012).

Exogenous FGF21 administration promotes many adaptations associated with energy insufficiency. In rodents, FGF21 has been shown to enhance hepatic gluconeogenesis, lipolysis in adipose tissue, and enhanced fatty acid oxidation and ketogenesis capacity in liver (Kharitonenkov et al., 2005, Badman et al., 2007, Inagaki et al., 2007, Potthoff et al., 2009, Chau et al., 2010, Chen et al., 2011, Vernia et al., 2014). Similarly, gluconeogenesis, fatty acids oxidation, and ketogenesis are known to be up-regulated in the liver of early lactating dairy cows (Graber et al., 2010, Schlegel et al., 2012, Akbar et al., 2015) Furthermore, it has been reported that FGF21 alleviates fasting induced accumulation of fat in liver by increasing TG clearance

(Badman et al., 2007, Inagaki, 2015) and that systemic administration of FGF21 decreases TG accumulation in liver of obese rodents (Coskun et al., 2008). Therefore, this hepatokine may contribute to a better coordination of metabolic function between liver and WAT during the NEB state of early lactation in dairy cows.

In summary, our experiments implicate elevated plasma NEFA is a key factor triggering hepatic FGF21 production and increased circulating levels in early lactation. Future work is needed to investigate the role of other candidate factors and to identify the functional consequences of increased plasma FGF21 at the onset of lactation.

REFERENCES

Adams, A. C., C. C. Cheng, T. Coskun, and A. Kharitonenkov. 2012. FGF21 requires betaklotho to act in vivo. PloS one 7(11):e49977.

Akbar, H., F. Batistel, J. K. Drackley, and J. J. Loor. 2015. Alterations in Hepatic FGF21, Co-Regulated Genes, and Upstream Metabolic Genes in Response to Nutrition, Ketosis and Inflammation in Peripartal Holstein Cows. PloS one 10(10):e0139963.

Arafat, A. M., P. Kaczmarek, M. Skrzypski, E. Pruszynska-Oszmalek, P. Kolodziejski, D. Szczepankiewicz, M. Sassek, T. Wojciechowicz, B. Wiedenmann, A. F. Pfeiffer, K. W. Nowak, and M. Z. Strowski. 2013. Glucagon increases circulating fibroblast growth factor 21 independently of endogenous insulin levels: a novel mechanism of glucagon-stimulated lipolysis? Diabetologia 56(3):588-597.

Badman, M. K., A. Koester, J. S. Flier, A. Kharitonenkov, and E. Maratos-Flier. 2009. Fibroblast growth factor 21-deficient mice demonstrate impaired adaptation to ketosis. Endocrinology 150(11):4931-4940.

Badman, M. K., P. Pissios, A. R. Kennedy, G. Koukos, J. S. Flier, and E. Maratos-Flier. 2007. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. Cell metabolism 5(6):426-437.

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63(9):1514-1529.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 73(9):2804-2819.

Bell, A. W. and D. E. Bauman. 1997. Adaptations of glucose metabolism during pregnancy and lactation. Journal of mammary gland biology and neoplasia 2(3):265-278.

Berglund, E. D., L. Kang, R. S. Lee-Young, C. M. Hasenour, D. G. Lustig, S. E. Lynes, E. P. Donahue, L. L. Swift, M. J. Charron, and D. H. Wasserman. 2010. Glucagon and lipid interactions in the regulation of hepatic AMPK signaling and expression of PPARalpha and FGF21 transcripts in vivo. American journal of physiology. Endocrinology and metabolism 299(4):E607-614.

Bernal-Santos, G., J. W. Perfield, 2nd, D. M. Barbano, D. E. Bauman, and T. R. Overton. 2003. Production responses of dairy cows to dietary supplementation with conjugated linoleic acid (CLA) during the transition period and early lactation. Journal of dairy science 86(10):3218-3228.

Block, S. S., W. R. Butler, R. A. Ehrhardt, A. W. Bell, M. E. Van Amburgh, and Y. R. Boisclair. 2001. Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. The Journal of endocrinology 171(2):339-348.
Bobe, G., B. N. Ametaj, J. W. Young, and D. C. Beitz. 2003a. Effects of exogenous glucagon on lipids in lipoproteins and liver of lactating dairy cows. Journal of dairy science 86(9):2895-2903.

Bobe, G., R. N. Sonon, B. N. Ametaj, J. W. Young, and D. C. Beitz. 2003b. Metabolic responses of lactating dairy cows to single and multiple subcutaneous injections of glucagon. Journal of dairy science 86(6):2072-2081.

Bobe, G., J. C. Velez, D. C. Beitz, and S. S. Donkin. 2009. Glucagon increases hepatic mRNA concentrations of ureagenic and gluconeogenic enzymes in early-lactation dairy cows. Journal of dairy science 92(10):5092-5099.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. Journal of dairy science 94(10):4897-4903.

Chau, M. D., J. Gao, Q. Yang, Z. Wu, and J. Gromada. 2010. Fibroblast growth factor 21 regulates energy metabolism by activating the AMPK-SIRT1-PGC-1alpha pathway. Proceedings of the National Academy of Sciences of the United States of America 107(28):12553-12558.

Chen, W., R. L. Hoo, M. Konishi, N. Itoh, P. C. Lee, H. Y. Ye, K. S. Lam, and A. Xu. 2011. Growth hormone induces hepatic production of fibroblast growth factor 21 through a mechanism dependent on lipolysis in adipocytes. The Journal of biological chemistry 286(40):34559-34566.

Coskun, T., H. A. Bina, M. A. Schneider, J. D. Dunbar, C. C. Hu, Y. Chen, D. E. Moller, and A. Kharitonenkov. 2008. Fibroblast growth factor 21 corrects obesity in mice. Endocrinology 149(12):6018-6027.

Cyphert, H. A., X. Ge, A. B. Kohan, L. M. Salati, Y. Zhang, and F. B. Hillgartner. 2012. Activation of the farnesoid X receptor induces hepatic expression and secretion of fibroblast growth factor 21. The Journal of biological chemistry 287(30):25123-25138.

De Koster, J. D. and G. Opsomer. 2013. Insulin resistance in dairy cows. The Veterinary clinics of North America. Food animal practice 29(2):299-322.

Domouzoglou, E. M. and E. Maratos-Flier. 2011. Fibroblast growth factor 21 is a metabolic regulator that plays a role in the adaptation to ketosis. The American journal of clinical nutrition 93(4):901s-905.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? Journal of dairy science 82(11):2259-2273.

Duffield, T. F., K. D. Lissemore, B. W. McBride, and K. E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. Journal of dairy science 92(2):571-580.
Dutchak, P. A., T. Katafuchi, A. L. Bookout, J. H. Choi, R. T. Yu, D. J. Mangelsdorf, and S. A. Kliewer. 2012. Fibroblast growth factor-21 regulates PPARgamma activity and the antidiabetic actions of thiazolidinediones. Cell 148(3):556-567.

Etherton, T. D., D. E. Bauman, and J. R. Romans. 1977. Lipolysis in subcutaneous and perirenal adipose tissue from sheep and dairy steers. Journal of animal science 44(6):1100-1106.

Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. The Journal of biological chemistry 226(1):497-509.

Galman, C., T. Lundasen, A. Kharitonenkov, H. A. Bina, M. Eriksson, I. Hafstrom, M. Dahlin, P. Amark, B. Angelin, and M. Rudling. 2008. The circulating metabolic regulator FGF21 is induced by prolonged fasting and PPARalpha activation in man. Cell metabolism 8(2):169-174.

Graber, M., S. Kohler, T. Kaufmann, M. G. Doherr, R. M. Bruckmaier, and H. A. van Dorland. 2010. A field study on characteristics and diversity of gene expression in the liver of dairy cows during the transition period. Journal of dairy science 93(11):5200-5215.

Hanigan, M. D., L. A. Crompton, C. K. Reynolds, D. Wray-Cahen, M. A. Lomax, and J. France. 2004. An integrative model of amino acid metabolism in the liver of the lactating dairy cow. Journal of theoretical biology 228(2):271-289.

Herdt, T. H. 2000. Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. The Veterinary clinics of North America. Food animal practice 16(2):215-230, v.

Inagaki, T. 2015. Research Perspectives on the Regulation and Physiological Functions of FGF21 and its Association with NAFLD. Frontiers in endocrinology 6:147.

Inagaki, T., P. Dutchak, G. Zhao, X. Ding, L. Gautron, V. Parameswara, Y. Li, R. Goetz, M. Mohammadi, V. Esser, J. K. Elmquist, R. D. Gerard, S. C. Burgess, R. E. Hammer, D. J. Mangelsdorf, and S. A. Kliewer. 2007. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell metabolism 5(6):415-425.

Kharitonenkov, A., J. D. Dunbar, H. A. Bina, S. Bright, J. S. Moyers, C. Zhang, L. Ding, R. Micanovic, S. F. Mehrbod, M. D. Knierman, J. E. Hale, T. Coskun, and A. B. Shanafelt. 2008. FGF-21/FGF-21 receptor interaction and activation is determined by betaKlotho. Journal of cellular physiology 215(1):1-7.

Kharitonenkov, A., T. L. Shiyanova, A. Koester, A. M. Ford, R. Micanovic, E. J. Galbreath, G. E. Sandusky, L. J. Hammond, J. S. Moyers, R. A. Owens, J. Gromada, J. T. Brozinick, E. D.

Hawkins, V. J. Wroblewski, D. S. Li, F. Mehrbod, S. R. Jaskunas, and A. B. Shanafelt. 2005. FGF-21 as a novel metabolic regulator. The Journal of clinical investigation 115(6):1627-1635.
Kharitonenkov, A., V. J. Wroblewski, A. Koester, Y. F. Chen, C. K. Clutinger, X. T. Tigno, B. C. Hansen, A. B. Shanafelt, and G. J. Etgen. 2007. The metabolic state of diabetic monkeys is regulated by fibroblast growth factor-21. Endocrinology 148(2):774-781.

Kinoshita, K., N. Ozaki, Y. Takagi, Y. Murata, Y. Oshida, and Y. Hayashi. 2014. Glucagon is essential for adaptive thermogenesis in brown adipose tissue. Endocrinology 155(9):3484-3492.

Kuro-o, M. 2012. Klotho and betaKlotho. Advances in experimental medicine and biology 728:25-40.

Longuet, C., E. M. Sinclair, A. Maida, L. L. Baggio, M. Maziarz, M. J. Charron, and D. J. Drucker. 2008. The glucagon receptor is required for the adaptive metabolic response to fasting. Cell metabolism 8(5):359-371.

Lundasen, T., M. C. Hunt, L. M. Nilsson, S. Sanyal, B. Angelin, S. E. Alexson, and M. Rudling. 2007. PPARalpha is a key regulator of hepatic FGF21. Biochemical and biophysical research communications 360(2):437-440.

McArt, J. A., D. V. Nydam, and G. R. Oetzel. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. Journal of dairy science 95(9):5056-5066.

McCarthy, M. M., S. Mann, D. V. Nydam, T. R. Overton, and J. A. McArt. 2015. Short communication: concentrations of nonesterified fatty acids and beta-hydroxybutyrate in dairy cows are not well correlated during the transition period. Journal of dairy science 98(9):6284-6290.

McCarthy, M. M., T. Yasui, M. J. Felippe, and T. R. Overton. 2016. Associations between the degree of early lactation inflammation and performance, metabolism, and immune function in dairy cows. Journal of dairy science 99(1):680-700.

Muise, E. S., B. Azzolina, D. W. Kuo, M. El-Sherbeini, Y. Tan, X. Yuan, J. Mu, J. R. Thompson, J. P. Berger, and K. K. Wong. 2008. Adipose fibroblast growth factor 21 is up-regulated by peroxisome proliferator-activated receptor gamma and altered metabolic states. Molecular pharmacology 74(2):403-412.

Ogawa, Y., H. Kurosu, M. Yamamoto, A. Nandi, K. P. Rosenblatt, R. Goetz, A. V. Eliseenkova, M. Mohammadi, and M. Kuro-o. 2007. BetaKlotho is required for metabolic activity of fibroblast growth factor 21. Proceedings of the National Academy of Sciences of the United States of America 104(18):7432-7437.

Ohtani, Y., T. Takahashi, K. Sato, A. Ardiyanti, S. H. Song, R. Sato, K. Onda, Y. Wada, Y. Obara, K. Suzuki, A. Hagino, S. G. Roh, and K. Katoh. 2012. Changes in circulating adiponectin and metabolic hormone concentrations during periparturient and lactation periods in Holstein dairy cows. Animal science journal = Nihon chikusan Gakkaiho 83(12):788-795.

Osman, M. A., P. S. Allen, G. Bobe, J. F. Coetzee, A. Abuzaid, K. Koehler, and D. C. Beitz. 2010. Chronic metabolic responses of postpartal dairy cows to subcutaneous glucagon injections, oral glycerol, or both. Journal of dairy science 93(8):3505-3512.

Osman, M. A., P. S. Allen, N. A. Mehyar, G. Bobe, J. F. Coetzee, K. J. Koehler, and D. C. Beitz. 2008. Acute metabolic responses of postpartal dairy cows to subcutaneous glucagon injections, oral glycerol, or both. Journal of dairy science 91(9):3311-3322.

Ospina, P. A., J. A. McArt, T. R. Overton, T. Stokol, and D. V. Nydam. 2013. Using nonesterified fatty acids and beta-hydroxybutyrate concentrations during the transition period for herd-level monitoring of increased risk of disease and decreased reproductive and milking performance. The Veterinary clinics of North America. Food animal practice 29(2):387-412.

Pires, J. A., A. H. Souza, and R. R. Grummer. 2007. Induction of hyperlipidemia by intravenous infusion of tallow emulsion causes insulin resistance in Holstein cows. Journal of dairy science 90(6):2735-2744.

Potthoff, M. J., T. Inagaki, S. Satapati, X. Ding, T. He, R. Goetz, M. Mohammadi, B. N. Finck, D. J. Mangelsdorf, S. A. Kliewer, and S. C. Burgess. 2009. FGF21 induces PGC-1alpha and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response. Proceedings of the National Academy of Sciences of the United States of America 106(26):10853-10858.

Schlegel, G., R. Ringseis, J. Keller, F. J. Schwarz, W. Windisch, and K. Eder. 2012. Expression of fibroblast growth factor 21 in the liver of dairy cows in the transition period and during lactation. Journal of animal physiology and animal nutrition.

Schoenberg, K. M., S. L. Giesy, K. J. Harvatine, M. R. Waldron, C. Cheng, A. Kharitonenkov, and Y. R. Boisclair. 2011. Plasma FGF21 is elevated by the intense lipid mobilization of lactation. Endocrinology 152(12):4652-4661.

Vernia, S., J. Cavanagh-Kyros, L. Garcia-Haro, G. Sabio, T. Barrett, D. Y. Jung, J. K. Kim, J. Xu, H. P. Shulha, M. Garber, G. Gao, and R. J. Davis. 2014. The PPARalpha-FGF21 hormone axis contributes to metabolic regulation by the hepatic JNK signaling pathway. Cell metabolism 20(3):512-525.

CHAPTER 7: Conclusion

The transition from late gestation to early lactation is a challenging period for dairy cows. During this period, energy and mineral requirements are increased to support colostrum and milk production demands without an appropriate increment in dry matter intake (Bell, 1995, DeGaris and Lean, 2008), consequently dairy cows experience a period of negative energy and mineral balance (**NEMB**). In order to overcome this challenge several metabolic adaptations, triggered by key metabolic hormones, take place around parturition leading to the mobilization of non-esterified fatty acids (**NEFA**) and amino acids as alternative fuel source for various tissues, as well as mobilization of bone calcium reserves (Bauman and Currie, 1980, Bell, 1995, Bell and Bauman, 1997, Drackley, 1999, DeGaris and Lean, 2008). Such adaptations are a hallmark of early lactation but some cows do not adapt to this new physiological state properly. In this situation, elevated blood NEFA and low blood calcium concentrations are encountered and have been associated with increased disease occurrence (i.e. retained fetal membranes, metritis, displaced abomasum, fatty liver, and clinical ketosis), decreased milk production, and decrease reproductive performance (Duffield et al., 2009, Ospina et al., 2010a, b, Chapinal et al., 2011, McArt et al., 2012).

Therefore, the main objectives of this dissertation were: 1) To investigate the mobilization of body reserves during early lactation; 2) To characterize blood calcium concentration during the first 3 days in milk; 3) to evaluate the association between calcium concentration and changes in body weight as well as reproductive performance; 4) to assess the role of glucagon and NEFA on liver expression of the novel regulatory hormone fibroblast growth factor 21 (**FGF21**).

In Chapter 3, the association between BW change and subclinical hypocalcemia was investigated through an observational study. Our results demonstrated that low blood calcium concentration and diseases in early lactation influence BW change in multiparous dairy cows, but

only the latter influences the BW change in primiparous animals. Additionally, we reported the dynamics of blood calcium concentration during the first three days in milk. Using a cut-off point of 8.0 mg/dL we determined that 17% of first lactation animals, 55% of second lactation animals, and 73% of third and greater lactation animals presented low blood calcium concentration during the first 3 days of lactation. This results were in agreement with subclinical hypocalcemia prevalence and dynamics of blood calcium concentration previously reported by other (Goff, 2008, Reinhardt et al., 2011, Martinez et al., 2012). The results of our experiment will contribute to further investigations connecting mineral and energy imbalances during the transition period.

As previously mentioned subclinical hypocalcemia (**SHPC**) has been associated with impaired reproductive performance (Chapinal et al., 2012, Martinez et al., 2012, Chamberlin et al., 2013). Traditionally, SHPC has been defined as low blood calcium concentration within the first three days of lactation irrespective of the number of days the animals have low blood calcium concentration (Reinhardt et al., 2011, Martinez et al., 2012). In Chapter 4 we introduced the concept of chronic SHPC, which was defined as blood calcium concentrations below 8.6 mg/dL in all 3 first days post-calving. Despite the more conservative disease definition used during our experiment, almost 50% of third and greater lactation animals were classified as chronic subclinical hypocalcemic with 97% of the animals in this parity group presenting blood calcium concentration below the cut-off point within 24 hours of calving. Moreover, chronic SHPC was associated with longer time to return to cyclicity during voluntary waiting period, more days open, and lower odds of pregnancy at first service when compared to eucalcemic animals. These findings suggest that subclinical hypocalcemia is highly prevalent in dairy cows and is one of the factors influencing productivity. Additionally, by measuring blood calcium

concentration during the first 3 days of lactation and reporting its dynamics we can speculate that the current methods used to determine SHPC, e.g. measuring blood calcium concentration within 48 hours of calving, might be inflating the prevalence of the disease and might not be the best recommended time to test for SHPC. The data generated by this experiment can lead to the development of a more accurate SHPC testing scheme that can be used by the dairy industry at large.

Additional large epidemiological studies are necessary to determine the best cut-off points when defining subclinical hypocalcemia for the different parity groups. Currently, the cut-off points are based on results of studies that did not account for the natural variation of blood calcium concentration within the first few days of lactation. Therefore, understanding the variation of blood calcium concentration during the first week post-partum is imperative for the determination of the thresholds to be used when assessing the epidemiology of hypocalcemia in commercial herds. The determination of a more accurate cut-off points based on the results of a scientific study using a large number of individuals will provide information about the association of blood calcium concentrations in early lactation and possible negative impact of such condition and downstream production outcomes.

The mobilization of body reserves to fulfil energy can be measured using several different methods such as visual body condition score (**BCS**) (Edmonson et al., 1989, Ferguson et al., 1994), back-fat thickness (**BFT**)(Schroder and Staufenbiel, 2006) and body weight (**BW**)(Thorup et al., 2013). Amongst the different methodologies, body condition score and back-fat thickness mainly measure the mobilization of subcutaneous adipose tissue (Brethour, 1992, Bruckmaier et al., 1998), while body weight might be the only method that also accounts for the protein mobilization happening in early lactation. Extreme mobilization of body reserves can be

assessed using all three methods and has been associated with decreased milk production and impaired reproductive performance (Mosenfechtel et al., 2002, Berry et al., 2003, Lopez-Gatius et al., 2003, Roche et al., 2007, Sakaguchi, 2009). Therefore, monitoring the mobilization of body reserves can be used as a management tool to determine when interventions should be launched to prevent metabolic problems and potential loss of production. In Chapter 5, it is reported that BCS, BFT, and BW are correlated to each other and change similarly in early lactation across parity groups. Additionally, our results show that daily BW change and BW change over the first 30 days in milk are a better predictor of milk production then BCS and BFT. Therefore, when possible, sequential BW measurements, as often as weekly, during early lactation can replace BCS as a management tool. This could be a valuable tool to dairy producers because it eliminates the subjectivity of BCS and accounts for protein mobilization in addition to adipose tissue mobilization during the transition period.

As previously stated, the mobilization of body reserves is essential to dairy cows during early lactation because of the decreased energy availability characteristic of this period. Recently FGF21 plasma concentration have been reported to peak on the day of calving in dairy cows and to remain elevated levels throughout the first weeks of lactation when cows are in NEB (Schoenberg et al., 2011). Chapter 6 describes our experiments investigating the effects of glucagon and circulating fatty acids on FGF21 production in dairy cows. Glucagon administration during the experimental period enhanced liver capacity to express FGF21 but had little effect on plasma concentration of FGF21. In contrast, elevated plasma NEFA led to a substantially greater increment of hepatic FGF21 mRNA and to a 10 fold increase in plasma FGF21 concentration. These findings implicate plasma NEFA as a significant driver of the plasma FGF21 surge in early lactating dairy cows.

In future experiments, it will be important to determine whether FGF21 concentrations in early lactation dairy cows is associated with different liver fatty acid oxidation and ketogenesis capacity. Additionally, further research is necessary to determine if administration of FGF21 decreases liver triglyceride accumulation similarly to what is observed in rodents. The understanding of FGF21 in liver fatty acid oxidation, ketogenesis, and fatty liver will provide information on the importance of FGF21 to the successful transition into lactation by high producing dairy.

Overall, the research described in this dissertation contributes to a better understanding of metabolic adaptations in transition dairy cows. Nonetheless, the frequency of unsuccessful transition into lactation remains unacceptably high with substantial negative impact on animal welfare and the profitability of dairy enterprises. Thus, further research is necessary to improve our understanding of physiologic adaptations in transition dairy cows so that metabolic problems can be prevented.

REFERENCES

Bauman, D. E. and W. B. Currie. 1980. Partitioning of nutrients during pregnancy and lactation: a review of mechanisms involving homeostasis and homeorhesis. Journal of dairy science 63(9):1514-1529.

Bell, A. W. 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. Journal of animal science 73(9):2804-2819.

Bell, A. W. and D. E. Bauman. 1997. Adaptations of glucose metabolism during pregnancy and lactation. Journal of mammary gland biology and neoplasia 2(3):265-278.

Berry, D. P., F. Buckley, P. Dillon, R. D. Evans, M. Rath, and R. F. Veerkamp. 2003. Genetic relationships among body condition score, body weight, milk yield, and fertility in dairy cows. Journal of dairy science 86(6):2193-2204.

Brethour, J. R. 1992. The repeatability and accuracy of ultrasound in measuring backfat of cattle. Journal of animal science 70(4):1039-1044.

Bruckmaier, R. M., L. Gregoretti, F. Jans, D. Faissler, and J. W. Blum. 1998. Longissimus dorsi muscle diameter, backfat thickness, body condition scores and skinfold values related to metabolic and endocrine traits in lactating dairy cows fed crystalline fat or free fatty acids. Zentralblatt fur Veterinarmedizin. Reihe A 45(6-7):397-410.

Chamberlin, W. G., J. R. Middleton, J. N. Spain, G. C. Johnson, M. R. Ellersieck, and P. Pithua. 2013. Subclinical hypocalcemia, plasma biochemical parameters, lipid metabolism, postpartum disease, and fertility in postparturient dairy cows. Journal of dairy science 96(11):7001-7013.

Chapinal, N., M. Carson, T. F. Duffield, M. Capel, S. Godden, M. Overton, J. E. Santos, and S. J. LeBlanc. 2011. The association of serum metabolites with clinical disease during the transition period. Journal of dairy science 94(10):4897-4903.

Chapinal, N., S. J. Leblanc, M. E. Carson, K. E. Leslie, S. Godden, M. Capel, J. E. Santos, M. W. Overton, and T. F. Duffield. 2012. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. Journal of dairy science 95(10):5676-5682.

DeGaris, P. J. and I. J. Lean. 2008. Milk fever in dairy cows: a review of pathophysiology and control principles. Veterinary journal (London, England : 1997) 176(1):58-69.

Drackley, J. K. 1999. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? Journal of dairy science 82(11):2259-2273.

Duffield, T. F., K. D. Lissemore, B. W. McBride, and K. E. Leslie. 2009. Impact of hyperketonemia in early lactation dairy cows on health and production. Journal of dairy science 92(2):571-580.

Edmonson, A., I. Lean, L. Weaver, T. Farver, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. Journal of dairy science 72(1):68-78.

Ferguson, J. D., D. T. Galligan, and N. Thomsen. 1994. Principal descriptors of body condition score in Holstein cows. Journal of dairy science 77(9):2695-2703.

Goff, J. P. 2008. The monitoring, prevention, and treatment of milk fever and subclinical hypocalcemia in dairy cows. Veterinary journal (London, England : 1997) 176(1):50-57.

Lopez-Gatius, F., J. Yaniz, and D. Madriles-Helm. 2003. Effects of body condition score and score change on the reproductive performance of dairy cows: a meta-analysis. Theriogenology 59(3-4):801-812.

Martinez, N., C. A. Risco, F. S. Lima, R. S. Bisinotto, L. F. Greco, E. S. Ribeiro, F. Maunsell, K. Galvao, and J. E. Santos. 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.

McArt, J. A., D. V. Nydam, and G. R. Oetzel. 2012. Epidemiology of subclinical ketosis in early lactation dairy cattle. Journal of dairy science 95(9):5056-5066.

Mosenfechtel, S., M. Hoedemaker, U. J. Eigenmann, and P. Rusch. 2002. Influence of back fat thickness on the reproductive performance of dairy cows. The Veterinary record 151(13):387-388.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010a. Association between the proportion of sampled transition cows with increased nonesterified fatty acids and beta-hydroxybutyrate and disease incidence, pregnancy rate, and milk production at the herd level. Journal of dairy science 93(8):3595-3601.

Ospina, P. A., D. V. Nydam, T. Stokol, and T. R. Overton. 2010b. Associations of elevated nonesterified fatty acids and beta-hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the northeastern United States. Journal of dairy science 93(4):1596-1603.

Reinhardt, T. A., J. D. Lippolis, B. J. McCluskey, J. P. Goff, and R. L. Horst. 2011. Prevalence of subclinical hypocalcemia in dairy herds. Veterinary journal (London, England : 1997) 188(1):122-124.

Roche, J. R., J. M. Lee, K. A. Macdonald, and D. P. Berry. 2007. Relationships among body condition score, body weight, and milk production variables in pasture-based dairy cows. Journal of dairy science 90(8):3802-3815.

Sakaguchi, M. 2009. Differences between body condition scores and body weight changes in postpartum dairy cows in relation to parity and reproductive indices. The Canadian veterinary journal. La revue veterinaire canadienne 50(6):649-656.

Schoenberg, K. M., S. L. Giesy, K. J. Harvatine, M. R. Waldron, C. Cheng, A. Kharitonenkov, and Y. R. Boisclair. 2011. Plasma FGF21 is elevated by the intense lipid mobilization of lactation. Endocrinology 152(12):4652-4661.

Schroder, U. J. and R. Staufenbiel. 2006. Invited review: Methods to determine body fat reserves in the dairy cow with special regard to ultrasonographic measurement of backfat thickness. Journal of dairy science 89(1):1-14.

Thorup, V. M., S. Hojsgaard, M. R. Weisbjerg, and N. C. Friggens. 2013. Energy balance of individual cows can be estimated in real-time on farm using frequent liveweight measures even in the absence of body condition score. Animal : an international journal of animal bioscience 7(10):1631-1639.