Transition Nutrition for Improved Fertility, Productivity, and Health of Dairy Cattle

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A thesis submitted in fulfillment
of the requirements for the Degree of
Doctor of Philosophy

Faculty of Veterinary Science
School of Life and Environmental Sciences
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DECLARATION

This thesis is submitted to the University of Sydney in fulfilment of the requirements for the Doctor of Philosophy.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Rachael M. Rodney

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THESIS SUMMARY

The transition period, from approximately three weeks before to three weeks after calving, is characterised by sudden and substantial changes in nutrient requirements as dairy cattle respond to the metabolic challenges of late pregnancy and early lactation. The success by which cows meet these challenges determines their productivity, health and reproductive success not only over transition, but also throughout the following lactation. Manipulations of the pre-calving and early lactation diet have led to persistent improvements in milk production, health and reproduction, leading us to pose the question, *how can a relatively short intervention, applied during the per-partum or early lactation period have long lasting effects on fertility, health and productivity throughout lactation?*

This thesis compiles a collection of research that was undertaken to better understand the influence of nutritional intervention during the peri-parturient period on dairy cow fertility, health and production. Increased milk protein production and rapidly fermentable sugar intake were associated with a decreased proportion pregnant while increased fatty acid and starch intake, and metabolisable energy balance were associated with an increased proportion pregnant (Chapter 2). Inclusion of fats in the early lactation diet increased the pregnancy to service by 27% and tended to reduce calving to pregnancy interval (Chapter 3) and the magnitude of these responses to fat interventions varied among types of fat, possibly reflecting different roles, or provision of different fatty acids, from the different fat types. Feeding fats during transition may be an essential component of an integrated response to the challenges of controlling tissue mobilization in early lactation and limiting the amount of readily fermentable carbohydrate fed. Effects of dietary protein degradability and genetic merit on milk casein composition and yield were examined (Chapter 4) and variables including pre-calving metabolisable protein balance that predicted milk protein and casein yield and composition were identified. Increased pre-calving body weight and plasma alpha amino nitrogen and cholesterol concentrations were associated with increased milk, protein, and casein yields, but decreased milk protein and casein contents. Cows producing lower milk protein and casein content were less likely to become pregnant. Supplementary calcidiol and negative dietary cation-anion difference (DCAD) diets in the pre-partum period have beneficial effects on mineral and energy metabolism (Chapter 6 and 7), milk production (Chapter 7), and health and reproductive outcomes (Chapter 8). Supplementation with calcidiol improved composition and yield of colostrum and early lactation milk yield, when compared with cows supplemented with cholecalciferol (Chapter 7).

Additionally, calcidiol supplementation decreased incidence of retained placenta and metritis while negative DCAD diets increased calcium concentration in the blood around calving and eliminated incidence of clinical milk fever in transition cows. The hypothesis that interactions between bone and energy metabolism observed in other species are present in dairy cattle and have feedback over time was also supported (Chapter 9), identifying relationships among concentrations of vitamin D metabolites, insulin-like growth factor 1, osteocalcin and glucose over time.

Through the collection of research presented, this thesis is able to demonstrate that nutritional interventions applied during the peri-parturient period have homestatic, but also homeorhetic effects on metabolism, and can substantially influence reproductive, productive and health outcomes of dairy cows well into lactation. This work highlighted the complexity and interrelated nature of transition nutrition, and importantly showed that productivity does not have to come at the cost of fertility and health where diets are properly integrated. The insights gathered regarding fat, protein, energy, minerals and vitamin D interventions and the relationships of metabolites underlying these, gives us tools to move closer to identifying the components of optimal transition diets and understanding the mechanisms by which these relatively short term interventions can have powerful and long lasting effects of dairy cow fertility, productivity and health.

QUOTATIONS

The Cow

by Jane Taylor

Thank you, pretty cow, that made
Pleasant milk to soak my bread,
Every day and every night,
Warm, and fresh, and sweet, and white.

Do not chew the hemlock rank,
Growing on the weedy bank;
But the yellow cowslips eat;
They perhaps will make it sweet.

Where the purple violet grows,
Where the bubbling water flows,
Where the grass is fresh and fine,
Pretty cow, go there to dine.

ACKNOWLEDGEMENTS

First, I must thank my supervisor Ian, who is incredibly generous with his time and in sharing his knowledge, both on veterinary science and nutrition but also on life in general. I am very appreciative of the way he always makes himself available, no matter the time of day or day of the week. Ian genuinely cares about us as his students, welcoming us into his family more as surrogate children rather than students.

It was wonderful spending the majority of my candidature based in the Scibus office in Camden, and I am very thankful to Ian and Mossy for giving me this opportunity. Being based here, I was exposed to many different areas of the industry, got meet a lot of interesting people, and was involved with new and different areas of research. I was also surrounded by a great group of people who are not just colleagues but friends; Carole, Dawn, Joanne, Pete, Vicky, Yatza, (and their families) who I very much enjoyed having morning tea and vegemite biscuits with most days. I also must say a huge thank you to Helen, who was a wealth of PhD related knowledge, a sounding board for both ideas and frustrations and a good friend.

I was lucky to have a large and diverse group of research supervisors who all brought different areas of expertise to my research. My favourite memory of Pietro is with his blood tube belt, ready for sampling during the Brownlow Hill trials, and I thank him for his help with multiple areas of my research. I enjoyed talking with David; his selfless sharing of knowledge taught me so much and opened my mind to new avenues to explore with my research. Peter was ever patient and persisted with me through some tricky analyses, putting up with my silly questions. Jeff and Yani helped me to fit into the research community, especially the Dairy Research Group at Sydney University, not always an easy task when I was based off campus.

Professor Jose Santos gave me the opportunity to work on a fantastic trial conducted at the University of Florida. I think him for welcoming me into his research group and allowing me to work on such exciting research. I am also incredibly lucky to have worked on this trial with Dr Natalia Martinez, as well as being a knowledgeable scientist she was a pleasure to work with. Despite the long hours, sleep deprivation and blisters she was always smiling. I am also thankful to the other students who helped us through this trial, who are acknowledged in the related paper.

I am also very thankful to Rachel who gave me a home so far away from mine, and taught me to drive on the wrong side of the road.

A very big thank you must also go to the people at Brownlow Hill, where I did two of my research trials. Especially Edgar and Allan who got used to the ideas of having the cows painted all different colours, and were very accommodating of all the strange things I asked them to do and record in the name of research. They made going to the farm each day enjoyable. Thank you also to Jaime and Hannah who helped with the trial, and the numerous other students, friends and colleagues who pulled on their boots and lent a hand. I also enjoyed working with the vets and university employees during the time I spent teaching.

To my other co-authors, contributors and sponsors, many of who are formally acknowledged in the *Acknowledgement of contribution to the research work and/or authorship* but also others who supported in other ways. By sharing their knowledge and providing support, they allowed me to do research that I hope will benefit the dairy industry. I was also thankful to the farmers and researchers that welcomed me into their farms and labs in many parts of the country, and the world.

The postgraduate community was a pleasure to be involved with, and I thank my fellow postgrads for the shared dinners, moral support and research tips. I also recognise my many friends who at times who put up with tube labelling or proofreading in exchange for a catch up and who were usually patient when I showed up late and/or smelling like cow. A special mention must go to Aaron who let me take over the dining room and generally just be in the way for the final month of my writing process.

One of my favourite things about the time I spent doing this PhD was the time I get to spend at home with my family in Camden. I always looked forward to dinner on Tuesdays with Grandpa and Dad. My parents and sister Danielle really could not have done more to support me.. I am also incredibly thankful to Harry; chief proofreader and cheerleader, who was always patient and unconditionally encouraging. On the great days and the hard ones, they were always encouraging me to do my best. I know the PhD process was sometimes stressful for them too and at one point or another they all ended up in overalls on the end of a shovel or feed bin. I am so thankful for them, both through this process, and always. While completing my PhD I lost my beautiful Grandma. I know she would have loved to see me finish and I dedicate this to her.

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PUBLICATIONS FROM WORK IN THIS THESIS

PEER-REVIEWED PUBLICATIONS

Rodney, R. M., J. K. Hall, C. T. Westwood, P. Celi, and I.J. Lean. 2016. Precalving and early lactation factors that predict milk casein and fertility in the transition dairy cow. J. Dairy Sci. 99:7554-7567.

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Lean, I., P. DeGaris, P. Celi, D. McNeill, R. Rodney, and D. Fraser. 2014. Influencing the future: interactions of skeleton, energy, protein and calcium during late gestation and early lactation. Anim. Prod. Sci. 54:1177-1189.

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Rodney, R. M., P. Celi, . Scott, K. Breinhild, and I.J. Lean. Submitted 2017. Effects of transition nutrition on the fertility of lactating dairy cows: a meta-analysis. J. Dairy Sci.

Rodney, R. M., N. Martinez, E. Block, L. L. Hernandez, C. D. Nelson, P. Celi, J. E. P. Santos, and I. J. Lean. Submitted 2017. Effects of pre-partum dietary cation-anion difference and source of vitamin D on dairy cows: Vitamin D, mineral and bone metabolism. J. Dairy. Sci.

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Rodney, R. M., J. K. Hall, C. T. Westwood, P. Celi, and I.J. Lean. 2013. Effects of nutrient balance during the transition period on reproduction, production and health in the dairy cow. Faculty of Veterinary Science Postgraduate Conference, 6-7 November, Camden, New South Wales.

Lean. I.J., and R. Rodney. 2014. Nutrition and Reproduction: What really has an effect? Page 159-167 in Proc.of the XXVIII World Buiatrics Congress, Cairns, Queensland Australia.

Lean, I. J., H. Raadsma, M. Khatkar, J. House, and R. Rodney. 2013. Reproductive research: some past findings and future directions. Page 19 in Proc. *InCalf Reproduction Symposium 2013, May, Melbourne, Australia,*

Lean, I. J., Rabiee, A. R., and R.M. Rodney. 2013. Nutrition as an Intervention – How Nutrition Affects Reproduction. In Australian Cattle Veterinarians Conference 2014 Focus on Fertility – "Sex in the Long Grass" Darwin, Northern Territory, Australia.

Lean, I. J., Rabiee, A. R., and R.M. Rodney. 2013. Nutrition as an Intervention – Specific Interventions. In Australian Cattle Veterinarians Conference 2014 Focus on Fertility – "Sex in the Long Grass" Darwin, Northern Territory, Australia.

INVITED TALKS AND PRESENTATIONS

April 2016	Australian Association of Ruminant Nutrition Seminar, Melbourne VIC
August 2016	Future Ready Dairy Farms Expo, Bega NSW
June 2016	Dairy NSW Industry Forum, Wagga Wagga NSW
March 2015	Dairy Moving Foward, Melbourne VIC

OTHER MEDIA RELATED TO THIS RESEARCH

Interview on transition nutrition in dairy cows, ABC Country Hour- 2 September 2016

Dairy cow health: Vitamin D can go a long way to improve production. The Weekly Times, March 2017 <http://www.weeklytimesnow.com.au/agribusiness/dairy/dairy-cow-health-vitamin-d-can-go-a-long-way-to-improve-production/news-story/cc2d9fe5dc0ad5a4a4471dc771e717d6>

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AWARDS OBTAINED DURING THIS THESIS

2017	Australian Dairy Conference Young Dairy Science Communication Award
2016	Dairy Research Foundation Emerging Dairy Scientist Program Best Presentation
2016	Dairy Science Travel Grant
2016	ASAP Postgraduate Student Workshop Best Late Stage Poster Presentation
2015	Dairy Research Foundation Emerging Dairy Scientist Program Participant
2014	Animal Production Science ASAP Young Scientist's Award

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author

Rachael Rodney, October 2017

ACKNOWLEDGEMENT OF CONTRIBUTION TO THE RESEARCH WORK AND/OR AUTHORSHIP

This thesis examines aspects of the nutrition of dairy cows during the transition period on fertility, health, productivity, and vitamin D metabolism. This thesis contains eight original research papers published in, or submitted to peer-reviewed journals.

The development of the papers in this thesis, other than Chapters 7 and 8, has been the primary responsibility of the candidate, working within the Faculty of Veterinary Science, School of Life and Environmental Sciences under the supervision of Adj. Prof. Ian Lean, Dr Pietro Celi, Em. Prof. David Fraser, Dr Jeff Downing, Prof. Sergio (Yani) Garcia, and Assoc. Prof. Peter Thomson. The research conducted at the University of Florida (Chapters 6, 7, 8, and 9) was also supervised by Prof. Jose Santos.

Chapters 6, 7, and 8 are presented as a series of papers reporting the findings from one experiment. The candidate had responsibilities in designing and conducting this experiment, sample collection, and finalizing of manuscript prior to publication. The collaborative nature of research however meant that the writing of the papers presented in Chapters 7 and 8 was the primary responsibility of Dr Natalia Martinez, at the time, a PhD candidate within the University of Florida, and Prof. Jose Santos. The inclusion of these papers in Chapters 7 and 8 of this thesis is necessary to represent the full contribution of the work conducted by the candidate during the completion of this PhD. The original submission of this thesis included these papers in the form in which they were originally submitted to the journal for review. Updated papers reflecting the journal review process have been developed with assistance from Prof Jose Santos and Dr Ian Lean and the most recent versions at the time of submission have been included in this thesis.

The inclusion of co-authors for other papers reflects the fact that the work came from active collaboration between Australian and international researchers and with industry, and acknowledges input into team-based research.

The following details the contribution of each of the co-authors to one or more peer-reviewed publications within the thesis.

Adj. Prof. Ian Lean contributed substantially to study design, field work, statistical analysis, finalizing manuscripts prior to publication and responding to reviewers' comments (All Chapters).

Dr Pietro Celi contributed to the study design, field work, and finalizing of manuscripts prior to publication (Chapters 2, 3, 4, 5, 6, and 9).

Mr William Scott and Ms Kamilla Breinhild assisted with entering and modelling the diets in CPM-Dairy and contributed to finalizing of manuscript prior to publication (Chapters 2 and 3).

Prof. Jose Santos contributed substantially to the study design, field work, statistical analysis and finalizing of manuscripts prior to publication and responding to reviewers' comments (Chapters 6 and 9), statistical analysis and writing of manuscripts and responding to reviewers' comments (Chapters 7 and 8), and finalizing manuscripts prior to publication (Chapter 2).

Dr Jenianne Hall and Dr Charlotte Westwood collected the milk composition and fertility base data during their respective PhD studies, and contributed to finalizing of manuscript prior to publication (Chapter 4).

Dr Joe McGrath contributed to the study design, coordinated the vitamin D assays, and contributed to finalizing of manuscript prior to publication (Chapter 5).

Emeritus Prof. David Fraser provided advice on study design, interpretation of results, and contributed to review of manuscripts (Chapters 5, 6 and 9).

Dr Helen Golder assisted with field and laboratory work and manuscript editing prior to publication (Chapter 5).

Dr Stephen Anderson conducted the insulin, IGF-1, and osteocalcin assays and contributed to finalising the manuscript prior to publication (Chapter 5).

Dr David McNeill provided correspondence regarding sample analysis and contributed to finalising the manuscript prior to publication (Chapter 5).

Dr Natalia Martinez contributed substantially to the study design and field work (Chapters 6 and 9) and statistical analysis and writing of manuscripts (Chapters 7 and 8).

Dr Elliot Block contributed to study design and finalizing manuscripts prior to publication (Chapters 6,7,8, and 9).

Dr Laura Hernandez conducted the serotonin assays and contributed to finalizing manuscript prior to publication (Chapter 6).

Dr Corwin Nelson contributed to study design (Chapters 6, 7, and 8).

Dr Gene Wijffels and group conducted adiponectin and leptin assays, and provided methods for inclusion in the publications (Chapter 6 and 9).

Assoc. Professor Peter Thomson assisted with statistical advice and design of analysis (Chapter 9).

I, as a co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate.

Adj. Prof. Ian Lean Date 16/3/2017

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LIST OF ABBREVIATIONS

The following abbreviations have been used throughout this thesis.

%	percent
ºC	degrees Celsius
aa/AA	amino acid
AAN	α amino nitrogen
ABV	Australian Breeding Value
ADF	acid detergent fibre (fiber)
AI	artificial insemination
AIC	Akaike's information criteria
ALA	alpha-linolenic acid
AR1	first-order autoregressive
BCS	body condition score
BE	base excess
BHB	beta-hydroxybutyrate
BW	body weight
Ca	calcium
CI	confidence interval (also 95% CI)
Cl	chlorine
CLA	conjugated linoleic acid
CN	casein
cOC	carboxylated osteocalcin
CP	crude proetin
CPM	Cornell-Penn-Miner
CSFA	calcium salts of fatty acids
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CTX-1	C-terminal telopeptide of type 1 collagen
Cu	copper
CV	coefficient of variation
CYP27B1	cytochrome p450 27B1 enzyme (also known as 1-α hydroxylase)
d	day(s)
DCAD	dietary cation-anion difference
DIM	days in milk

DM	dry matter
DMI	dry matter intake
EAA	essential amino acid
ECM	energy corrected milk
EDTA	Ethylenediaminetetraacetic acid
ES	effect size
FA	fatty acid
FCM	fat corrected milk
g	gram(s)
GM	genetic merit
GnRH	Gonadotropin-releasing hormone
h	hour(s)
HCO_3^-	bicarbonate
HR	hazard ratio
I^2	statistic for impact of heterogeneity
iCa	ionised (ionized) calcium
IGF-1	insulin-like growth factor-1
IGg	Immunoglobulin G
IRR	incident rate ratio
IU	international units
K	potassium
Ln	natural logarithm
LSM	least squares means
ME	metabolisable (metabolizable) energy
mEq	milliequivalents
MFI	mean fluorescence intensity
Mg	magnesium
Mn	manganese
mo	month(s)
MP	metabolisable (metabolizable) protein
N	nitrogen
Na	sodium
NDF	neutral detergent fibre
NE	net energy

NEB	negative energy balance
NEFA	non-esterified fatty acid
NEL	net energy lactation
NFC	Non-Fiber Carbohydrate
NRC	National Research Council
NSW	New South Wales
OB	osteoblast
OC	osteocalcin
P	phosphrous/phosphate
pCO2	partial pressure of CO2
peNDF	physically effective NDF
$PGF2_{\alpha}$	Prostaglandin F2alpha
pH	hydrogen ion concentration
PHT	partially hydrogenated tallow
PMN	polymorphonuclear leukocyte
pO_2	partial pressur of O_2
PTH	parathyroid hormone
RDP	rumen degradable protein
RR	relative risk
RUP	rumen undegradable protein
S	sulfur
SCC	somatic cell count
SCS	somatic cell score
SD	standard deviation
SE	standard error
SMD	standardized mean difference
SNF	solids-not-fat
TMR	total mixed ration
uOC	undercarboxylated osteocalcin
USA	United States of America
VDR	vitmain D receptor
wk	week(s)
WMD	weighted mean difference
Zn	zinc

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CHAPTER ONE: INTRODUCTION

The transition period, from approximately three weeks before to three weeks after calving, is characterised by sudden and substantial changes in nutrient requirements as dairy cattle respond to the metabolic challenges of late pregnancy and early lactation (Bell, 1995). The success by which cows meet these challenges determines their productivity, health and reproductive success not only over transition, but also throughout the following lactation. Manipulations of the pre-calving and early lactation diet have led to persistent improvements in milk production, health and reproduction (Overton and Waldron, 2004, Thatcher et al., 2011), leading us (Lean et al., 2015) to pose the question; *How can a relatively short intervention, applied during the per-partum or early lactation period have long lasting effects on fertility, health and productivity?*

THE TRANSITION PERIOD

When cows do not adapt well to the onset of lactation, poor fertility and a high incidence of metabolic disease occur, leading to losses in cow productivity and negative animal welfare implications. Approximately 80% of disease in adult cows occurs during the transition period, and diseases are often interrelated (Curtis et al., 1983, Drackley, 1999). Poor reproductive performance of lactating dairy cattle is a complex disorder that has increased in prevalence over time and reflects associations with intensification of production and increased milk production (Butler, 2000, Lucy, 2001, Lean et al., 2008, Berry et al., 2014). Although some of the decline is associated with genetic selection for greater milk production, the heritability of reproductive disorders is weak (Pryce et al., 1997, Berry et al., 2014). This suggests that much of the decline is associated with environmental change and interactions of environment with genetics.

Good nutritional management plays a key role in dairy cows' successful transition to lactation. DeGaris et al. (2010a, b) found that cattle exposed to well-balanced transition diets for 20 days had increased risk of pregnancy, greater persistence within the herd, and improved milk and milk solids yield compared to cattle that had not been exposed to transition diets. Nutritional influences on fertility have been examined and frequently reviewed (Ferguson, 1991, Butler, 2000, Leroy et al., 2008a, Leroy et al., 2008b, Bisinotto et al., 2012, Friggens et al., 2013), but optimum diets are still to be determined.

An understanding of the underlying physiological changes resulting from manipulation of transition diets is needed in order to refine optimal diets. The long lasting effects of peri-parturient nutritional intervention imply that it not only the homeostatic responses to increased nutrient demand that are altered by these interventions, but possibly also homeorhetic adaptations that are required to upregulate metabolism to meet the ongoing demands of lactation. Homeorhetic changes are defined as the 'coordinated changes in metabolism of body tissues necessary to support a physiological state' (Bauman and Currie 1980). The key aims of this thesis are to examine the effects of nutritional interventions applied during the periparturient period, especially on fertility, and improve understandings around vitamin D and related metabolism in the dairy cow.

PROTEIN

Dietary protein intake is one of the more comprehensively studied areas of dairy cattle nutrition, and clear associations have been seen between improved protein nutrition and improved production, fertility, and health (Van Saun et al., 1993). Yet, increasing the amount of soluble protein or degradability of protein in the diet reduces fertility (Lean et al., 2012). Increased protein intake, dietary percentage, or degradability can be associated with increased calving to pregnancy interval (Folman et al., 1981, Carroll et al., 1994, Butler, 2000, Westwood et al., 2002, Lean et al., 2012). The degradability of protein is important, and protein sources should be considered in terms of metabolisable protein (**MP**) provided rather than crude protein. Changes in dietary protein content may not simply affect MP balance, but also specific amino acid (**AA**) composition and supply. Specific roles for AA in reproductive performance are not well defined but may be important.

ENERGY AND CARBOHYDRATES

The availability of nutrients is determined by dry matter intake (**DMI**), digestibility and metabolizability of the diet, and the endogenous body tissue reserves, reflected in body weight (**BW**) and body condition (Lean and Rabiee, 2006). The irreversible loss of nutrients in milk production and requirements for maintenance and growth diminishes the nutrient pool available for reproduction (Baldwin et al., 1987, Friggens et al., 2013). The difference between dietary intake and such expenditure determine the nutrient balance and if a negative balance occurs endogenous reserves are depleted. Many studies have examined the effects of estimated negative

energy balance (**NEB**) on fertility (Wathes et al., 2007, Butler, 2012). A positive energy balance in early lactation decreases the interval to first ovulation and increases the probability of pregnancy at the following breeding (Butler and Smith, 1989). Excessively low or high body condition score (**BCS**) at calving, or extreme losses of BW or BCS in early lactation, are usually associated with impaired reproductive outcomes (Heuer et al., 1999, Pryce et al., 2001, Buckley et al., 2003, Lopez-Gatius et al., 2003). Whereas BW and BCS change are often used as proxies for energy balance, they may more correctly be interpreted as a reflection of nutrient balances of protein, minerals, specific fatty acids and vitamins, as well as energy.

Substantial energy deficits contribute to incidence of metabolic disease, decreased milk production and poor reproductive efficiency (Butler, 2000). Energy deficiency reduces or impairs gonadotropin-releasing hormone (**GnRH**) and gonadotrophn secretions essential for follicle development (Butler, 2003, Formigoni and Trevisi, 2003). Changes in, or absolute values of, blood metabolite concentrations including glucose, non-esterified fatty acids (**NEFA**), beta-hydroxybutyrate (**BHB**), insulin, and insulin-like growth factor (**IGF**) 1 have been associated with changes in reproductive performance (Leroy et al., 2005, Leroy et al., 2008b, LeBlanc, 2010, Ospina et al., 2010, Chapinal et al., 2012), however, these indicators are not uniformly associated with adverse reproductive outcomes (Chapinal et al., 2012). Carbohydrates are important sources of energy for cows, as well as for rumen microorganisms and generally increase the efficiency of protein utilization and microbial protein production (Hoover and Stokes, 1991, Aldrich et al., 1993, Hristov et al., 2005). However, increased concentrations of rapidly fermentable carbohydrates can increase the risk of ruminal acidosis (Plaizier et al., 2008, Golder et al., 2012), which may reduce feed intake or produce a metabolic acidosis leading to detrimental alteration of uterine environment (Moore et al., 1991, Zebeli et al., 2015). Consequently, there could be positive and negative effects of carbohydrates on fertility, but relatively little work exists examining the effects of carbohydrate intervention of reproduction.

FATS

Recent understandings of the role of fats in metabolism open new opportunities for improving production, health and reproduction in cattle. Traditionally, supplementation with fats during transition has not been recommended (Santos et al. 2003) due to the potential for DMI to be reduced, particularly in heifers (Hayirli et al. 2002). Increasing the fat content of diets also has the potential to adversely effect rumen microbiota. However, including fat can increase energy

density of the diet without increased dependence on rapidly fermentable carbohydrates which, when fed at high levels, can compromise rumen and metabolic health. Fats not only provide an energy source, but also essential fatty acids (Mattos et al., 2000) which are precursors for steroid hormones. The effects of feeding fat during transition have not been clear, with studies reporting mixed results (Grummer and Carroll, 1991, Rabiee et al., 2012). More recently however, studies have shown beneficial effects of dietary fat on improved reproductive performance (Thatcher et al., 2006, De Veth et al., 2009), milk yeld (Rabiee et al., 2012, Boerman, 2014), energy balance (Von Soosten et al., 2012), and reduced the incidence of metabolic diseases. The beneficial effects of fat have been observed independently of the provision of energy (Staples et al., 1998).

VITAMIN D AND DIETARY CATION-ANION DIFFERENCE (DCAD)

The study of vitamin D in cattle has traditionally focused on changes in calcium (**Ca**) and mineral metabolism in response to hypocalcemia, particularly parturient paresis (Olson et al., 1973, Julien et al., 1977, Horst et al., 2003). However, there is increasing evidence of a wider role vitamin D plays in integrated metabolism, in particular, linkages with skeletal and energy metabolism. The main stages of vitamin D activation were depicted by Deeb et al. (2007) in the following figure.

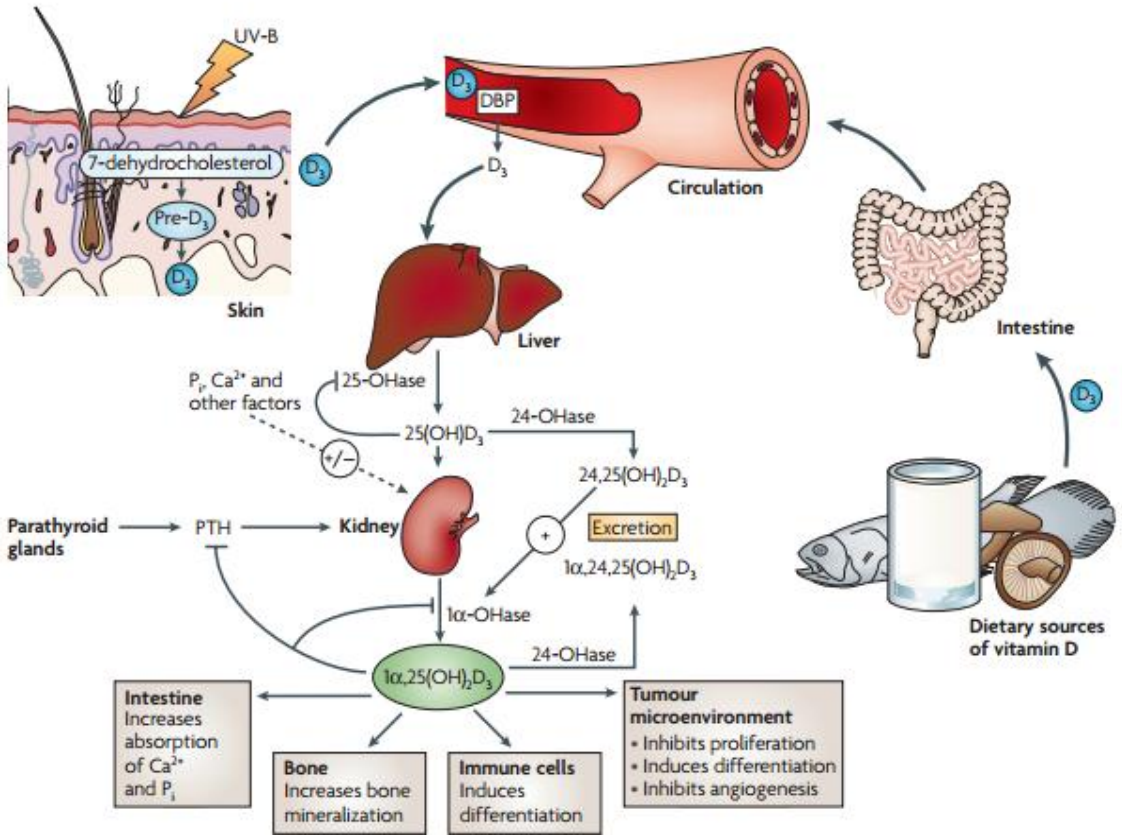

Figure 1. Vitamin D metabolism. Photochemical synthesis of vitamin D_3 (cholecalciferol, D_3) occurs cutaneously where pro-vitamin D_3 (7-dehydrocholesterol) is converted to pre-vitamin D_3 (pre-D_3) in response to ultraviolet B (sunlight) exposure. Vitamin D_3 , obtained from the isomerization of pre-vitamin D_3 in the epidermal basal layers or intestinal absorption of natural and fortified foods and supplements, binds to vitamin D-binding protein (DBP) in the bloodstream, and is transported to the liver. D_3 is hydroxylated by liver 25-hydroxylases (25-OHase). The resultant 25-hydroxycholecalciferol ($25(OH)D_3$) is 1α-hydroxylated in the kidney by 25-hydroxyvitamin D_3-1α-hydroxylase (1α-OHase). This yields the active secosteroid $1\alpha,25(OH)_2\ D_3$ (calcitriol), which has different effects on various target tissues. The synthesis of $1\alpha,25(OH)_2\ D_3$ from $25(OH)D_3$ is stimulated by parathyroid hormone (PTH) and suppressed by $Ca^{2+,}$ Pi and $1\alpha,25(OH)_2\ D_3$ itself. The rate-limiting step in catabolism is the degradation of $25(OH)D_3$ and $1\alpha,25(OH)_2\ D_3$ to $24,25(OH)D_3$ and $1\alpha,24,25(OH)_2\ D_3$, respectively,which occurs through 24-hydroxylation by 25-hydroxyvitamin D 24- hydroxylase (24-OHase), encoded by the CYP24A1 gene. $24,25(OH)D_3$ and $1\alpha,24,25(OH)_2\ D_3$ are consequently excreted. The main effects of $1\alpha,25(OH)_2\ D_3$ on various target tissues are highlighted above. Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS CANCER] Deeb et al., 2007, copyright 2007.

Low blood calcium triggers a cascade of metabolic responses including, mediated by parathyroid hormone (**PTH**), the hydroxylation of calcidiol to calcitriol. Calcitriol facilitates the active absorption of Ca in the intestine (Fraser and Kodicek, 1973), and in combination with PTH, stimulates bone reabsorption. Studies in mice and man have identified a pivotal role for osteocalcin in a feedback loop between bone and energy metabolism (Lee et al., 2007, Wolf, 2008). Understandings of the relationships between bone and energy metabolism were summarized by Lean et al. (2015) in the following Figure.

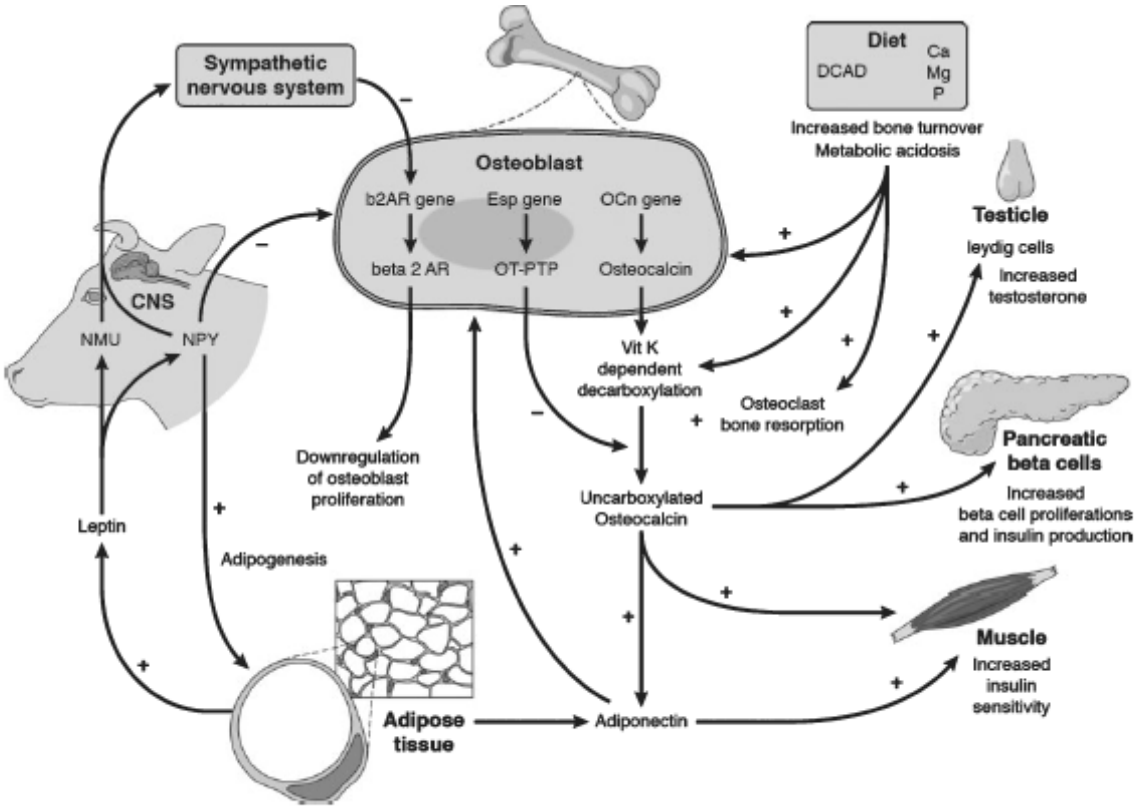

Figure 2. This figure modified from Wolf (2008) shows the relationship between skeleton and energy metabolism. The potential role for acidifying or negative dietary cation anion difference diets has been included and the effects of skeleton on reproductive metabolism, identified to date, are outlined. Reprinted from "Influencing the future: interactions of skeleton, energy, protein and calcium during late gestation and early lactation," by I.J. Lean, P.J. DeGaris, P. Celi, D.M. McNeill, R.M. Rodney, and D.R. Fraser, 2014, Animal Production Science,54, p. 1179.

Calcidiol stimulates osteoblasts to produce and excrete osteocalcin (**OC**). There are two forms of OC; an undercarboxylated (**uOC**) inactive form, and following a series of vitamin-K facilitated carboxylations, a carboxylated (**cOC**) biologically active form. Calcitriol supplementation elevated plasma Ca, total OC and uOC (Kim et al., 2011) although these results were not seen following introduction of calcidiol or cholecalciferol (Taylor et al., 2008). Murine studies

identified metabolic effects orchestrated by osteocalcin that influence the regulation of energy metabolism including, control of insulin release, tissue sensitivity of insulin and the integration of these process with other metabolic pathways (Lee et al., 2007). Osteocalcin stimulates the secretion of insulin by the pancreas, which inhibits osteoblast (**OB**) activity, thereby enhancing bone resorption (Lee et al., 2007). Further, bone appears to influence lipid metabolism and vice versa as OC stimulates the production of adiponectin by adipose cells. Adiponectin increases sensitivity to insulin, increases OB proliferation and differentiation (Berner et al. 2004) and increases glucose uptake by skeletal muscle (Yamauchi et al. 2002). Calcium may also play a direct role in energy metabolism, and insulin secretion (Capen and Rosol 1989). Cows in an induced sub-clinical hypocalcaemic state had reduced DMI and impaired energy metabolism, with lower concentrations of insulin and higher concentrations of glucose and NEFA in their plasma (Martinez et al., 2014).

One of the most powerful tools to influence metabolism of dairy cows has been use the use of negative DCAD diets, which in creating mild metabolic acidosis, improve mineral metabolism, and increase sensitivity to calcitrophic hormones. These have primarily been employed pre-partum as a means of reducing incidence of hypocalcemia, but beneficial effects on reproductive performance have also been seen (Beede, 1992, Morton, 2004).

THESIS PRESENTATION

The collection of work presented in this thesis examines the potential for dietary interventions in the peri-parturient period to improve dairy cow metabolism to enhance fertility, health and productivity outcomes. To do this several approaches are utilized to analyse data from new field and desktop studies as well as reanalysis of existing data to explore new hypotheses. This thesis contains two systematic reviews utilizing the global literature on early lactation nutrition and reproduction. The first examines the effects of diet as a whole (Chapter 2), followed by a specific examination of the effects of dietary fat interventions on proportion pregnant to insemination and time to pregnancy (Chapter 3). Reproductive and production outcomes related to peri-parturient protein nutrition are explored by utilizing and building on existing data in Chapter 4. This thesis also includes a series of randomized clinical experiments related to vitamin D supplementation in mid-lactation (Chapter 5) and peri-parturient (Chapters 6 to 9) cows. The results discussed in Chapters 6 to 8 are presented as a series of papers reporting the findings from one experiment. These reflect the collaborative nature of research, as responsibility for writing the manuscripts

was shared between the project's co-investigators. Chapter 9 builds on this further, with a more detailed examination of relationships between metabolites associated with bone and energy metabolism using time-series analysis. Research Chapters (Chapters 2 to 9) are written in the style of manuscripts; each with its own abstract, introduction, materials and methods, results, discussion and conclusions. As such, there is repetition between some areas of the methods (e.g. description of diets) across multiple chapters. Some papers were submitted to a North American journal (Journal of Dairy Science) and as a result, some words in these chapters (Chapters 2-4 and 6-9) are spelled using American spelling. The collected findings and implications of this series of work are summarized and discussed in Chapter 10.

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CHAPTER TWO: EFFECTS OF TRANSITION NUTRITION ON THE FERTILITY OF LACTATING DAIRY CATTLE: A META-ANALYSIS

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OVERVIEW OF CHAPTER TWO

The introduction to this thesis (Chapter 1) identified some of the nutritional interventions that can have beneficial outcomes on dairy cow fertility when fed during the peri-parturient period. However, the effects of such interventions are complex and interrelated, and the optimum dietary requirements for this period are not yet determined. This Chapter presents a systematic review and meta-analysis of early lactation diets on the proportion pregnant to service and calving to pregnancy interval of dairy cows. Traditionally, interventions have been studied in isolation, but this analysis examines contributions of individual components rather than differences betweentreatment and control diets that influence reproductive outcomes.

ABSTRACT

This meta-analysis of 39 experiments containing 118 treatments explored the effects of diet interventions in early lactation on the proportion of dairy cows pregnant to artificial insemination (AI; pregnancy to AI) and on calving to pregnancy interval. It also identified factors that may explain variation in these responses. The objectives were to identify effects of diet on reproduction, rather than differences between specific dietary interventions. The examination of calving to pregnancy interval utilized the more traditional method of analyzing difference between a treatment and the reference treatment used for comparison within a given experiment.

The systematic review identified fewer experiments (n = 39) than had been expected. Four different multivariable models including the random effect of experiment were used to examine the effects of CPM-Dairy (version 3.08) estimated diet and production variables on proportion pregnant to AI These models examined; i) output of products, ii) balance or duodenal availability of nutrients, iii) intake of nutrients, or iv) percentage of nutrients in the diet. The multivariable models identified positive associations between estimated increased fatty acid intake g/d (incidence rate ratio (IRR) 1.0003; SE 0.0001), starch intake kg/d (IRR 1.061; SE 0.029), metabolizable energy balance MJ/d (IRR 1.004; SE 0.002), and duodenal c14:0 g/d (IRR 1.008; SE 0.004) availability with the proportion of cows pregnant to AI, whereas rapidly fermentable sugar intake kg/d (IRR 0.813; SE 0.054), percentage of sugar in the diet (IRR 0.960 (SE 0.015), and milk protein yield 100g/d (IRR 0.922; SE 0.022) were associated with a redcued proportion of cows pregnant to AI.

There was no multivariable model developed to assess variables associated with calving to pregnancy interval but, univariably, increased metabolizable energy balance was associated with a shorter calving to pregnancy interval ($P = 0.020$), whereas increased milk production was associated with longer time to pregnancy ($P = 0.004$). Increased intake of some AA, particularly threonine and lysine, were associated with a longer calving to pregnancy interval ($P < 0.05$). It is clear nutritional management around calving can influence reproductive success. The importance of dietary fats and positive energy and protein balances in early lactation for improved fertility outcomes is supported and suggests that starch and sugars may have different effects on the proportion of cows that are pregnant to AI. This work also highlighted a need for further focused field studies exploring the roles of specific fatty acids, AA, phosphorus, and carbohydrates on reproduction.

Keywords: carbohydrate, fats, fertility, protein

INTRODUCTION

Poor reproductive performance of lactating dairy cattle is a complex disorder that reflects associations with intensification of production and increased milk production (Butler, 2000, Lucy, 2001, Lean et al., 2008, Berry et al., 2014). However, it is difficult to determine a causal basis for the decrease in fertility as genetics and environment have changed markedly over the last decades. Although some of the decline in fertility observed in the past is associated with genetic selection for greater milk production, the heritability of reproductive disorders is weak (Pryce et al., 1997, Berry et al., 2014). This suggests that much of the decline previously observed was associated with environmental change and interactions of environment with genetics.

Nutritional influences on fertility have been examined and frequently reviewed (Ferguson, 1991, Butler, 2000, Bisinotto et al., 2012, Friggens et al., 2013), but difficulties and inconsistencies in study design occur. Studies must have large numbers of experimental units to identify biologically and economically important differences in proportion of cows pregnant (Lean et al., 2016). Nutritional influences during the transition period (± 4 weeks of calving) may be of particular importance (Overton and Waldron, 2004), but it is clear that the effect of diet on fertility during this period is complex, and multi-factorial. Further, confounding is present in nutritional experiments, as the addition of one nutrient inherently alters concentrations of other nutrients in the diet (Lean et al., 2012). Nutrients may also interact to alter availability, uptake, or production of other nutrients and influence energy partitioning and future milk production beyond the period of immediate intervention (Block, 1984, Van Saun et al., 1993, Degaris et al., 2008, Lean et al., 2014). Therefore, although experiments examining single nutritional interventions are essential to develop understandings of dietary components on reproductive outcomes, responses can be difficult to evaluate due to the potential for confounding. Meta-analysis, using published literature, provides the opportunity to combine studies and overcome the confounding caused by changes in single diets. It also allows the use of existing data to address hypotheses which could not be addressed previously, increases study power, and provides measures of the variation, or heterogeneity, of results among a group of experiments (Lean et al., 2009).

The effects of some nutritional interventions on pregnancy and time to pregnancy have been examined using meta-analysis (De Veth et al., 2009, Rabiee et al., 2010, Lean et al., 2012,

Rodney et al., 2015 – Chapter 3). These studies used randomised controlled experiments that evaluated either the addition of fats (De Veth et al., 2009, Rodney et al., 2015 – Chpater 3), organic trace minerals (Rabiee et al., 2010) or protein (Lean et al., 2012) on reproductive outcomes. Few experiments have been identified that examine the effects of carbohydrate fractions on fertility, which has previously limited the ability to examine these specific dietary components using meta-analysis. Consequently, the effects of carbohydrate fractions were of interest in this study.

The concept underpinning this study is that reproductive failure in a group of cows is, in part, a metabolic disorder reflecting the inability of diets to supply adequate intakes, concentrations, or ratios of nutrients that are required for optimal reproductive performance. The objective of this study was to utilize carefully described dietary information from the available literature to explore the effects of diet during the transition period on measures of pregnancy and calving to pregnancy interval as well as identifying factors that may explain variation in these responses. It was hypothesized that dietary formulation and intake of nutrients during the transition period affects the probability of pregnancy to AI and interval from calving to pregnancy in dairy cows. Previous meta-analyses examining the effects of nutritional interventions during transition on reproduction identified surprisingly few papers that were suitable for inclusion (Lean et al., 2012, Rodney et al., 2015 – Chapter 3). This study of the proportion pregnant to AI differs from previously conducted meta-analyses in that the proportion pregnant for each treatment represents a single observation, whereas previous studies have examined the difference in proportion pregnant between a reference and treatment group. The former approach allows a focus on the effects of overall diet on reproduction, rather than differences between specific interventions. As such, all nutrients, and potential interactions among these, could be examined, irrespective of whether they were the intended intervention of a particular diet. The examination of calving to pregnancy interval utilized the more traditional method of using the difference between treatment and a reference group for each variable in analyses.

MATERIALS AND METHODS

Literature Search

A systematic review across three databases (PubMed, Web of Science CABI, and Google Scholar) and references in papers was used to identify experiments exploring nutritional interventions during transition and fertility outcomes that were published in English between

1970 and 2015 in a peer-reviewed journal, conference proceedings, or as an accepted thesis. Combinations of the following search terms were used; cow, cattle, dairy, fertility, pregnancy, conception, reproduction, b-vitamin, beta-carotene, biotin, calcium, CLA, cobalt, conjugated linoleic acid, copper, cottonseed, energy, fat, fiber/fibre, manganese, magnesium, minerals, molybdenum, omega-3, organic minerals, phosphorous, protein, selenium, soy, soya bean meal, vitamin A, vitamin ADE, vitamin D, vitamin E, Zinpro, zinc. Results were ordered by "relevance" according to the database and articles were assessed until there were 500 chronological papers that, from the title, appeared unrelated to the topic and unworthy of further review.

Inclusion and Exclusion Criteria

For clarity of reporting these results, individual nutritional treatments will be referred to as "treatments" and manuscripts or published papers that report one or more treatments will be referred to as "experiments". For calving to pregnancy interval, the difference between two treatments is used and is referred to as a "comparison". Treatments were suitable for inclusion if they were from randomized controlled experiments using *Bos taurus* dairy cows in their first or later lactation during the dry/lactating period (i.e. nulliparous non-lactating heifers were excluded). Experiments had to evaluate the effect of individually feeding cows during the transition period (up to 4 weeks pre- and/or postpartum), and describe the diet in enough detail that it could be evaluated using CPM-Dairy (version 3.08; Cornell-Penn-Miner, https://cahpwww.vet.upenn.edu/archives/9-Purchase-CPM-3.0.8.1.html). Experiments reporting both pre- and post-calving interventions were identified in the systematic review; however, the low number of experiments reporting pre-calving diets meant analysis was confined to post-calving treatments only. The number of cows in each treatment and, for continuous variables, measures of variance or *P*-values that allowed an estimate of SD to be derived, must have been reported. Fertility measures must have been reported as i) proportion of cows pregnant to first AI, second AI or within 28 d of breeding season, before 126 DIM, or within the lactation (labeled throughout the manuscript as "proportion pregnant to AI"); and/or ii) calving to pregnancy interval or days open (labeled throughout the manuscript as "calving to pregnancy interval"), and these are detailed for each experiment in Table 1. The numerator for the proportion of cows pregnant was the cows pregnant and the denominator was the number of cows in the group with experiments reporting pregnancy to a single service (Table 1). Appropriate experimental design including details of randomization, suitable analysis, and elimination of bias or confounding was also evaluated.

Table 1. Summary of experiments included in the meta-analysis

Reference	N	Dietary intervention[1]	Duration of treatment (d)	Prop preg to AI (%)[2]	Calving to preg interval (days)
Ambrose et al.,	59	Sunflower seed (0.1% alpha-linolenic acid)	From 28 d before to 32	32[A]	-
2006	62	Flaxseed (56.7% alpha-linolenic acid)	d after AI	48	-
Barton et al.,	32	13% CP	1 to 100 d postpartum	41[A]	71[F]
1996	32	20% CP	(or 120 d postpartum if not pregnant)	44	81
Bruckental et al.,	12	Basal diet (9.2% CP)	Throughout lactation	-	79[G]
1986	12	Soybean meal (14.3 % CP)		-	110
	12	Soybean meal (18.0% CP)		-	88
	12	Urea (14.3% CPg/kg CP)		-	94
	12	Urea phosphate		-	93
Bruckental et al.,	78	Low soybean meal (17% CP) – multiparous	0 to 168 d postpartum	49[B]	-
1989	66	High soybean meal (21% CP) – multiparous		42	-
	70	Fish meal (21% CP) – multiparous		50	-
	30	Low soybean meal (17% CP) – primiparous	0 to 112 d postpartum	47	-
	29	High soybean meal (21% CP) – primiparous		45	-
	28	Fish meal (21% CP) – primiparous		57	-
Canfield et al.,	15	Moderate protein (16% CP) – primiparous	0 d postpartum to 20 d	47[A]	-
1990	16	High protein (19 % CP) – primiparous	after first AI	31	-
	16	Moderate protein (16% CP) – multiparous		50	-
	16	High protein (19% CP) – multiparous		31	-
Carroll et al.,	29	13% CP	5 to 100 d postpartum	62[A]	72[F]
1988	28	20% CP	(or 126 d if not pregnant)	54	82
Carroll et al.,	23	Control	Fed for 100 d starting at 5 ± 2 DIM	50[A]	-
1990	23	5% added prilled long-chain fatty acids		50	-
Carroll et al.,	9	Control (0% fish meal)	12 to 125 d postpartum	67[A]	-
1994	9	Fish meal (3.5%)		33	-
Castaneda-	16	CSFA (approximately 1%)	2 wk prepartum to 9 wk	19[C]	160[H]
Gutierrez et al.,	16	CLA isomers (approximately 0.2%) + CSFA (approximately 0.9%)	postpartum	31	135
2005	15	CLA isomers (approximately 0.3%) + CSFA (approximately 0.8%)		33	151
Chester-Jones et	25	67% inorganic sulfate and 33% AA complex	Fed for the entire	28[A]	159[F]
al., 2013	24	67% inorganic sulfate and 33% polysaccharide complex	lactation and some cows remained on treatment	71	114
	24	100% polysaccharide complex	for subsequent lactation	29	135
	26	100% inorganic sulfate		27	139
deFeu et al.,	20	Standard TMR		50[A]	117[G]
2009	20	High-quality TMR		21	120
Edwards et al.,	18	Crude protein (13% CP)	Entire lactation	44[A]	123[F]
1980	18	Crude protein (15% CP)		39	141
	18	Crude protein (17% CP)		33	139
Ferguson et al.,	52	Control (0 kg fat) – herd 1	1 to 150 d postpartum	33[A]	105[F]
1990	44	0.5 kg fat – herd 1		57	99
	34	Control (0 kg fat) – herd 2		38	93
	27	0.5 kg fat – herd 2		63	88
	33	Control (0 kg fat) – herd 3		52	95
	24	0.5 kg fat – herd 3		67	82
Ferris et al.,	40	Low phosphorous diet (4.4 g P/kg DM in winter and 3.6 g P/kg DM in summer) – year 1	From parturition for up to four lactations)	38[A]	-
2010	40	High phosphorous diet (7.0 g P/kg DM in winter and 6.8 g P/kg DM in summer) – year 1		40	-
	50	Low phosphorous diet (4.4 g P/kg DM in winter and 3.6 g P/kg DM in summer) – year 2		32	-

Table 1 (Continued). Summary of experiments included in the meta-analysis

Reference	N	Dietary intervention[1]	Duration of treatment (d)	Prop preg to AI (%)[2]	Calving to preg interval (days)
Ferris et al., 2010 cont.	50	High phosphorous diet (7.0 g P/kg DM in winter and 6.8 g P/kg DM in summer) – year 2		46	-
	50	Low phosphorous diet (4.4 g P/kg DM in winter and 3.6 g P/kg DM in summer) – year 3		32	-
	50	High phosphorous diet (7.0 g P/kg DM in winter and 6.8 g P/kg DM in summer) – year 3		26	-
	50	Low phosphorous diet (4.4 g P/kg DM in winter and 3.6 g P/kg DM in summer) – year 4		24	-
	50	High phosphorous diet (7.0 g P/kg DM in winter and 6.8 g P/kg DM in summer) – year 4		8	-
Garcia Bojalil et al., 1998	11	0% CSFA and 11.1% DM ruminally degradable intake protein	1 to 120 d postpartum	27[A]	80[F]
	11	2.2% CSFA and 11.1% DM ruminally degradable intake protein		45	83
	10	0% CSFA and 15.7% of DM ruminally degradable intake protein		40	73
	11	2.2% CSFA and 15.7% DM ruminally degradable intake protein		45	86
Garnsworthy et al., 2009	14	Low starch (9.8% starch)	0 to 120 d postpartum	21[A]	66[G]
	11	High starch (18.2% starch)		9	81
Gilmore et al., 2011	27	Low protein and methionine (14% CP, 40 g/d methionine)	0 to 120 d postpartum	33[A]	134[G]
	27	Recommended protein (17% CP)		29	142
Holter et al., 1992	18	Control	0 to 112 d postpartum	33[A]	-
	19	Linted whole cottonseed (15%)		47	-
	19	Linted whole cottonseed and CSFA at ~ 3%		42	-
Howard et al., 1987	54	Moderate protein (15% CP)		-	80[F]
	47	High protein (20% CP)		-	80
Hutchinson et al., 2011	36	CSFA (60 g/d)	0 to 60 d postpartum	39[A]	111[G]
	33	Lipid-encapsulated CLA (80 g/d)		52	109
Lucy et al., 1991	9	Unbalanced basal diet formulated to be deficient in nutrients supplied by 2.9kg of long bermudagrass hay offered separately	7 to 60 d postpartum	33[D]	-
	8	Balanced basal diet with long bermudagrass hay offered separately		50	-
	9	0% CSFA, bermudagrass hay chopped and fed with TMR		33	-
	9	2.2% CSFA, bermudagrass hay chopped and fed with TMR		67	-
	8	Alfalfa hay chopped and mixed with TMR		100[R]	-
	9	Alfalfa hay cubed and mixed with TMR		33	-
Lucy et al., 1992	9	Control (0% CSFA) no niacin	15 to 98 d postpartum	44[A]	63[F]
	8	CSFA (3.0%) no niacin		25	63
	9	Control (0% CSFA) niacin added		44	70
	8	CSFA (3.0%) - niacin added		0[R]	0
Markus et al., 1996	11	Control – multiparous	~23 d postpartum for 16 wk	73[D]	163[G]
	10	Tallow (2.7%) – multiparous		90	124
	10	Whole sunflower seeds (7.1%) – multiparous		90	172
	6	Control – primiparous		100[R]	115
	6	Tallow (2.7%) – primiparous		50	112
	6	Whole sunflower seeds (7.1%) – primiparous		67	155
Mikula et al., 2011	12	Maize (2.5 kg/d)	14 d prepartum to parturition, parturition to 120 d	75[A]	115[F]
	12	Triticale then maize (2.5 kg/d of triticale from 14 d prepartum to parturition then 2.5 kg/d of maize to 120 d postpartum)		75	104
	12	Maize then triticale (2.5 kg/d of maize from 14 d prepartum to parturition then 2.5 kg/d of triticale to 120 d postpartum)		33	156
	12	Triticale (2.5 kg/d)		42	130

Table 1 (Continued). Summary of experiments included in the meta-analysis

Reference	N	Dietary intervention[1]	Duration of treatment (d)	Prop preg to AI (%)[2]	Calving to preg interval (days)
Moallem et al., 2010	20	CSFA (approx 0.2%)	21 to 100 d postpartum	35[A]	88[F]
	20	Encapsulated lipid supplement (approx 0.2%)		35	82
Moussavi et al., 2008	5	Control (0% fish meal)	21 d prepartum to 35 d postpartum	-	99[F]
	5	Fish Meal (3.5% prepartum, 1.95% postpartum)		-	111
Petit and Twagiramungu., 2006	36	Whole flaxseed (10.6%)	6 wk prepartum to 120 d postpartum.I If cows were diagnosed pregnant, the diet was fed for 50 d of gestation	44[A]	-
	34	CSFA (3.9%)		56	-
	40	Micronised soybeans (18.05%)		40	-
Ruegsegger and Schultz., 1985	27	Soybean meal (20%)	10 to 115 d postpartum	-	115[G]
	20	Heat-treated whole soybean (25%)		-	109
Salfer et al., 1995	16	Control (0% PHT pre- and postpartum)	14 d prepartum to 151 d postpartum	-	88[F]
	16	1% PHT at d-14 to parturition, 2% PHT 1 to 151 DIM		-	102
	16	0% PHT at d-14 to parturition, 2% PHT 1 to 151 DIM		-	95
Senatore et al., 1996	40	Single diet	0 to 100 d postpartum	43[A]	-
Siciliano-Jones et al., 2008	125	Minerals provided in sulfate form	3 wk prepartum through to 35 wk of lactation	40[A]	115[F]
	125	Minerals (Zn, Mn, and Cu) supplied as AAcomplexes		28	124
Sklan et al., 1989	54	Control	0 to 170 d postpartum	28[A]	-
	54	Calcium soaps of fatty acids (2.4%)		43	-
Sklan et al., 1994	29	Control (0% CSFA) – multiparous	0 to 120 d postpartum	41[E]	-
	33	CSFA (2.5%) – multiparous		33	-
	19	Control (0% CSFA) – primiparous		74	-
	21	CSFA (2.5%) – primiparous		33	-
Soltan et al., 2010	60	Single diet	3 wk prepartum to 12 wk postpartum	44[B]	-
Son et al., 1996	17	Tallow (3%) and low supplementary escape protein	14 to 84 d postpartum	24[A]	-
	15	Tallow (3%) and high supplementary escape protein		67	-
	15	Control (Tallow 0%, replaced with corn) and high supplementary escape protein		27	-
	15	Control (Tallow 0%, replaced with corn) and low supplementary escape protein		40	-
Son et al., 2000	11	Control	1 to 98 d postpartum	36[A]	-
	14	CSFA		36	-
Toni et al., 2007	90	Inorganic minerals	60 d prepartum to 200 d postpartum	19[A]	111[F]
	90	AA complexes of Zn, Mn, and Cu		21	112
Westwood et al., 2000	40	High degradable (19.3% CP, 15% RUP: 85% RDP)	21 d prepartum to 150 d postpartum	45[A]	102[G]
	42	Low degradable (19.3% CP, 35% RUP: 65% RDP)		67	84
Wu et al., 2000	26	0.31% P	From beginning to end of lactation	88[A]	78[F]
		0.40% P		44	106
		0.49% P		33	112

[1]CLA – conjugated linoleic acids; CSFA – calcium salts of fatty acids; PHT – partially hydrogenated tallow

[2] Measures that contributed to proportion pregnant to AI were reported as: conception to first service ([A]); conception to first two services or pregnant after 28 d of breeding season ([B]); pregnancy before 126 DIM ([C]); pregnancy within the lactation ([D]); or pregnancy to first service ([E]). Diets that had 0 or 100% conception or pregnancy destabilised the model and were removed from analysis of this measure ([R]).

[3] Measures that contributed to calving to pregnancy interval were: days open ([F]); days from calving to conception ([G]); or days from calving to pregnancy ([H]).

Data and Diet Extraction

Data were extracted from each published paper including authors, year of publication, reference (including journal) of the publication, title of the publication, parity of cows (first or multiple lactations), and number of cows in treatment and reference groups. As stated, reproductive variables examined were defined as proportion of cows pregnant to AI and calving to pregnancy intervals for each treatment. A summary of experiments and treatments included in the analysis is provided in Table 1.

To extract and model dietary information, data from accepted experiments were entered into CPM-Dairy (version 3.08) following the standard operating procedure described in Rabiee et al. (2012). Ration ingredients and intake in the experiments were entered using ingredients selected from the standard CPM-Dairy feed bank and calibrated to the specifications described in the experiment. Preference was given to revising nutrient compositions of forages. No nutrient content revisions beyond expected typical biological variation were identified. Once calibrated for the reference treatments, no further corrections were made to the analyses of individual components during modelling of the treatment diet. It was assumed that the same forages were used in all treatments of a given experiment, and any tested difference in the comparison of diet analyses within an experiment would likely reflect the distribution of errors around feed sampling and analysis. These data were combined with information on cows, production, housing, and environment from the paper to predict diet composition and outcomes. Individual dietary components and production factors were extracted. Throughout this paper, the international system of units was used, and full list of variables assessed and units used are presented in Table 2.

Table 2. Summary of mean, standard deviation, minimum, and maximum values observed in the experiments and estimated by CPM

Variable	Mean	SD	Minimum	Maximum
Milk production (kg/d)	33.4	6.1	19.1	52.9
Milk protein (%)	3.1	0.2	2.7	3.7
Milk protein yield (kg/d)	1.0	0.2	0.6	1.6
Milk fat (%)	3.5	0.4	2.3	4.3
Milk fat yield (kg/d)	1.2	0.2	0.6	1.7
Dry matter intake (kg/d)	20.6	2.7	10.2	25.1
Metabolizable energy balance (MJ/d)	7.1	26.0	-58.5	84
Metabolizable protein balance (g/d)	-102.9	291.9	-799	712.3
Crude protein (%)	18.0	2.4	10.8	25.6
Crude protein eaten (kg/d)	3.7	0.7	1.7	6.0
Rumen undegradable protein (% CP)	36.2	5.5	20.4	50.4
Rumen undegradable protein eaten (kg/d)	1.4	0.4	0.6	2.2
Rumen degradable protein (% CP)	63.8	5.5	49.6	79.6
Rumen degradable protein eaten (kg/d)	2.4	0.5	1.1	4.3
Soluble protein (% CP)	34.1	6.5	13.0	57.9
Soluble protein eaten (kg/d)	1.3	0.4	0.5	3.1
Urea cost (mJ/d)	0.8	2.0	0	11.6
Methionine (g/d)	45.4	6.7	24.5	58.7
Lysine (g/d)	147.3	24.0	62.5	188.9
Arginine (g/d)	143.0	24.4	59.5	198
Threonine (g/d)	108.0	16.2	49.8	136.9
Leucine (g/d)	184.0	32.5	104.5	260.4
Isoleucine (g/d)	111.8	17.2	54.9	139.9
Valine (g/d)	126.6	21.2	60.2	179.6
Histidine (g/d)	58.2	10.2	26.9	79.7
Phenylalanine (g/d)	114.9	18.7	57.5	148.9
Tryptophan (g/d)	34.5	5.9	15	51.2
Total essential amino acids (g/d)	1073.7	167.3	515.3	1345.1
Ether extract (%)	4.8	1.6	1.8	8.6
Long-chain fatty acids (%)	3.8	1.5	1.3	7.5
C12:0 intake (g/d)	6.5	6.3	0	37.5
C14:0 intake (g/d)	7.8	6.5	1.0	31.4
C16:0 intake (g/d)	164.3	98.1	36.4	482.2
C16:1 intake (g/d)	5.1	6.6	0.8	41.9
C18:0 intake (g/d)	35.4	48.1	5.0	367.8
C18:1t intake (g/d)	1.9	5.8	0	40.2
C18:1c intake (g/d)	160.6	93.5	9.6	494.6
C18:2 intake (g/d)	322.0	164.5	92.8	876.7
C18:3 intake (g/d)	65.2	61.5	16.1	531.2
C other intake (g/d)	17.9	10.4	6.4	64.4
C12:0 duodenal availability (g/d)	6.5	6.3	0	37.5
C14:0 duodenal availability (g/d)	7.8	6.5	1.0	31.4
C16:0 duodenal availability (g/d)	178.4	94.1	9.8	486.5

Table 2 (Continued). Summary of mean, standard deviation, minimum, and maximum values observed in the experiments and estimated by CPM

Variable	Mean	SD	Minimum	Maximum
C16:1 duodenal availability (g/d)	4.3	0.9	2.2	8.8
C18:0 duodenal availability (g/d)	456.6	147.0	175.5	869.2
C18:1t duodenal availability (g/d)	71.6	52.7	12.2	315.3
C18:1c duodenal availability (g/d)	44.2	34.9	5.3	206.7
C18:2 duodenal availability (g/d)	39.6	23.9	6.1	170.9
C18:3 duodenal availability (g/d)	3.9	4.0	0.5	27.2
c other duodenal availability (g/d)	52.1	8.5	26.1	84.9
Sugar (%)	5.1	2.3	2.1	13
Starch (%)	26.2	7.6	8.9	47.9
NDF (%)	31.9	4.7	14.4	46.1
Physically effective NDF (%)	23.2	3.9	5.7	31.8
Non-fibrous carbohydrate (%)	40.8	5.6	27.8	56.5
Fermentable carbohydrate total intake (kg/d)	8.7	1.2	4.8	11.2
NDF intake (kg/d)	2.1	0.5	0.2	3.5
Starch intake (kg/d)	4.3	1.3	1.5	8.5
Soluble fiber intake (kg/d)	1.2	0.5	0.3	2.5
Sugar intake (kg/d)	1.0	0.5	0.2	2.6
Calcium (%)	0.9	0.3	0.3	1.5
Phosphorous (%)	0.5	0.1	0.3	1.0
Magnesium (%)	0.3	0.1	0.2	0.5

Statistical Analysis

All statistical analyses were conducted using Stata (Intercooled Stata v.13 and v.14, USA). Following initial exploration of the data, a Poisson regression indicated that the data were over-dispersed and use of a negative binomial model for data analysis was indicated. Negative binomial meta-regression analyses (NBREG) were used to explore the effect of each predictive variable (e.g. dietary component or output) extracted on proportion of cows pregnant, and the contribution of each observation was weighted by the number of cows on the treatment. The unit of interest was each individual treatment within paper. Variance in the proportion of pregnant cows was examined by Generalized Linear Latent And Mixed Models (GLLAMM) (Skrondal and Rabe-Hesketh, 2004, Grilli and Rampichini, 2006). The variance attributable to treatment was small (<3% of total variance) and the variance attributable to experiment was approximately 30% of the total variance. Consequently, the random effect of experiment, but not treatment, was included in the statistical models developed. Variables with univariable $P < 0.20$ in the negative binomial analysis were assessed in population averaging, multivariable models (XTNBREG) with backward elimination, in which the random effect of experiment was accounted for.

The population averaging model is an equal within-panel (experiment) robust correlation model for the dispersion parameter (δ_i). As described by Hausman et al. (1984) for a random-effects overdispersion model, δ_i is allowed to vary randomly across groups; namely, it is assumed that that $1/(1 + \delta_i) \sim$ Beta (r, s where r, s are parameters for δ_i. The joint probability of the counts for the ith group is:

$$\Pr(Y_{i1} = y_{i1}, \ldots, Y_{in_i} = y_{in_i} | \mathrm{X}_i) = \int_0^{\infty} \prod_{t=1}^{n_i} \Pr(Y_{it} = y_{it} | x_{it}, \delta_i)\ f(\delta_i)\ d\delta_i$$

$$= \frac{\Gamma(r+s)\Gamma\left(r + \sum_{t=1}^{n_i} \lambda_{it}\right)\Gamma\left(s + \sum_{t=1}^{n_i} y_{it}\right)}{\Gamma(r)\Gamma(s)\Gamma\left(r + s + \sum_{t=1}^{n_i} \lambda_{it} + \sum_{t=1}^{n_i} y_{it}\right)} \prod_{t=1}^{n_i} \frac{\Gamma(\lambda_{it} + y_{it})}{\Gamma(\lambda_{it})\Gamma(y_{it} + 1)}$$

for $\mathrm{X}_i = (\mathrm{x}_i1, \ldots, \mathrm{x}_{ini})$ and where f is the probability density function for δ_i. The Y_{yt} is the count of the tth observation in the ith group and Γ is the gamma function.

The resulting log likelihood is:

$$\ln L = \sum_{i=1}^{n} \omega_i \left[\ln\Gamma(r+s) + \ln\Gamma\left(r + \sum_{k=1}^{n_i} \lambda_{ik}\right) + \ln\Gamma\left(s + \sum_{k=1}^{n_i} y_{ik}\right) - \ln\Gamma(r) - \ln\Gamma(s) \right.$$
$$- \ln\Gamma\left(r + s + \sum_{k=1}^{n_i} \lambda_{ik} + \sum_{k=1}^{n_i} y_{ik}\right)$$
$$\left. + \sum_{t=1}^{n_i} \{\ln\Gamma(\lambda_{it} + y_{it}) - \ln\Gamma(\lambda_{it}) - \ln\Gamma(y_{it} + 1)\} \right]$$

where $\lambda_{it} = \exp(\mathrm{x}_{it}\beta + \mathrm{offset}_{it})$ and ω_i is the weight for the ith group. Four models were examined; i) output of products, ii) balance or duodenal availability of nutrients, iii) intake of nutrients, or iv) percentage of nutrients in the diet. Statistical significance was accepted if $P < 0.05$. Diets (n = 3) that resulted in 0% or 100% pregnancy (Lucy et al., 1991, Lucy et al., 1992, Markus et al., 1996) created instability in the models and were removed from analyses. Collinearity among variables was explored for all models developed using the "Collin" function in Stata that provides the variance inflation index and condition number. Collinearity as indicated by a condition index > 50 (data not provided) for starting models for multivariable analysis for CPM estimated metabolizable AA and duodenal fatty acids was present and model coefficients were unstable during backward stepping procedures. If more than one strongly collinear variable qualified for entry into a model, one variable was chosen based on biological significance, rather than solely on *P*-values. Plausible quadratic relationships for variables were evaluated. Because of collinearity observed among CPM predicted fatty acid intakes that had individual positive

associations with the proportion pregnant, these were combined (for those individual fatty acids that had $P < 0.20$) into "positive fatty acids intake" (including C14:0, C16:0, C16:1, C18:0, C18:1t, C18:1c, and other fatty acids intake) and "positive fatty acids duodenal availability" (including C14:0, C16:0, C16:1, C18:0, C18:1c, other fatty acids duodenal, Table 3). These variables were tested in intake and balance or duodenal availability multivariable models, respectively. Similarly, CPM predicted metabolizable AA were combined and assessed as a single variable as these were also collinear. Model fit during development of the final model was evaluated using the Wald statistic. No final models had a condition index > 10 and mean variance inflation factors were < 4 (data not shown).

Dietary or production variables were used as predictors to explore their associations with calving to pregnancy interval. For those analyses, the difference in interval to pregnancy between a given treatment and the reference treatment within an experiment was the response of interest. Random effects standardized mean difference (**SMD**), also called effect size (**ES**) meta-analysis (METAN), was used following the method of DerSimonian and Laird (1986) as outlined by Rodney et al. (2015 – Chapter 3). If the experiment reported separate estimates of the measure of variance (SE or SD) for each treatment, these were recorded as such. Many experiments reported a common SE or SD and these estimates were used for the multiple treatments. In experiments in which SE was reported, a SD was derived before analysis. Some experiments reported only exact *P*-values and these were used to estimate SD, or in experiments in which non-significance was indicated without an exact *P*-value, $P = 0.50$ was used to determine the related SD. To explore sources of heterogeneity of response arising from diet, for each dietary variable the differences between a treatment and the reference treatment within an experiment were calculated and a random effects meta-regression analysis (METAREG) (Higgins and Thompson, 2002) was used to screen individual variables. For fat, starch or energy, and mineral interventions, the reference treatment was defined as the diet containing less of the ingredient of interest, or the most commonly used variety of the ingredient, based on the *a priori* hypothesis that increasing fat, starch or energy, or mineral intake would reduce calving to pregnancy interval. Alternatively, increasing the amount of CP or degradability of protein in the diet has negative effects on fertility (Lean et al., 2012). Consequently, the reference treatment for experiments using protein comparisons was classified as that with the most CP or RDP inclusion. The individual relative risk was used for each diet as the outcome and the associated SE was used as the measure of variance. There were a smaller number of comparisons available for inclusion in this analysis as a result of the fewer diets identified reporting calving to pregnancy interval and the unit of

interest being the difference between a treatment and the reference treatment for each comparison. As a result, a multivariable analysis was not conducted for calving to pregnancy interval. A random-effects weighted mean difference (**WMD**) between the treatment group and reference group is provided for the interval to pregnancy with the weighting reflecting the inverse of the variance of the treatments included according to the *nostandard* method in the metan model of Stata to allow an interpretation of treatment effects in familiar units (d), rather than ES. A P-value of <0.05 was accepted as being significant and P values >0.05 and <0.1 were identified as trends.

RESULTS

Literature Review and Assessment

The detailed systematic review identified more than 60,000 results across the three databases. If a reason for exclusion could be clearly identified in the title of a paper, the experiment was excluded during the screening phase. Such exclusions included experiments not in English, experiments that used *Bos taurus indicus*, crossbred cows or nulliparous non-lactating cows, experiments unrelated to cattle or transition nutrition, abstracts, and reviews. Some experiments contained a single treatment whereas others reported two or more treatments which were assessed separately. Of the 334 experiments that remained for eligibility, the main reasons treatments were excluded from the meta-analysis were that they were not completely randomized experiments with a single treatment assigned per cow (ie. were reviews, case studies, Latin-square or cross-over designs) that assessed nutrition and reproduction during the transition period (65 experiments); included changes in nutritional intervention during the transition period that could not be adequately quantified from the data reported, including those that used an injectable intervention (160 experiments); used *Bos taurus indicus*, crossbred, beef, or only nulliparous cows (36 experiments); or did not report the reproductive variables specified for inclusion (e.g. ovulation or reproductive hormone concentrations) or unit of interest was the oocyte or conceptus (34 experiments). Some of the treatments excluded after assessment for eligibility examined valid interventions, but the experiment contained a lack of detail about the diet or feed intake was not accurately measured (e.g. pasture or group feeding), making the diets unsuitable for extraction and inclusion in this study.

The systematic review identified 39 experiments containing 118 individual treatments that were suitable for inclusion in this analysis. Of these, 35 experiments with 109 treatments reported

proportion of pregnant cows and 23 experiments containing 42 comparisons reported calving to pregnancy interval. The treatments examined a range of dietary interventions with varying inclusion rates and timings of intervention. Few experiments provided details on prepartum diets, and hence the meta-analysis focused on postpartum, early lactation diets. A further three experiments (Colazo et al., 2009, Nowak et al., 2013, Badiei et al., 2014) provided details of pre-calving diets only and were not included in this analysis. If an experiment reported multiple pre-calving treatments and a common post-calving diet, the post-calving treatment was used for the multiple groups, but these were included in the analysis as separate treatments. Descriptive statistics of dietary components and production outputs is provided in Table 2.

Reproduction Outcomes

The systematic review identified 35 experiments, reporting 109 treatments that provided details of the proportion pregnant. The mean proportion pregnant was 0.44 (SD 0.16). Because of the complex nature of, and likely collinearity between components of the diets, dietary variables and production outputs were classified as reporting the i) output of nutrient including production, ii) balance or duodenal availability of nutrient, iii) intake amount, or iv) percentage of nutrient in the diet, and these were explored as four separate models. Univariable analyses of the associations between each variable and fertility outcomes are detailed in Table 3 and multivariable models including the random effect of experiment for factors affecting the proportion pregnant are described in Table 4.

Proportion Pregnant to AI

i) Outputs. In the univariable model, milk fat yield (0.996 ±0.002 kg/d) was negatively associated (P = 0.009) and milk fat percentage (0.86 ± 0.071) and milk protein yield (0.996 ± 0.002, kg/d) tended to be negatively associated with pregnancy to AI (P = 0.066). Although milk production (kg/d) and milk protein percentage were not associated ($P \geq 0.15$) with pregnancy to AI, they were both included in the multivariable models. There was a tendency for urea cost (MJ/d) to be positively associated (1.03 ± 0.017, P = 0.082) with pregnancy to AI (Table 3). The multivariable model, accounting for the random effect of experiment, resulted in a negative association of milk protein yield (0.922 ± 0.022 100g/d, P = 0.001) with pregnancy to AI (Table 4).

ii) Nutrient balance and duodenal availability. The nutrient balance and duodenal availability model assessed the associations of CPM estimated ME balance (MJ/d), MP balance (g/d), and duodenal availabilities of several individual fatty acids (C14:0, C16:0, C16:1, C18:0, C18:1c,

and C other g/d; Table 3) with pregnancy to AI. The negative association for CPM estimated metabolizable EAA availabilities (g/d) with pregnancy to AI were also considered in the development of the multivariable model. When examined in the multivariable model, CPM estimated ME balance (1.004 ± 0.002 MJ/d) and duodenal C14:0 (1.008 ± 0.004 g/d) availability remained in the model, both having positive relationships with pregnancy to AI (P = 0.017 and 0.037, respectively, Table 4). The combined positive association between duodenal fatty acid availability (g/d) was also considered for inclusion in this model, but resulted in poorer model fit than the final model that included only C14:0 (g/d).

iii) Intake. The intake model identified positive associations between CPM estimated individual fatty acid intakes (C14:0, C16:0, C16:1, C18:0, C18:1t, C18:1c, and C other intake (g/d)) or starch intake (kg/d) with pregnancy to AI (Table 3). The CPM estimated neutral detergent fiber (0.82 ± 0.071 kg/d), soluble fiber (0.81 ± 0.054 kg/d) and sugar intakes (0.79 ± 0.053 kg/d) were negatively associated with pregnancy to AI (P = 0.022, 0.002 and 0.001, respectively, Table 3). The multivariable model included combined positive fatty acids intake (g/d), starch intake (kg/d), and sugar intake (kg/d). Fatty acid intake (1.0003 ± 0.0001 g/d) and starch intakes (1.061 ± 0.029 kg/d) were positively associated with pregnancy to AI (P = 0.05 and 0.029, respectively), whereas increasing sugar intake (0.813 ± 0.54 kg/d) was associated with decreased pregnancy to AI (P = 0.002, Table 4).

iv) Diet Percentage. In the univariable model, positive associations of the CPM estimated dietary percentage of ether extract, long chain fatty acids, starch, and P, and negative associations of the dietary percentage of physically effective NDF and sugar were identified with pregnancy tor AI (Table 3). Sugar percentage alone remained in the multivariable model, having a negative association with pregnancy to AI (0.960 ± 0.015, P = 0.01, Table 4).

Table 3. Univariable effects of production and dietary factors affecting pregnancy to AI and calving to pregnancy interval. Factors associated with proportion pregnant to service were examined using a negative binomial regression while factors associated with calving to pregnancy interval were assessed using a random effects standardized mean difference (SMD) (effect size) meta-analysis and meta-regression. Differences between a treatment and the reference treatment within experiment for each production and dietary variable were calculated for use in the meta-regression

Item (values on DM basis)	Pregnancy to AI			Calving to pregnancy interval		
	Relative Risk (SE)	95% CI	P-value	Coefficient (SE)	95% CI	P-value
Milk production (kg/d)	0.99 (0.006)	0.981 to 1.004	0.199	0.07 (0.024)	0.024 to 0.121	0.004
Milk protein (%)	0.75 (0.148)	0.513 to 1.108	0.150	0.59 (0.485)	-0.393 to 1.568	0.233
Milk protein yield (100g/d)	0.996 (0.002)	0.992 to 1.0002	0.066	0.05 (0.026)	-0.006 to 0.097	0.082
Milk fat (%)	0.86 (0.071)	0.730 to 1.010	0.066	-0.27 (0.233)	-0.742 to 0.198	0.249
Milk fat yield (kg/d)	0.996 (0.002)	0.993 to 0. 999	0.009	0.14 (0.137)	-0.140 to 0.412	0.325
Dry matter intake (kg/d)	1.00 (0.014)	0.975 to 1.029	0.909	0.09 (0.075)	-0.067 to 0.237	0.266
Metabolizable energy balance (MJ/d)	1.01 (0.001)	1.002 to 1.008	0.001	-0.01 (0.005)	-0.021 to -0.002	0.020
Metabolizable protein balance (g/d)	1.00 (0.0001)	0.999994 to 1.001	0.055	0.0001 (0.0002)	-0.0003 to 0.001	0.614
Crude protein (%)	0.99 (0.015)	0.962 to 1.019	0.507	0.04 (0.022)	-0.004 to 0.084	0.077
Crude protein intake (kg/d)	0.98 (0.050)	0.888 to 1.086	0.727	0.23 (0.115)	-0.006 to 0.459	0.056
Rumen undegradable protein (% CP)	1.01 (0.007)	0.998 to 1.023	0.115	0.002 (0.007)	-0.011 to 0.015	0.783
Rumen undegradable protein intake (kg/d)	1.078 (0.109)	0.882 to 1.312	0.470	0.15 (0.130)	-0.113 to 0.415	0.254
Rumen degradable protein (% CP)	0.99 (0.006)	0.977 to 1.002	0.114	-0.002 (0.007)	-0.015 to 0.011	0.781
Rumen degradable protein intake (kg/d)	0.92 (0.070)	0.796 to 1.070	0.289	0.16 (0.142)	-0.122 to 0.450	0.254
Soluble protein (% CP)	0.998 (0.006)	0.986 to 1.009	0.707	-0.003 (0.006)	-0.014 to 0.009	0.646
Soluble protein intake (kg/d)	0.96 (0.084)	0.811 to 1.142	0.657	0.01 (0.143)	-0.276 to 0.304	0.923
Urea cost (MJ/d)	1.03 (0.017)	0.996 to 1.061	0.082	-0.002 (0.035)	-0.073 to 0.069	0.953
Methionine intake (g/d)	0.995 (0.005)	0.985 to 1.006	0.357	0.02 (0.010)	-0.004 to 0.037	0.105
Lysine intake (g/d)	0.999 (0.002)	0.996 to 1.002	0.482	0.01 (0.003)	-0.001 to 0.012	0.082
Arginine intake (g/d)	0.9998 (0.002)	0.997 to 1.003	0.903	0.004 (0.002)	-0.001 to 0.008	0.109
Threonine intake (g/d)	0.998 (0.002)	0.994 to 1.003	0.508	0.01 (0.005)	-0.0004 to 0.019	0.061
Leucine intake (g/d)	0.999 (0.001)	0.997 to 1.001	0.424	0.003 (0.002)	-0.001 to 0.007	0.138
Isoleucine intake (g/d)	0.999 (0.002)	0.994 to 1.003	0.570	0.01 (0.004)	-0.003 to 0.014	0.162
Valine intake (g/d)	0.999 (0.002)	0.996 to 1.003	0.781	0.01 (0.003)	-0.002 to 0.011	0.155
Histidine intake (g/d)	0.998 (0.004)	0.990 to 1.005	0.564	0.01 (0.006)	-0.003 to 0.023	0.114
Phenylalanine intake (g/d)	0.999 (0.002)	0.995 to 1.003	0.576	0.01 (0.003)	-0.001 to 0.012	0.116
Tryptophan intake (g/d)	0.99 (0.006)	0.980 to 1.004	0.186	0.01 (0.012)	-0.010 to 0.038	0.254
Total essential amino acids intake (g/d)	0.9998 (0.0002)	0.9994 to 1.0003	0.536	-0.003 (0.004)	-0.001 to 0.001	0.448
Ether extract (%)	1.05 (0.025)	1.001 to 1.097	0.047	-0.02 (0.047)	-0.110 to 0.078	0.734
Long-chain fatty acids (%)	1.05 (0.026)	1.002 to 1.103	0.039	-0.02 (0.051)	-0.124 to 0.082	0.678
C12:0 intake (g/d)	0.998 (0.006)	0.986 to 1.009	0.687	-0.02 (0.047)	-0.110 to 0.081	0.760
C14:0 intake (g/d)	1.02 (0.005)	1.007 to 1.026	0.001	0.01 (0.013)	-0.020 to 0.034	0.593
C16:0 intake (g/d)	1.00 (0.0003)	0.9999 to 1.001	0.116	-0.0003 (0.001)	-0.002 to 0.001	0.645
C16:1 intake (g/d)	1.01 (0.004)	1.005 to 1.023	0.001	0.01 (0.014)	-0.018 to 0.038	0.490
C18:0 intake (g/d)	1.00 (0.001)	1.001 to 1.003	0.001	-0.001 (0.001)	-0.003 to 0.001	0.367
C18:1t intake (g/d)	1.01 (0.005)	1.001 to 1.021	0.024	0.0003 (0.021)	-0.042 to 0.042	0.987
C18:1c intake (g/d)	1.00 (0.0004)	1.0004 to 1.002	0.003	0.00003 (0.001)	-0.002 to 0.002	0.972
C18:2 intake (g/d)	1.00 (0.0002)	0.9996 to 1.00049	0.933	0.0001 (0.001)	-0.001 to 0.002	0.850
C18:3 intake (g/d)	0.9999 (0.00048)	0.999 to 1.001	0.788	-0.002 (0.005)	-0.012 to 0.009	0.764
C other intake (g/d)	1.01 (0.003)	1.003 to 1.014	0.004	-0.001 (0.004)	-0.009 to 0.007	0.767

Table 3 (Continued). Univariable effects of production and dietary factors affecting pregnancy to AI and calving to pregnancy interval. Factors associated with proportion pregnant to service were examined using a negative binomial regression while factors associated with calving to pregnancy interval were assessed using a random effects standardized mean difference (SMD) (effect size) meta-analysis and meta-regression. Differences between a treatment and the reference treatment within experiment for each production and dietary variable were calculated for use in the meta-regression

Item (values on DM basis)	Pregnancy to AI			Calving to pregnancy interval		
	Relative Risk (SE)	95% CI	P-value	Coefficient (SE)	95% CI	P-value
C12:0 duodenal availability (g/d)	0.998 (0.006)	0.986 to 1.009	0.688	-0.01 (0.047)	-0.109 to 0.083	0.789
C14:0 duodenal availability (g/d)	1.02 (0.005)	1.007 to 1.026	0.001	0.01 (0.013)	-0.020 to 0.034	0.593
C16:0 duodenal availability (g/d)	1.00 (0.0004)	0.9999 to 1.001	0.102	-0.0003 (0.001)	-0.002 to 0.001	0.659
C16:1 duodenal availability (g/d)	1.08 (0.038)	1.011 to 1.158	0.023	-0.05 (0.121)	-0.292 to 0.196	0.693
C18:0 duodenal availability (g/d)	1.00 (0.0002)	1.000003 to 1.001	0.048	-0.0003 (0.001)	-0.001 to 0.001	0.642
C18:1t duodenal availability (g/d)	1.00 (0.001)	0.999 to 1.002	0.601	0.001 (0.002)	-0.003 to 0.005	0.610
C18:1c duodenal availability (g/d)	1.00 (0.001)	1.00004 to 1.004	0.045	-0.00001 (0.002)	-0.004 to 0.004	0.995
C18:2 duodenal availability (g/d)	1.00 (0.002)	0.997 to 1.005	0.511	-0.0004 (0.002)	-0.005 to 0.004	0.834
C18:3 duodenal availability (g/d)	1.00 (0.009)	0.986 to 1.020	0.763	-0.004 (0.016)	-0.037 to 0.028	0.780
c other duodenal availability (g/d)	1.01 (0.004)	0.999 to 1.015	0.087	-0.003 (0.005)	-0.014 to 0.008	0.606
Sugar (%)	0.95 (0.013)	0.929 to 0.981	0.001	-0.01 (0.064)	-0.136 to 0.123	0.919
Starch (%)	1.02 (0.005)	1.006 to 1.025	0.001	-0.01 (0.011)	-0.032 to 0.012	0.358
NDF (%)	0.99 (0.009)	0.974 to 1.011	0.400	0.01 (0.020)	-0.029 to 0.052	0.555
Physically effective NDF (%)	0.97 (0.011)	0.945 to 0.989	0.003	-0.03 (0.036)	-0.097 to 0.047	0.487
Non-fibrous carbohydrate (%)	1.01 (0.008)	0.992 to 1.022	0.399	-0.03 (0.015)	-0.055 to 0.006	0.113
Total fermentable carbohydrate intake (kg/d)	0.97 (0.032)	0.907 to 1.032	0.311	-0.07 (0.097)	-0.267 to 0.124	0.464
NDF intake (kg/d)	0.82 (0.071)	0.694 to 0.972	0.022	0.21 (0.227)	-0.245 to 0.672	0.353
Starch intake (kg/d)	1.09 (0.034)	1.023 to 1.155	0.007	-0.05 (0.071)	-0.194 to 0.091	0.469
Soluble fiber intake (kg/d)	0.81 (0.054)	0.715 to 0.927	0.002	-0.06 (0.235)	-0.532 to 0.418	0.809
Sugar (kg/d)	0.79 (0.053)	0.689 to 0.896	0.000	-0.07 (0.310)	-0.698 to 0.555	0.818
Calcium (%)	1.16 (0.161)	0.881 to 1.519	0.294	-0.50 (0.514)	-1.535 to 0.541	0.339
Phosphorous (%)	1.96 (0.577)	1.099 to 3.487	0.023	2.10 (1.185)	-0.295 to 4.495	0.084
Magnesium (%)	1.09 (0.649)	0.338 to 3.505	0.887	3.27 (2.449)	-1.678 to 8.221	0.189

Table 4. Multivariable negative binomial regression models assessing associations between dietary components or production factors and pregnancy to AI. Models accounted for the random effect of experiment

Model	Covariable	Relative Risk (SE)	95% CI	*P*-value
Outputs	Milk protein yield (100 g/d)	0.992 (0.022)	0.881 to 0.965	0.001
Balance and duodenal availability	Metabolizable energy balance (MJ/d)	1.004 (0.002)	1.001 to 1.008	0.017
	C14:0 duodenal (g/d)	1.008 (0.004)	1.0005 to 1.015	0.037
Intake	Fatty acid intake (g/d)	1.0003 (0.0001)	1.000 to 1.001	0.050
	Starch (kg/d)	1.061 (0.029)	1.006 to 1.118	0.029
	Sugar (kg/d)	0.813 (0.054)	0.713 to 0.927	0.002
Diet percentage	Sugar (% DM)	0.960 (0.015)	0.930 to 0.990	0.010

Calving to Pregnancy Interval. Twenty three experiments containing 42 comparisons reported calving to pregnancy interval. The meta-analysis indicated that interventions designed to improve reproductive performance during the transition period tended to numerically reduce the calving to pregnancy interval (SMD = -0.05; WMD = 0.71 d less 95% CI = -3.40 to 1.97d), and this result was homogeneous (I^2 = 0.0%) (Figure 1). As expected from previous meta-analyses (Lean et al., 2012, Rodney et al., 2015 – Chapter 3), diets that increased the amount of fat (WMD = 1.64 less d 95% CI = -5.28 to 2.01d) or decreased the amount or degradability of protein in the diet (WMD 7.41 d less 95% CI = -13.18 to – 1.63) were associated with a shorter calving to pregnancy interval (Figure 1), although this was only a tendency for the diets reporting interventions using fats. Increasing dietary starch or energy density tended to increase calving to pregnancy interval, although there were only three diets examining the latter intervention and the confidence interval was large (95% CI -0.14 to 0.70; Figure 1). There were mixed associations with mineral based interventions increasing the calving to pregnancy interval (WMD = 6.77 d increased 95% CI = 0.82 to 12.54), reflecting varying responses between and within the same experiment. When examined using meta-regression, increased CPM estimated difference in ME balance (MJ/d) between the treatment and reference treatments was associated with shorter calving to pregnancy interval (-0.01 ± 0.005, P = 0.02) whereas increased difference in milk production (kg/d) was associated with longer calving to pregnancy interval (0.07 ± 0.024, P = 0.004) (Table 3). Increased differences between the treatment and reference treatments in CP percentage (0.04 ± 0.022), CP intake (0.23 ± 0.115 kg/d), P percentage (2.10 ± 1.185), and milk protein yield (0.05 ± 0.026 100g/d) tended to be associated (P < 0.09) with longer calving to pregnancy interval (Table 3). Increased CPM estimated metabolizable AA intake, particularly of lysine (0.01 ± 0.003 g/d) and threonine (0.01 ± 0.005 g/d), tended to be associated with increased calving to pregnancy interval (P = 0.082 and 0.061, respectively) as did the difference in Mg (3.27 ± 2.449, P = 0.189) percentage, while the differences in non-fibrous carbohydrate (-0.03 ± 0.015, P = 0.113) tended to be associated with decreased interval (Table 3).

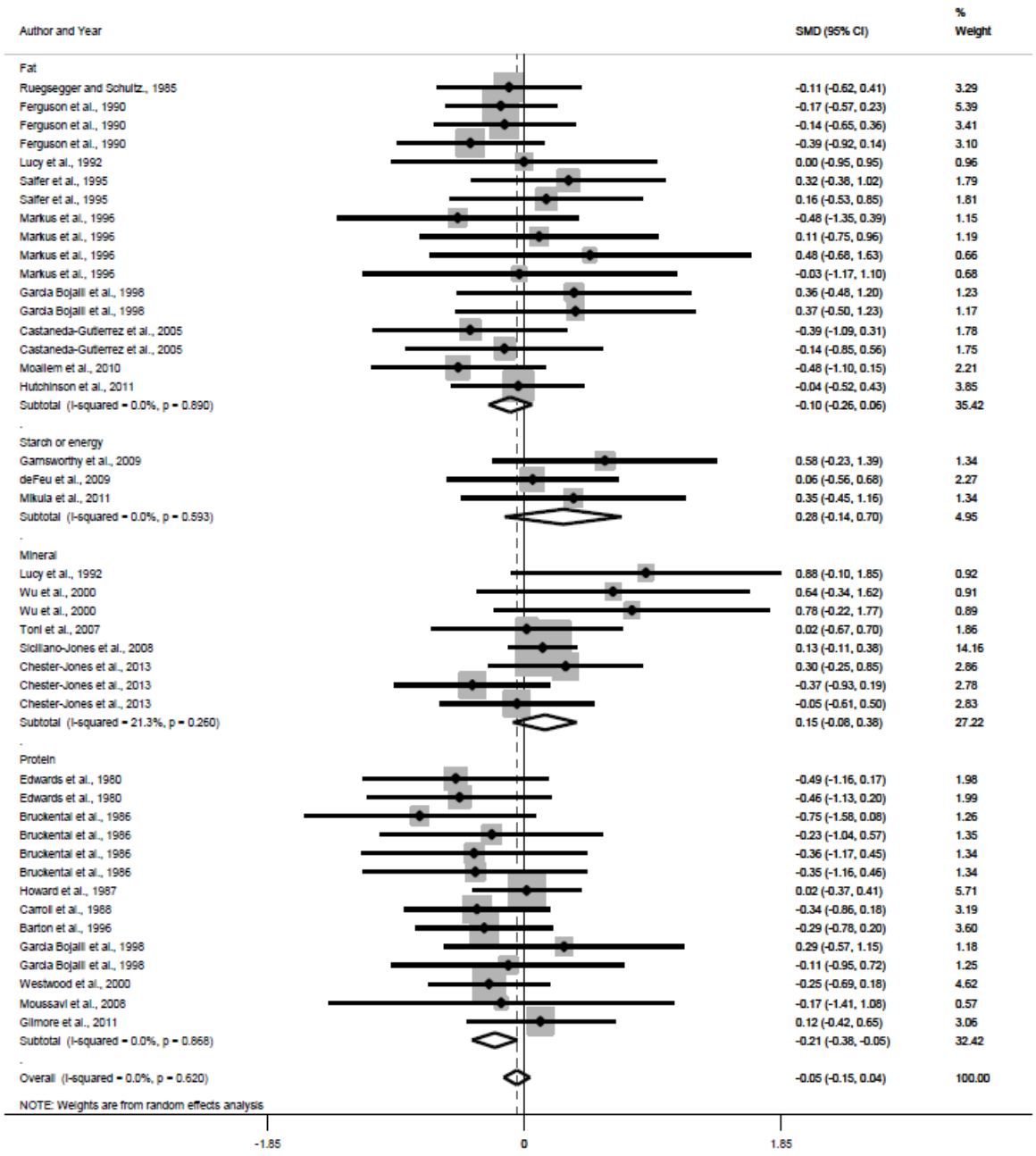

Figure 1. Forest plot of individual standardized mean difference (SMD), 95% CI and weights for comparisons of calving to pregnancy interval of cows fed different nutritional interventions during the early lactation period (Weighted mean differences are provided in results). For fat, starch or energy, and mineral comparisons, the reference treatment was the one with less of the ingredient of interest. For protein-based comparisons, the reference treatment was the one with more CP or RDP. Estimates were made of the SMD using a random effects method (DerSimonian and Laird, 1986). The weights that each comparison contributed are in the right-hand column and are indicated by the size of the box. The larger the box, the greater the contribution of that comparison to the overall estimate. The solid vertical grey line represents a mean difference of zero or no effect. Points to the left of the line represent a reduction in days to pregnancy, whereas points to the right of the line indicate an increase. The upper and lower limit of the line connected to the square represents the upper and lower 95% CI for the effect size. The overall pooled effects size and 95% CI is indicated by the diamonds at the bottom of each fat group. The overall effect was highly homogeneous, as indicated by the I^2 of 0.00%, indicating little error attributable to measurement error.

DISCUSSION

This study provides a quantitative evaluation of dietary factors and production outputs that are associated with the probability of pregnancy to service and time to pregnancy from 118 diets contained within 39 experiments. The dietary data were extracted rigorously according to previously described methods (Rabiee et al., 2012) using feed data from the experiments themselves and from the CPM-Dairy (v 3.08) feedbank and calibrating these according to tested data from the experiments. Notwithstanding the rigor applied to diet extraction, and the choice of the CPM model as it is the most extensively tested (Bateman et al., 2001, Moate et al., 2004, Moate et al., 2006, Tedeschi et al., 2008), limitations in the estimates of dietary components exist. These include limitations or errors of the model, variations in composition and timing of the diets fed, and feed analysis estimations. It is anticipated that these errors are likely to be non-differential, that is neither favoring nor supporting the key hypotheses. Under these conditions, errors in estimation are likely to drive hypotheses towards the null and, therefore, findings in this study are likely to be robust. Although interventions in the prepartum and early lactation period can have long lasting effects on metabolism and fertility, the proximity of dietary changes, for example of protein, to the time of conception/pregnancy can also be crucial. This study focuses on nutritional interventions fed during the early post-partum period, but it is worth noting that the majority of the diets were fed not just in this early lactation but also into the breeding period. The choice of statistical models used was determined by the use of count data, that is, the proportion of cows pregnant. The decision was made to use a negative binomial, random-effects model based on the clustering of treatments within experiments, and over-dispersion of the data (Neuhaus et al., 1991). The term over-dispersion reflects an evaluation that the variance estimated is larger than the expected count (Rabe-Hesketh and Skrondal, 2008).

There is extensive literature that has observed that cows with greater milk production generally have poorer fertility (Spalding et al., 1975, Lean et al., 1989, Butler, 2000, Westwood et al., 2002, Pryce et al., 2004), and that genetic selection for increased production can reduce fertility (Hageman et al., 1991, Buckley et al., 2000, Horan et al., 2005, Pollott and Coffey, 2008). While genetic differences were not examined in this data set, associations between increased milk fat (kg/d) and protein production (kg/d) with reductions in the proportion pregnant, and actual milk yield (kg/d) and milk protein yield (100g/d) with longer calving to pregnancy interval (Table 3) were identified. Milk protein yield (100g/d) remained in the final model, having a greater significance in than in the univariable model. The multivariable model accounted for the random

effect of experiment, which was an important factor influencing relationships in proportion pregnant, being estimated as contributing approximately 30% of the variance in this outcome However, protein yield in very early lactation (first 3 weeks of lactation) was positively associated with proportion of first services that resulted in pregnancy (Rodney et al., 2016 – Chapter 4), and others identified positive associations between milk protein percentage and improved reproductive performance (Buckley et al., 2003, Madouasse et al., 2010, Morton et al., 2016). These findings highlight possible differences between experiments conducted at the level of the individual and those at the group level. As milk protein production increases in a group of cows, it may be expected that nutrients intakes will need to be more closely aligned with nutrient losses, whereas the individual within the herd with greater production may have better phenotypic adaptation to the environment allowing greater milk protein yield and percentage.

The availability of nutrients that can be allocated to reproduction is not just determined by immediate diet, that is intake of nutrients, but also by endogenous body tissue reserves, reflected in BW and BCS (Lean and Rabiee, 2006). Hence, DMI is a key determinant of exogenous nutrient availability. The irreversible loss of nutrients in milk production and use of nutrients for maintenance and growth diminishes the nutrient pool available for reproduction (Baldwin et al., 1987, Friggens et al., 2013). The difference between dietary intake and such expenditure determine the nutrient balance, and if a negative balance occurs endogenous reserves are depleted. Many studies have examined the effects of estimated negative energy balance (**NEB**) on fertility (Wathes et al., 2007). The length and severity of a NEB at the onset of lactation is largely determined by DMI around calving (Villa-Godoy et al., 1988) and milk yield (Westwood et al., 2002). Estimated energy balance (MJ/d) was, as anticipated, positively associated with improved proportion pregnant and shorter calving to pregnancy interval (Tables 2 and 3). A better energy balance during the first 3 to 4 weeks of lactation reduces the interval to first ovulation and increases the probability of pregnancy at the following breeding (Butler and Smith, 1989). Excessively low or high BCS at calving, or extreme losses of BW or BCS in early lactation, are usually associated with impaired reproductive outcomes (Heuer et al., 1999, Pryce et al., 2001, Buckley et al., 2003, Lopez-Gatius et al., 2003). Body weight and BCS change are often used as proxies for energy balance, although they may more correctly be interpreted as a reflection of nutrient balances of protein, minerals, specific fatty acids and vitamins, as well as energy.

Although less well explored than ME balance, MP balance is also important for successful reproduction. In this study, improved early lactation MP balance tended (P = 0.055) to increase the proportion pregnant (Table 3). Rodney et al. (2016 – Chapter 4) found that increased prepartum MP balance slightly decreased the proportion of first services that resulted in pregnancy, highlighting the difference in pre- and postpartum metabolism. Notably, the prepartum MP balance (Rodney et al., 2016 – Chapter 4) was markedly positive, exceeding 400 g/d on average. In contrast, Van Saun et al. (1993) found greater milk protein percentage and a trend to fewer services per conception in cows fed more RUP before calving. Those cows did not gain weight, suggesting a lesser MP balance than those of Rodney et al. (2016 – Chapter 4). Increasing CP intake may increase nutrient loss via increased milk production, and have negative effects on fertility. Increasing CP content of the diet does not necessarily increase MP availability, but decreasing the degradability of protein, or increasing the fermentability of the diet may be more effective in increasing MP availability.

Alterations in dietary protein may not simply affect MP balance, but also specific AA composition and supply of metabolizable AA. Specific roles for AA in reproductive performance are not well defined. Lysine and methionine have been suggested to be the most co-limiting AA for production and supplementation of these may increase milk yield, but results are inconsistent (Rogers et al., 1989, Doepel et al., 2004, Ardalan et al., 2010). Supplementation of lactating cows with rumen protected methionine and/or lysine has had positive or negligible effects on reproductive outcomes (Rogers et al., 1989, Ardalan et al., 2010). The current study focused on diets in the early lactation period, whereas these other studies (Rogers et al., 1989, Ardalan et al., 2010) supplemented cows later in lactation (from 4 weeks). It is notable that the combined EAA (g/d) reduced the proportion of cows pregnant in a univariable model when the effect of experiment was considered (data not shown). Limitations in these data, including a lack of specifically detailed AA profiles of the feeds, may have in part, prevented stronger relationships being observed. Given the importance of protein and the need to increase understandings of roles of specific AA, this should be an area for further investigation.

The tendency for increased protein intake, dietary percentage, or degradability to be associated with reduced calving to pregnancy interval (Figure 1) is consistent with other studies (Folman et al., 1981, Carroll et al., 1994a, Butler, 2000, Westwood et al., 2002, Lean et al., 2012). Butler (1998) reviewed the means by which feeding excess protein can be detrimental. The tendency (P = 0.084) for CPM estimated urea cost (MJ/d) to be positively associated with the proportion of

cows pregnant was surprising, and a similarly positive relationship between estimated urea costs and fertility was identified in a meta-analysis examining the effects of feeding fats on fertility (Rodney et al., 2015 – Chapter 3). This relationship may indicate interactions between dietary components, in particular between microbial protein production and rumen available fats, rather than simply the effect of protein content of the diet *per se*. There is a need to review optimal levels, composition and quality of proteins and nitrogen in diets containing additional fat. Further studies must also consider the way protein is measured as well as the contribution of microbial protein to meeting protein requirements.

Microbial lipolysis and biohydrogenation in the rumen ensure that intake of fatty acids and those available for absorption in the duodenum differ, and hence were explored separately. Fats not only provide an energy source, but also are essential precursors for steroid hormones and the beneficial effects of fat have been observed independently of the provision of energy (Staples et al., 1998). There was a strong correlation between individual fatty acids and substantial instability identified, as indicated by marked changes in co-efficients, in the development of multivariable models when stepwise removal of individual fatty acids was conducted. In the current study, intakes of many of the fats (C14:0, C16:1, C18:0, C18:1t, C18:1c and C other (g/d)) were univariably associated with an increased proportion of cows pregnant (Table 3). These were combined into a single variable for the multivariable intake analysis to account for the collinearity among the fats. Strong correlation and collinearity among individual fatty acids was also indentified in the balance and duodenal availability model and while a positive association between duodenal myristic acid (C14:0) (g/d) and proportion pregnant was identified, this finding may indicate a positive effect of fats on pregnancy, in general, rather than a specific effect of myristic acid intake *per se*. The positive effects of feeding fats on reproductive outcomes, both proportion pregnant and calving to pregnancy interval (Tables 2 and 3), observed in this study are consistent with a previous meta-analysis (Rodney et al., 2015 – Chapter 3) that found feeding fats improved (27% increase in relative risk) the proportion of cows pregnant and tended to decrease calving to pregnancy interval. In particular, positive effects of CLA on fertility have been identified (De Veth et al., 2009) however the limited number of diets that fed CLA in this data set and outputs from CPM-Dairy precluded individual investigation, and further examination of this area may be beneficial.

Carbohydrates are important sources of energy for cows, as well as for rumen microorganisms and generally increase the efficiency of protein utilization and microbial protein production

(Hoover and Stokes, 1991, Aldrich et al., 1993, Hristov et al., 2005). However, increased concentrations of rapidly fermentable carbohydrates can increase the risk of acidosis (Plaizier et al., 2008). Type of carbohydrate also influences the risk of acidosis, with sugars posing a greater risk than starches (Golder et al., 2012). Consequently, there could be positive and negative effects of carbohydrates on fertility. The positive associations of starch percentage and intake (kg/d), possibly because of a generally slower fermentation rate than sugars, and negative associations of soluble fiber and sugar percentage and intake (kg/d) with proportion pregnant were identified in univariable analyses (Tables 2 and 3). There was also a negative association between increased CPM estimated peNDF intake (kg/d) and proportion pregnant in the univariable analysis. The only variable that remained in the multivariable models was the effect of sugar intake (kg/d), negatively associated with the proportion of cows pregnant. Ruminal acidosis may decrease fertility by reducing feed intake, producing a metabolic acidosis leading to detrimental alteration of uterine environment, stimulating inflammation that induces prostaglandin release, and resulting in luteolysis in a process analogous to that of mastitis (Moore et al., 1991, Zebeli et al., 2015).

The positive univariable association between P percentage and the proportion of cows pregnant is consistent with studies in beef cattle that found improved fertility outcomes with increased dietary P concentration (Hart and Michell, 1965). However, studies in dairy cattle have not identified an influence of dietary P on reproduction (Carstairs et al., 1980, Wu and Satter, 2000, Wu et al., 2000), possibly because of the body's ability to buffer P from skeletal stores or because a smaller study power did not allow a rigorous assessment. Ferris et al. (2010) examined cattle over 4 lactations in a study designed to examine long term depletion of P stores, and found numerical, yet non-statistical improvements in several fertility parameters, although this work lacked the study power to examine these robustly.

CONCLUSIONS

This study highlights several important findings for future research on the effects of transition nutrition on fertility. It confirms that nutritional management of cows during the transition period can have substantial effects on reproductive success and this finding is consistent with previous meta-analytical studies in this area. The number of well described diets that were able to be included in the analysis was limited, and less than expected. This lack of large numbers of suitable treatments and the complexity of responses of the cattle to these treatments strongly

suggest that there is a crucial need for further focused field experiments exploring the roles of nutrition on reproduction and interactions among dietary components.

Overall, this study confirmed earlier findings that excessive protein intake can impair fertility, but that a positive MP balance is consistent with better fertility. However, there may be a need to increase protein intake when feeding fats and other work suggests a need to control the MP balance before calving. The role of specific metabolizable AA needs further study. The study confirms also the positive effects of feeding fats, and highlights a need for detailed studies in this area. We also, critically, identified potential effects of specific carbohydrate fractions, especially sugar (kg/d), starch (kg/d) and peNDF (kg/d) on reproductive outcomes. The importance of positive energy and protein balances in early lactation for improved fertility outcomes was supported.

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CHAPTER THREE: EFFECTS OF DIETARY FAT ON FERTILITY OF DAIRY CATTLE: A META-ANALYSIS AND META-REGRESSION

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OVERVIEW OF CHAPTER THREE

Complementing the meta-analysis presented in Chapter 2 which focused on the transition diet as a whole, this Chapter presents a systemic review and meta-analysis of the effects of dietary fat interventions in the early post-partum period on reproductive outcomes. It considers different fat interventions, and aims to identify if particular sources of fats and fatty acids (as estimated using CPM-Dairy) had more substantial effects on the proportion pregnant and or calving to pregnancy interval of dairy cows when fed in the early lactation period.

ABSTRACT

There is increasing evidence of positive effects of feeding fats during transition on fertility and the adaptation to lactation. This study utilized meta-analytic methods to explore the effects of including fats in the transition diet on the risk of pregnancy to service ('proportion pregnant') and calving to pregnancy interval. Meta-analysis was used to integrate smaller studies, and increase the statistical power over that of any single study and explore new hypotheses. We explored the effect of fats and diet composition on fertility using meta-regression methods.

There were relatively few highly controlled studies providing detailed descriptions of the diets used that examined interactions between fat nutrition and reproductive outcomes. Only 17 studies containing 26 comparisons were suitable for inclusion in statistical evaluations. Reproductive variables evaluated were risk of pregnancy 'proportion pregnant', primarily to first service, and calving to pregnancy interval. Production variables examined were milk yield, milk composition, and body weight. The sources of heterogeneity in these studies were also explored. A 27% overall increase in pregnancy to service was observed (RR = 1.27; 95% Confidence interval Knapp Hartung 1.09 to 1.45) and results were relatively consistent (I^2 = 19.9%). A strong indication of a reduction in calving to pregnancy interval was also identified, which was consistent across studies (I^2 = 0.0%) supporting a conclusion that overall, the inclusion of fats does improve fertility. Further exploration of the factors contributing to proportion pregnant using bivariate meta-regression identified variables that reflected changes in diet composition or animal response resulting from inclusion of the fat interventions in the experimental diets fed. Increased fermentable neutral detergent fiber and soluble fiber intakes increased the proportion pregnant while increased milk yield of the treatment group decreased this measure. Unexpectedly, the estimated energy costs of urea production also had a positive association with proportion pregnant. The limited number of suitable studies for the analysis highlights the need for more work to improve understanding of the critical nutritional factors affecting fertility. These factors include specific fatty acids in dietary interventions that contribute to increasing fertility of cows in dairy production systems.

Keywords: dietary fat, fertility, conjugated linoleic acid

INTRODUCTION

Managing fertility of lactating dairy cattle is a challenge for dairy producers as poor fertility reduces productivity and profit. Declines in fertility have been noted and reflect associations with intensification of production and higher levels of milk production (Butler, 2000, Lucy, 2001, Lean et al., 2008, Thatcher et al., 2011). Studies are needed to identify which environmental factors, especially nutritional ones, may have a role in influencing the fertility of cattle.

The transition period, from approximately three weeks before to three weeks after calving, is characterized by changes in metabolism as dairy cattle respond to the metabolic challenges of late pregnancy and early lactation (Bell, 1995). Good management during the transition period, in particular nutritional strategies, can reduce the effects of this metabolic stress and improve production and reproduction (De Veth et al., 2009). DeGaris et al. (2010a, b) found that the risk of pregnancy increased by approximately 30% in cattle exposed to transition diets for 20 days compared to cattle not exposed.

Recent understandings of the role of fats in metabolism open new opportunities for improving metabolism, health and reproduction in cattle. Inclusion of fats in the diet during this transition period has improved reproductive performance (Thatcher et al., 2006, De Veth et al., 2009), improved energy balance (Von Soosten et al., 2012), reduced the incidence of metabolic diseases, and allowed energy density to be maintained in diets without increasing the use of rapidly fermentable carbohydrates.

The strength of meta-analytic methods is the ability to integrate smaller studies using effect size metrics, enhance the statistical power over that of any single study and provide the potential to explore new hypotheses (Lean et al., 2009). Further, there is a challenge in studies of nutrition and reproduction, specifically, that when a nutritional intervention is applied something else in the diet necessarily changes (Lean et al., 2012). Therefore, there is a need to consider the potential for confounding influences in interpreting studies of nutrition and reproduction. Meta-regression methods allow this type of investigation. This study was designed to utilize meta-analytic and meta-regression methods to explore the effects of including fats in the diet during the transition period on measures of pregnancy, calving to pregnancy interval, and milk yield and components and the factors that may explain sources of variation in these responses.

MATERIALS AND METHODS

Literature Search

A systematic review, across three databases (PubMed, Web of Science CABI and Google Scholar) and references in papers, was used to identify studies exploring fat nutrition during transition and fertility that were published in English between 1970 and 2014 in a peer-reviewed journal, conference proceedings or as an accepted thesis. Combinations of the following search terms were used; cow, cattle, dairy, fertility, pregnancy, reproduction, pregnancy, fat, CLA, conjugated linoleic acid, cottonseed, linoleic acid, linolenic acid, omega -6, omega-3, and energy.

Inclusion and Exclusion Criteria

Papers were deemed suitable for inclusion in the study if they were randomized controlled experiments using *Bos taurus* dairy cows in their first or later lactation during the dry/lactating period (i.e. primiparous non-lactating heifers were excluded). Studies evaluated the effect of feeding during the transition period, including the period 3 weeks before and after calving, and included sufficient dietary details for the diet to be evaluated using CPM-Dairy (version 3.08; Cornell-Penn-Miner, http://cahpwww.vet.upenn.edu/node/77) for cows that were fed as individuals or in appropriately replicated pens. Papers that had valid interventions, but did not provide adequate dietary detail or animals were group fed and not replicated were identified, but diets were not extracted. Measures of fertility were reported as i) first service conception or pregnancy to a defined number of services (‘proportion pregnant’); and/or ii) calving to pregnancy interval and a measure of dispersion suitable to provide a standard deviation. Studies also were assessed for quality of study design including details of randomization, appropriate analysis and elimination of bias or confounding. The number of cows in each treatment and control group and measures of variance or *P*-values for continuous variables that allowed an estimate of standard deviation to be derived must have been reported.

Data and Diet Extraction

Data extracted included authors, year, journal and type of publication, title of paper, feeding system, number of cows in treatment and control groups, parity, body weight, and body condition score for each group. Reproductive variables that were recorded were defined as proportion of cows pregnant to service (reported in the papers as first service pregnancy percentage or conception rate, pregnancy percentage (‘rate’) to first two services, or pregnancy)

Table 1. Summary of papers included in meta-analysis and meta-regression

		Dietary intervention			Results for control, treatment	
Study	No. of cows	Control	Treatment	Duration of treatment	Proportion pregnant to service (%)	Calving to pregnancy interval/days open (d)
Ambrose et al., 2006	121	Sunflower seed (0.1%, ALA[1])	Flaxseed (56.7% ALA)	28 d prepartum to 32 d postinsemination	32, 48	—
Boken et al., 2005	18	Control	Soybean oil refining product (approximately 2%)	0 d to 14 wk postpartum	22, 0	—
Castaneda-Gutierrez et al., 2005	32	Calcium salts of palm FA distillate (approximately 1%)	CLA isomers (approximately 0.2%) + calcium salts of palm FA distillate (approximately 0.9%)	2 wk prepartum to 9 wk postpartum	43, 75	—
	32	Calcium salts of palm FA distillate (approximately 1%)	CLA isomers (approximately 0.3%) + calcium salts of palm FA distillate (approximately 0.8%)	2 wk prepartum to 9 wk postpartum	43, 50	—
Ferguson et al., 1990	96	Control	Prilled FA (3%)	1 to 150 d postpartum	33, 57	105, 99
	61	Control	Prilled FA (6%)	1 to 150 d postpartum	38, 63	93, 88
	57	Control	Prilled FA (9%)	1 to 150 d postpartum	52, 67	95, 82
Garcia Bojalil et al., 1998	24	Control; fed low protein	Calcium salts of long-chain FA (2.2%); fed low protein	1 to 120 d postpartum	27, 45	80, 83
	21	Control; fed high protein	Calcium salts of long-chain FA (2.2%); fed high protein	1 to 120 d postpartum	40, 45	73, 86
Holter et al., 1992	67	Control	Linted whole cottonseed (15%)	0 to 112 d postpartum	33, 47	107, 96
	73	Control	Linted whole cottonseed and Megalac (approximately 3%)	0 to 112 d postpartum	33, 42	107, 123
Hutchinson et al., 2011	72	Calcium salts of palm FA (approximately 0.3%)	Lipid-encapsulated CLA (approximately 0.4%)	0 to 60 d postpartum	39, 52	111, 109
Lucy et al., 1991	18	Control	Ca FA (2.2%)	7 to 60 d postpartum	33, 67	—
Markus et al., 1996	33	Control	Tallow (2.7%)	~23 d postpartum for 16 wk	82, 75	—
	33	Control	Whole sunflower seeds (7.1%)	~23 d postpartum for 16 wk	82, 81	—
Moallem et al., 2010	42	Calcium salts of FA (approximately 0.2%)	Encapsulated lipid supplement (approximately 0.2%)	21 to 100 d postpartum	35, 35	88, 82
Petit and Twagiramungu., 2006	83	Whole flaxseed (10.6%)	Megalac (3.9%)	6 wk prepartum to 120 d prepartum, except where cows were diagnosed pregnant diet was fed for 50 d of gestation	44, 56	—
	89	Whole flaxseed (10.6%)	Micronized soybeans (18.05%)	6 wk prepartum to 120 d prepartum, except where cows were diagnosed pregnant diet was fed for 50 d of gestation	44, 40	—
Ruegsegger and Schultz, 1985	58	Soybean meal (20%)	Heat-treated whole soybean (25%)	10 to 115 d postpartum	—	115, 109
Salfer et al., 1995	32	Control	Partially hydrogenated tallow 1% prepartum and 2% postpartum;	14 d prepartum to 151 d postpartum	—	88, 102
Sklan et al., 1989	108	Control	Calcium soaps of FA (approximately	0 to 170 d postpartum	28, 43	88, 74

Table 1 (Continued). Summary of papers included in meta-analysis and meta-regression

Study	No. of cows	Dietary intervention		Duration of treatment	Results for control, treatment	
		Control	Treatment		Proportion pregnant to service (%)	Calving to pregnancy interval/days open (d)
Sklan et al., 1994	66	Control; multiparous	Calcium salts of FA (25%); multiparous	41, 33	—	
	56	Control; primiparous	Calcium salts of FA (25%); primiparous	74, 33	—	
Son et al., 1996	34	Control; high supplementary escape protein	Tallow (3%); high supplementary escape protein	2 to 12 wk postpartum	27, 67*	—
	34	Control; low supplementary escape protein	Tallow (3%); low supplementary escape protein	2 to 12 wk postpartum	40, 24	—
Son et al., 2000	25	Control	Calcium salts of FA (3%)	1 to 98 DIM	36, 36	—

[1]ALA = alpha-linolenic acid.
*Difference between groups ($P \leq 0.05$).

and calving to pregnancy intervals (also reported as calving to conception interval or days open) for each treatment. Milk production (kg/cow/d), milk fat percentage and yield (kg/cow/d) and milk protein percentage and yield (kg/cow/d) were also recorded. Data were extracted and entered into a spreadsheet (Excel, Microsoft Corp., Redmond, WA). A summary of studies included is provided in Table 1.

To extract and model dietary information, data from accepted papers were entered into CPM-Dairy (version 3.08; Cornell-Penn-Miner, http://cahpwww.vet.upenn.edu/node/77) following the standard operating procedure described in Rabiee et al. (2012). Ration ingredients and intake in the papers were entered into CPM-Dairy using ingredients selected from the feed bank (http://cahpwww.vet.upenn.edu/node/83) and edited to the specifications described in the paper. This was combined with information on cows, housing and environment from the paper to predict diet composition. If there was uncertainty in regard to the unit of interest or measures of dispersion reported in papers, authors were contacted to provide clarification of these measures.

Statistical Analysis

All statistical analyses were conducted using Stata (v 13 Intercooled Stata v.13, USA). The influence of fat nutrition during transition on production and reproductive performance was analyzed using

meta-analysis. Trials were grouped by type of fat intervention (oilseeds, calcium salts of fatty acids (**CSFA**), tallow, CLA or other) and meta-analyses were conducted for each group and overall. Meta-analyses were conducted to examine the effects of fat intervention on risk of pregnancy to service ('proportion pregnant to service'), primarily reported to first service; and days from calving to pregnancy; and milk yield and composition (milk protein yield, milk protein percentage, milk fat yield and milk fat percentage). Further meta-analyses, of reproductive variables only, were conducted that also included data from the papers identified as having valid intervention but lacking adequate detail. Dicotomous data were analyzed by using relative risk (**RR**) and continuous data by standardized mean difference (**SMD**) which is also called effect size **(ES**) analysis. The RR estimates were pooled using methods for random effects models to evaluate the effect of trial, and with the Hartung-Knapp-Sidik-Jonkman (Knapp-Hartung) method (IntHout et al., 2014). The use of this method for meta-analysis is more robust than alternative methods such as the DerSimonian and Laird method for discrete data, especially where there is heterogeneity (IntHout et al., 2014). As described by IntHout et al. (2014), the DerSimonian and Laird method uses the normal distribution to derive *P*-values and confidence intervals, while Knapp-Hartung method uses the t-distribution with k-1 degrees of freedom where k is the number of studies in the meta-analysis. IntHout et al. (2014) describes the estimated variance of $\hat{y}_\tau$ using the DerSimonian and Laird method as

$$var_{DL} = \frac{1}{\sum w_i^\tau}$$

while the Knapp-Hartung method estimated the variance is

$$var_{KH} = \frac{\sum w_i^\tau (y_i - \hat{y}_\tau)^2}{(k-1)\sum w_i^\tau}$$

where k is the number of studies, y_i is the effect size estimate from the i^{th} study, w_i is the fixed effect weight, and τ^2 is the heterogeneity of the effect size between studies. If the paper reported separate estimates of measures of variance (SE or SD) for each group, these were recorded as such. Many studies reported a common SE or SD and these estimates were used for both control and treatment groups. Where SE was reported, a SD was derived before analysis. Some studies reported exact P values which were used to estimate SD.

Random effects models (DerSimonian and Laird, 1986) were used to evaluate production outcomes (milk yield, milk protein yield, milk protein percentage, milk fat yield and milk fat percentage) and body weight, estimating the SMD, 95% confidence intervals, and statistical significance of SMD. Where there was only one comparison in a group, that group was not

reported individually and only included in the overall pooled result. The approximate predictive interval (Harris et al., 2008) for the treatment effect was also explored, but as this was very large in some cases, reflecting small numbers of studies in some groups, it was not included in forest plots. We recognize that there is a clustering effect that results from multiple comparisons to a single control group within a study. We have determined that the variance inflation effect resulting from high intra-class correlations from clustering will be minor unless there are very large numbers of repeated comparisons. The statistical methods for the meta-analytic procedures that were used in this paper have been based on those published by one of the authors of this study (Lean et al., 2009).

Forest Plots

The effects of treatments on proportion pregnant to service, calving to pregnancy interval and milk yield are displayed in the forest plots, using the estimated RR or SMD. The weighting of a study is estimated by the inverse of the variance of the effect size. Boxes draw attention to the studies with the greatest weight.

Assessment of Heterogeneity

Variations among the trial level RR or SMD were assessed using a chi-squared (Q) test of heterogeneity. Heterogeneity in studies reflects underlying differences in clinical diversity of the herds and treatments used, differences in study design and analytical methods, and statistical variation around responses. Identifying the presence and sources of the heterogeneity improves understanding of the responses to treatments. We used an α level of 0.10 because of the relatively poor power of the χ^2 test to detect heterogeneity among small numbers of trials. Heterogeneity of results among the trials was quantified using the I^2 statistic (Higgins and Thompson, 2002), who developed this measure of the impact of heterogeneity on a meta-analysis, from mathematical criteria, that are independent of the number of studies and the treatment effect metric. I^2 is a transformation of the square root of the χ^2 heterogeneity statistic divided by its degrees of freedom and describes the proportion of total variation in study estimates that is due to heterogeneity. Negative values of I^2 were assigned a value of zero, consequently the value I^2 lies between 0 and 100%. An I^2 value greater than 50% indicates moderate heterogeneity (Higgins et al., 2003).

Publication Bias

We investigated the presence of publication bias using funnel plots which are a scatter plot of the intervention effect estimates from individual studies plotted against study precision. The name 'funnel plot' arises because precision of the estimated intervention effect increases as the size and precision of a study increases. Effect estimates from small studies will scatter more widely at the bottom of the graph and the spread narrows for larger studies. In the absence of bias, the plot should approximately resemble a symmetrical (inverted) funnel. If there is bias, for example because smaller studies without statistically significant effects remain unpublished, this will lead to an asymmetrical appearance of the funnel plot and a gap will be evident in a bottom corner of the graph (Duval and Tweedie, 2000). In this situation, the effect calculated in a meta-analysis will tend to overestimate the intervention effect.

Mean Differences

A weighted mean by group was calculated for dietary variables to identify differences between treatment and control groups that may possibly cause confounding. Using the values calculated by modeling in CPM, the difference between treatment and control in each comparison was averaged across each fat type. A positive value indicates that the treatment group provides a greater value than the control group. "Other" fats were not explored as all comparisons in this group were from the same study. Lean et al. (2012) noted that in nutritional trials where there is an addition to the diet, there is inevitably a part of the diet that is replaced. This may unintentionally add or reduce other nutritive components other than the variable of interest (eg, a change in protein content) that could affect the outcome being measured. By examining these differences, potential sources of confounding can be identified.

Meta-regression

Meta-regression analyses were used to explore sources of heterogeneity of response arising from diet for reproductive outcomes, using the individual RR for each trial as the outcome and the associated standard error as the measure of variance. The differences between treatment and control groups for each variable were calculated and a random effects meta-regression analysis (Higgins and Thompson, 2002) was used to screen individual variables using a *P*-value of $\leq$ 0.20. A bivariate model, including the effect of fat group, was conducted to assess dietary factors that influenced the proportion pregnant. All variables with *P*-value of $\leq$ 0.20 in the bivariate meta-regression were further tested in a mixed model, including fat group, using a forward stepping meta-regression with explanatory variables with the lowest *P*-value entering the model

first. Minerals were explored separately to other factors. Model fit during development of the final model was evaluated using I^2, tau^2 and R^2 where I^2 describes the percentage of total variation across studies that is due to heterogeneity (Higgins et al., 2003), tau^2 is the variance of the standard deviation of the distribution of true effects across studies (Borenstein et al., 2011) and R^2 is the ratio of explained variance to total variance, or the proportion of variance explained by that covariate (Borenstein et al., 2011). The assessment of model fit using I^2, tau^2 and R^2 was conducted according to methods described by Harbord and Higgins (2008). Due to the low number of trials identified for calving to pregnancy interval, a multivariate analysis was not conducted.

RESULTS

Literature Review and Assessment

The detailed systematic review identified more than 5,000 papers. All papers were critically reviewed against the selection criteria. Some studies contained a single comparison whereas others reported two or more comparisons, which were assessed separately. Where a reason for exclusion could be clearly identified in the title of a paper, the study was excluded during the screening phase. Such exclusions included papers not in English, studies that used *Bos Indicus* or crossbred cows or primiparous non-lactating heifers, studies unrelated to cattle or fats, and reviews. Of the papers that remained for eligibility (n = 67), the main reasons studies were excluded from the meta-analysis were that they were not randomized controlled trials (ie. were reviews, case studies, Latin-square or cross-over designs) (6 papers); included changes in nutritional intervention during the feeding period that could not be adequately quantified (30 papers); or reproductive variables measured were not those specified for inclusion (e.g. ovulation or reproductive hormone concentrations) or unit of interest was the oocyte or conceptus (14 papers).

Many of the studies excluded after assessment for eligibility examined valid interventions but contained a lack of detail about the diet or feed intake was not accurately measured (e.g. pasture, ad libitum or group feeding), making the diets unsuitable for extraction. These interventions are still valid, although not able to be included in the analysis, and are detailed in Table 2.

After assessment, 17 studies containing 26 comparisons were found suitable for inclusion in the meta-analysis. A range of different fat sources were identified for this analysis and papers were classified by fat type; Oilseeds (n = 6), CSFA (n = 9), Tallow (n = 4), CLA (n = 4) and Other (n = 3). The Other group was comprised solely of comparisons of prilled fatty acids obtained from a single paper. Consequently, there is a limited ability to draw conclusions from this group. However, these data have been included in the overall pooled estimates. A summary of these comparisons is available in Table 1.

Table 2. Summary of papers containing valid interventions that were found unsuitable for extraction due to the dietary details provided or measured

Item	No. of cows	Dietary intervention		Duration of treatment	Reason for exclusion from meta-regression
		Control	Treatment		
Baldi et al., 2000	28	Corn (0.5 kg/d)	Calcium soaps (0.2 kg/d)	14 d prepartum to 7 DIM	Group fed
Colazo et al., 2009	48	Canola (8% DM)	Linola (8% DM)	34 to 0 d prepartum	No control group
	48	Canola (8% DM)	Flax (8% DM)		No control group
Dirandeh et al., 2013	40	Linseed (4.03% up to first heat > 40d postpartum, then 3.04%)	Palm oil (1.41% up to first heat >40 d postpartum, then 0.53%)	0 to 120 DIM	Diet not suitable for extraction
Grossi et al., 2013	17	Palm Oil	n-3 FA (fish oil; 75 g of FA/d)	21 to 1 d prepartum	Fish product
Hutchinson et al., 2012	369	Control	Lipid encapsulated CLA	0 to 60 DIM	No DMI
Juchem et al., 2010	344	Calcium salts (2%) DM palm oil	Calcium salts (2%) DM linoleic and *trans*-octadecenoic acids	25 d prepartum to 80 DIM	Group fed
Mandebvu et al., 2003	40	Calcium salts of long-chain FA from palm FA distillate (1.7%)	Calcium salts of long-chain FA from soybean oil (1.7%)	0 to 45 DIM fresh diet then up to 10 weeks in high-producing group	Group fed
McNamara et al., 2003	134	Control	Megalac plus 3%	10 to 103 DIM	Group fed
	134	Control	Megalac gold		Group fed
Moallem et al., 1997		Control	Calcium soaps of FA (0.5 kg/d)	0 to 150 DIM	Group fed
Petit and Benchaar, 2007	61	Micronized soybean	Whole flaxseed	6 wk prepartum to 50 d pregnancy for pregnant cows or 120 DIM for those not diagnosed as pregnant	Pooled results for different diets
	56	Micronized soybean	Megalac		Pooled results for different diets
Scott et al., 1995	220	Control	Rumen-inert fat (0.45 kg/d)	0 to 200 DIM	Group fed
Sklan et al., 1991	99	Control	Calcium soaps of FA (2.6% DM)	0 to 120 DIM	Group fed

Mean Differences

Mean differences between the nutritional composition of control and treatment diets are displayed by fat type in Table 3. There is variation between treatments but many of the differences are small. The difference in ME intake between treatment and control groups varies from -4.06 MJ/d for the tallow treatment vs. control to 0.3 MJ/d for CLA vs. control. The CLA studies were the only group with a lower average ME balance in the treatment group. The greatest

Table 3. Weighted means of the difference between treatment and control dietary variables by fat type[1]

	Fat type							
	Oilseed		Calcium salts of FA		Tallow		CLA	
Dietary variable	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
DMI (kg/cow per d)	0.15	0.06	−0.22	0.05	−0.72	0.10	−0.63	0.03
Estimated ME balance (MJ/cow/d)	−1.32	0.44	−1.21	0.51	−4.06	1.47	0.30	0.08
Estimated MP balance (g/cow per d)	−18.41	17.99	−118.71	6.90	−146.87	13.28	−59.20	5.09
Bacterial MP (% of MP intake)	−0.46	0.29	−0.57	0.07	0.58	0.18	0.32	0.01
CP (% of diet)	0.03	0.08	−0.18	0.01	−0.18	0.06	0.00	(omitted)
CP eaten (kg/cow per d)	0.07	0.02	−0.08	0.01	−0.17	0.02	−0.11	0.01
RUP (% of CP)	0.70	0.39	0.44	0.10	−0.97	0.23	−0.26	0.00
RUP eaten (kg/cow per d)	0.06	0.02	−0.01	0.01	−0.11	0.01	−0.05	0.00
RDP (% of CP)	−0.71	0.39	−0.44	0.10	0.94	0.23	0.26	0.00
RDP eaten (kg/cow per d)	0.01	0.01	−0.07	0.01	−0.06	0.02	−0.06	0.00
Soluble protein (% of CP)	1.05	0.34	−0.44	0.06	1.34	0.16	0.00	(omitted)
Soluble protein eaten (kg/cow per d)	0.04	0.01	−0.04	0.00	−0.01	0.01	−0.04	0.00
Urea cost (MJ/cow per d)	0.17	0.03	−0.24	0.03	−0.50	0.08	−0.01	0.00
Predicted PUN (mg/dL)	0.58	0.19	−1.37	0.07	−1.27	0.13	−0.59	0.05
Long-chain FA (% of diet)	0.75	0.08	1.55	0.06	2.03	0.03	0.10	0.01
Ether extract (% of diet)	1.08	0.07	1.40	0.07	2.36	0.04	0.06	0.01
NDF (% of diet)	0.82	0.13	0.38	0.16	2.77	0.39	−0.04	0.01
NFC (% of diet)	−1.73	0.15	−1.28	0.05	−5.00	0.45	−0.02	0.00
Sugar (% of diet)	−0.27	0.06	0.04	0.03	0.25	0.03	0.00	(omitted)
Starch (% of diet)	−1.20	0.16	−1.55	0.07	−5.91	0.63	−0.13	0.02
Peptides (% of requirement)	8.64	0.97	−0.33	0.59	5.28	1.17	0.77	0.04
Peptides and ammonia (% of requirement)	−0.23	1.01	2.00	0.17	3.49	0.65	0.00	(omitted)
Methionine (% of requirement)	−0.68	0.69	−6.41	0.34	−5.57	0.44	−2.32	0.19
Lysine (% of requirement)	−5.31	0.86	−6.57	0.29	−3.31	0.30	−2.32	0.19
C12:0 intake (g/cow per d)	0.56	0.04	0.80	0.03	0.54	0.06	−0.31	0.02
C14:0 intake (g/cow per d)	1.23	0.40	6.64	0.18	10.18	0.74	−0.10	0.08
C16:0 intake (g/cow per d)	16.47	4.47	218.34	3.87	107.33	6.79	−11.47	1.98
C16:1 intake (g/cow per d)	2.62	0.34	−0.33	0.04	13.04	1.00	−0.19	0.02
C18:0 intake (g/cow per d)	8.71	1.95	11.99	0.49	76.43	4.83	−1.64	0.11
C18:1*trans* intake (g/cow per d)	−2.99	0.43	−0.01	0.00	13.55	1.40	−0.21	0.03
C18:1*cis* intake (g/cow per d)	74.15	4.51	132.54	1.11	189.25	4.09	−10.89	1.14
C18:2 intake (g/cow per d)	−134.09	15.60	−3.64	3.52	19.26	14.97	−3.65	1.04
C18:3 intake (g/cow per d)	200.33	13.65	−83.72	11.33	6.47	0.68	−2.08	0.11
Other FA intake (g/cow per d)	0.53	0.60	0.71	0.18	7.87	0.86	27.42	2.74
C12:0 duodenal (g/cow per d)	0.56	0.04	0.80	0.03	0.54	0.06	−0.31	0.02
C14:0 duodenal (g/cow per d)	1.23	0.40	6.64	0.18	10.18	0.74	−0.10	0.08
C16:0 duodenal (g/cow per d)	15.55	4.03	212.49	3.71	100.61	8.00	−10.11	1.78
C16:1 duodenal (g/cow per d)	0.01	0.02	−0.16	0.01	0.66	0.09	−0.17	0.01
C18:0 duodenal (g/cow per d)	60.48	5.48	−20.41	10. 12	197.48	3.91	−9.93	0.58
C18:1*trans* duodenal (g/cow per d)	16.48	3.25	−22.26	4.99	25.69	1.01	−1.89	0.18
C18:1*cis* duodenal (g/cow per d)	16.67	1.27	95.94	1.99	42.21	1.56	−6.43	0.85
C18:2 duodenal (g/cow per d)	14.85	1.86	7.57	0.84	−8.47	1.99	1.47	0.36
C18:3 duodenal (g/cow per d)	11.33	0.59	−3.98	0.59	0.46	0.10	−0.17	0.03
Other FA duodenal (g/cow per d)	−0.31	0.27	−0.57	0.06	1.37	0.60	20.76	2.23
Total intake of fermented carbohydrate (kg/cow per d)	−0.20	0.04	−0.34	0.02	−0.61	0.04	−0.23	0.01
Carbohydrate fermented NDF intake (kg/cow per d)	0.06	0.01	−0.00	0.01	0.14	0.03	−0.06	0.00
Carbohydrate fermented starch intake (kg/cow per d)	−0.17	0.03	−0.33	0.02	−0.90	0.07	−0.09	0.01
Carbohydrate fermented soluble fiber intake (kg/cow per d)	−0.04	0.01	−0.01	0.01	0.12	0.01	−0.04	0.00
Carbohydrate fermented sugar intake (kg/cow per d)	−0.05	0.01	−0.00	0.01	0.03	0.00	−0.03	0.00
Ca (% of DM)	−0.05	0.00	0.20	0.01	0.04	0.01	−0.01	0.00
P (% of DM)	−0.01	0.00	−0.02	0.00	−0.03	0.00	0.00	(omitted)
Mg (% of DM)	−0.00	0.00	−0.01	0.00	0.00	(omitted)	0.00	(omitted)

[1]A positive result indicates a greater value in the treatment over control.

differences in MP balance between treatment and control groups were the diets including tallow (-146.87g), which were lower than those of oilseed based treatments (-18.41g).

The difference in palmitic acid (C16:0) intake between control and treatment varied between fat types. The control diets for CLA based interventions provided on average, a palmitic acid intake 11.47g lower than treatment diets and the CSFA treatment diets provided an average of 218.34g more palmitic acid than controls. This intake difference was reflected in daily duodenal flux (-10.11g vs 212.49g for CLA and CSFA respectively). Similar patterns were observed in the intake of oleic acid (C18:1cis) where the difference between control and treatment was very different for the CLA group (4.09g more), whereas the tallow or CSFA treatments provided an intake of 189.25g and 132.54g less, respectively. The differences between the control and treated cows for duodenal availability of C18:1cis were estimated to be -6.43g, 42.21g and 95.94g for CLA, Tallow and CSFA respectively. Differences in linoleic acid (C18:2) and linolenic acid (C18:3) intake were also noted between fat types (Table 3).

Reproduction Outcomes

The pooled estimates show that increasing dietary fat during the transition period increased the risk of pregnancy (proportion pregnant to service) by 27% when predicted using the method described by Knapp Hartung (95% CI 1.09 to 1.45) (Knapp and Hartung, 2003) (Figure 1, Table 4). All groups tended to show a positive effect, but individually none (excluding Other fats) showed an individually significant benefit. Only two comparisons showed individual significance in increased risk of pregnancy to service (Son et al., 1996, 2000). The RR for Boken et al. (2005) was individually negative (RR = 0.29) however, as indicated by the small grey square in Figure 1, the weighting was small, reflecting the low number of cows in the trial (control n = 9, treatment n = 6). Overall, there was a moderately high level of consistency among trials (I^2 = 19.9%), and the funnel plot is symmetrical (Figure 2), suggesting little publication bias. Investigation of the papers not suitable for meta-regression showed no notable change in effect size or direction of reproductive measures. As there was considerable variability and confounding in many of the experimental designs, a switching of fats, unsuitable fats (singular fats or fish oil) or unsuitable outcome variables, it negated their value to contribute, and these were not reported in the final meta-analysis.

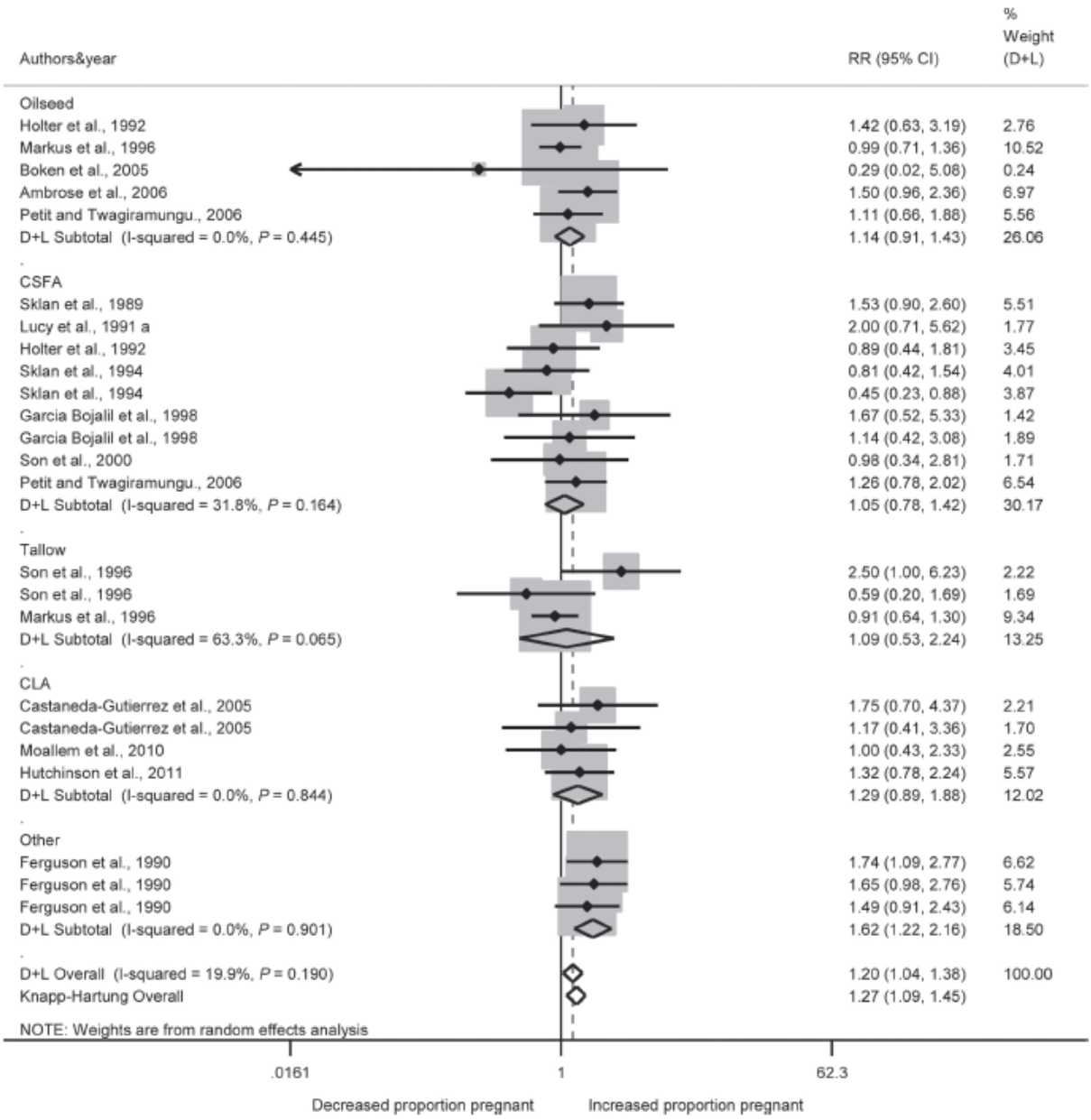

Figure 1. A forest plot of the studies that examined the relative risk of pregnancy to service (95% CI) for dietary fat interventions. Studies were grouped by fat type, with a pooled estimate calculated for each group. An overall pooled estimate was made of the SMD, in this case relative risk (RR), using a random effects model using the DerSimonian and Laird (D+L) method (DerSimonian and Laird, 1986) and Knapp Hartung prediction (IntHout et al., 2014). The weights that each study contributed are in the right-hand column and are indicated by the size of the box. The larger the box, the greater the study contribution to the overall estimate. The solid vertical gray line represents a mean difference of zero or no effect. Points to the left of the line represent a reduction in risk of pregnancy to service, whereas points to the right of the line indicate an increase. The upper and lower limit of the line connected to the square represents the upper and lower 95% confidence interval for the effect size. Where the interval crosses 1, shown by whiskers crossing the solid vertical line, the change is insignificant. The overall pooled effects size and 95% confidence interval is indicated by the diamond at the bottom. This effect was moderately homogeneous as indicated by the I^2 of 25.7%. D+L = DerSimonian and Laird.

The results of the bivariate meta-regression used to explore sources of heterogeneity of response arising from diet with the inclusion of fat groups are provided in Table 5. The proportion of cows pregnant increased with increasing intake of fermentable NDF and soluble fiber (kg/d) when assessed using a bivarite model accounting for fat group (P = 0.035 and 0.015 respectively). The estimated energetic cost of urea synthesis (MJ/d) (P = 0.022) was also positively associated with fertility. Increased actual milk yield (kg/d) for the treatment group decreased the proportion

pregnant (P = 0.036). These relationships were all significant and had the same point direction in univariate models. These factors, and those with P < 0.2 in the bivariate model (MP bal (g/d), NDF (%), starch (%), lysine (% rqd), C16:1 duodenal (g/d), fermentable sugar intake (g/d), and actual milk fat (%) (Table 5)) were assessed for inclusion in a multivariate model. However, no regression that combined more than two covariables with the effect of fat group resulted in significant covariables, apart from fat group.

Most studies indicated that increasing dietary fat during the transition period numerically decreased calving to pregnancy interval, but none were individually significant (Figure 3). Only 10 comparisons provided adequate data to be included in this meta-analysis, and as there was only one comparison for oilseeds, this reduces considerably the inference range for this group. Of the remaining groups, CLA had the greatest effect (SMD = -0.41), although this was not significant. Overall, there was a high level of consistency of response among trials (I^2 = 0.0%), but there appears to be some potential for publication bias in these data as the funnel plot is not symmetrical (Figure 4). The asymmetry may, however, reflect the limited number of studies. Only oleic acid (C18:1cis) intake and availability at the duodenum was associated with reduced calving to pregnancy interval, with a P-value < 0.2. Therefore no dietary measures were significantly associated with the calving to pregnancy interval (Table 6).

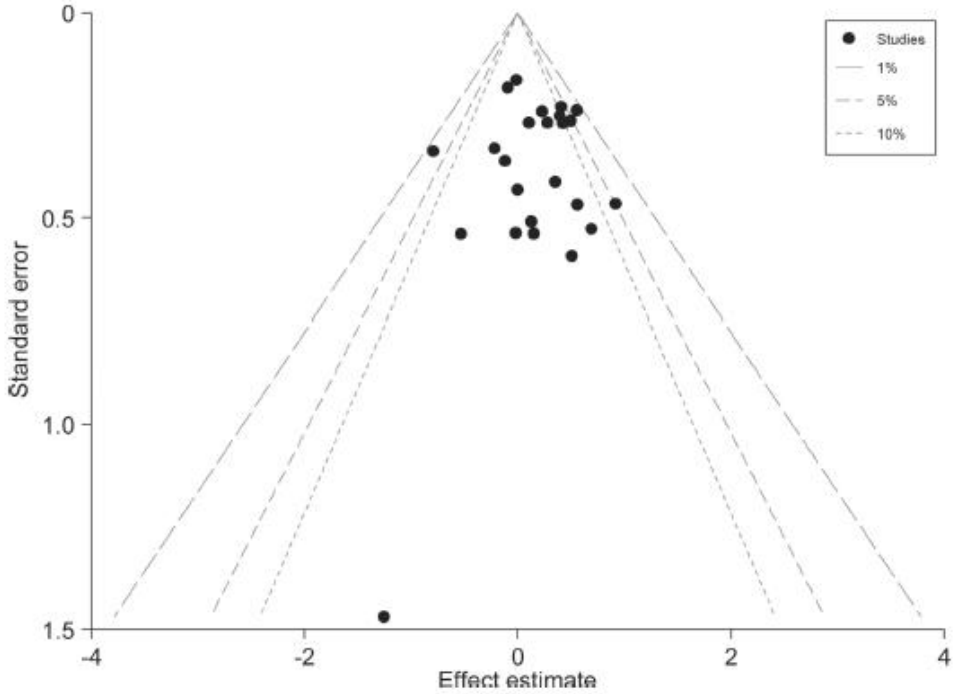

Figure 2. Contour-enhanced funnel plots for relative risk of pregnancy at first service for lactating dairy cows treated with different fats. Levels of significance for studies (●) within the gray broken lines are 0.01, 0.05, and 0.10.

Table 4. Effects of feeding fats on reproduction, milk yield and composition, and BW: meta-analysis outputs using DeSimonian and Laird random effects model unless specified[1]

Item	RR or SMD (95% CI)	I^2	*P*-value
Proportion pregnant to service[2]			
Overall	1.20 (1.04 to 1.38) 1.27 (1.09 to1.45) (Knapp-Hartung)	19.9	0.19
Oilseed	1.14 (0.91 to 1.43)	0.0	0.51
CSFA	1.05 (0.78 to 1.42)	31.8	0.16
Tallow	1.09 (0.53 to 2.24)	63.3	0.07
CLA	1.29 (0.89 to 1.88)	0.0	0.84
Calving to pregnancy interval			
Overall	−0.16 (−0.33 to 0.00)	0.0	0.82
Oilseed	—	—	—
CSFA	−0.04 (−0.28 to 0.36)	0.0	0.46
Tallow	—	—	—
CLA	−0.32 (−0.65 to 0.01)	0.0	0.88
Milk yield			
Overall	0.33(−0.1 to 0.67)	88.3	0.01
Oilseed	−0.10 (−0.97 to 0.77)	92.7	0.01
CSFA	0.73 (0.00 to 1.47)	92.1	0.01
Tallow	0.21 (−0.18 to 0.60)	0.00	0.90
CLA	0.52 (−0.35 to 1.39)	86.5	0.01
Protein %			
Overall	−0.14 (−0.38 to 010)	74.3	0.01
Oilseed	0.27 (−0.05 to 0.59)	41.6	0.16
CSFA	−0.26(−0.61 to 0.09)	58.9	0.02
Tallow	−0.25 (−0.65 to 0.14)	0.00	0.65
CLA	−0.45 (−0.87 to −0.03)	46.2	0.13
Protein yield			
Overall	0.34 (−0.07 to 0.75)	84.1	0.01
Oilseed	0.18 (−0.08 to 0.44)	0.0	0.79
CSFA	0.78 (−0.25 to 1.82)	92.5	0.01
Tallow	—	—	—
CLA	−0.11 (−0.41 to 0.19)	0.0	0.87
Fat yield			
Overall	0.04 (−0.39 to 0.47)	87.4	0.01
Oilseed	0.29 (0.03 to 0.55)	0.0	0.57
CSFA	0.64 (0.05 to 1.23)	85.1	0.01
Tallow	—	—	—
CLA	−1.00 (−1.55 to −0.44)	65.1	0.04
Fat %			
Overall	−0.03 (−0.32 to 0.26)	84.3	0.01
Oilseed	0.47 (−0.01 to 0.95)	76.9	0.01
CSFA	0.19 (−0.08 to 0.46)	47.0	0.07
Tallow	0.02 (−0.68 to 0.72)	67.3	0.05
CLA	−1.39 (−2.04 to −0.74)	71.6	0.01
BW			
Overall	−0.15 (−0.69 to 0.40)	90.1	0.01
Oilseed	−0.18 (−0.71 to 0.35)	81.2	0.01
CSFA	0.10 (−0.43 to 0.62)	64.7	0.06
Tallow	1.25 (−0.70 to 3.19)	92.0	0.01
CLA	—	—	—

[1] I^2 describes the percentage of total variation across studies that is due to heterogeneity (Higgins et al., 2003). CSFA = calcium salts of FA.

[2] Relative risk (RR) is reported, whereas standardized mean difference (SMD) is reported for categories not signified with an asterisk. These are standardized units and do not correspond to normal metrics.

Table 5. Bivariate meta-regression results controlling for the effect of fat group, for the effects of differences between treatment and control groups in dietary inputs on risk of proportion pregnant to service using Knapp Hartung (2003) methods

Dietary variable	Coefficient	SE	t	$P > \|t\|$	95% CI
DMI (kg/cow per d)	0.10	0.139	−0.72	0.48	−0.19 to 0.39
Estimated ME balance (MJ/cow/d)	0.01	0.014	−0.02	0.99	−0.03 to 0.03
Estimated MP balance (g/cow per d)	0.01	0.001	−1.51	0.15*	−0.01 to 0.01
Bacterial MP (% of MP intake)	0.12	0.102	−1.15	0.26	−0.10 to 0.33
CP (% of diet)	−0.04	0.206	0.19	0.85	−0.47 to 0.39
CP eaten (kg/cow per d)	0.36	0.638	−0.57	0.58*	−0.98 to 1.70
RUP (% of CP)	−0.06	0.070	0.88	0.39	−0.21 to 0.09
RUP eaten (kg/cow per d)	−0.39	1.172	0.33	0.74	−2.85 to 2.07
RDP (% of CP)	0.06	0.070	−0.89	0.39	−0.09 to 0.21
RDP eaten (kg/cow per d)	1.05	0.907	−1.16	0.26*	−0.86 to 2.96
Soluble protein (% of CP)	0.04	0.065	−0.62	0.55	−0.10 to 0.18
Soluble protein eaten (kg/cow per d)	1.52	1.338	−1.14	0.27	−1.29 to 4.33
Urea cost (MJ/cow/d)	0.53	0.209	−2.52	0.02*	0.09 to 0.97
Predicted PUN (mg/dl)	0.01	0.072	−0.01	0.99	−0.15 to 0.15
Long-chain FA (% of diet)	−0.09	0.109	0.84	0.41	−0.32 to 0.14
Ether extract (% of diet)	−0.08	0.100	0.79	0.44	−0.29 to 0.13
NDF (% of diet)	0.08	0.042	−1.77	0.09*	−0.01 to 0.16
NFC (% of diet)	−0.05	0.051	0.99	0.34*	−0.16 to 0.06
Sugar (% of diet)	0.29	0.262	−1.09	0.29	−0.27 to 0.83
Starch (% of diet)	−0.07	0.042	1.57	0.13*	−0.15 to 0.02
Peptides (% of requirement)	0.01	0.011	−0.53	0.60	−0.02 to 0.03
Peptides and ammonia (% of requirement)	−0.01	0.015	0.33	0.74	−0.04 to 0.03
Methionine (% of requirement)	0.02	0.020	−0.90	0.38	−0.02 to 0.06
Lysine (% of requirement)	0.03	0.018	−1.67	0.11*	−0.01 to 0.07
C12:0 intake (g/cow per d)	0.19	0.213	−0.89	0.39	−0.26 to 0.64
C14:0 intake (g/cow per d)	0.01	0.020	−0.16	0.87	−0.04 to 0.05
C16:0 intake (g/cow per d)	0.01	0.002	−0.41	0.69	−0.01 to 0.01
C16:1 intake (g/cow per d)	0.01	0.018	−0.23	0.82	−0.03 to 0.04
C18:0 intake (g/cow per d)	−0.01	0.003	0.03	0.98*	−0.01 to 0.01
C18:1*trans* intake (g/cow per d)	0.02	0.016	−0.94	0.36	−0.02 to 0.05
C18:1*cis* intake (g/cow per d)	−0.01	0.002	0.64	0.53*	−0.01 to 0.01
C18:2 intake (g/cow per d)	−0.01	0.001	1.29	0.21	−0.01 to 0.01
C18:3 intake (g/cow per d)	0.01	0.001	−0.18	0.86	−0.01 to 0.01
Other FA intake (g/cow per d)	−0.01	0.007	1.27	0.22	−0.02 to 0.01
C12:0 duodenal (g/cow per d)	0.19	0.213	−0.89	0.39	−0.26 to 0.64
C14:0 duodenal (g/cow per d)	0.01	0.020	−0.16	0.87	−0.04 to 0.05
C16:0 duodenal (g/cow per d)	0.01	0.002	−0.70	0.49	−0.01 to 0.01
C16:1 duodenal (g/cow per d)	0.36	0.257	−1.41	0.18	−0.18 to 0.90
C18:0 duodenal (g/cow per d)	−0.01	0.001	0.75	0.47	−0.01 to 0.01
C18:1*trans* duodenal (g/cow per d)	−0.01	0.002	0.21	0.84	−0.01 to 0.01
C18:1*cis* duodenal (g/cow per d)	−0.01	0.004	0.03	0.98	−0.01 to 0.01
C18:2 duodenal (g/cow per d)	−0.01	0.008	1.17	0.26*	−0.03 to 0.01
C18:3 duodenal (g/cow per d)	0.01	0.013	−0.19	0.85	−0.03 to 0.03
Other FA duodenal (g/cow per d)	−0.01	0.011	0.80	0.43	−0.03 to 0.01
Total intake of fermented carbohydrate (kg/cow per d)	0.24	0.269	−0.87	0.39	−0.33 to 0.80
Carbohydrate fermented NDF intake (kg/cow per d)	1.17	0.512	−2.28	0.04*	0.09 to 2.24
Carbohydrate fermented starch intake (kg/cow per d)	−0.27	0.294	0.92	0.37*	−0.89 to 0.35
Carbohydrate fermented soluble fiber intake (kg/cow per d)	2.18	0.812	−2.69	0.02*	0.48 to 3.89
Carbohydrate fermented sugar intake (kg/cow per d)	1.34	0.960	−1.40	0.18*	−0.68 to 3.36
Ca (% of DM)	1.46	1.152	−1.27	0.22	−0.96 to 3.88
P (% of DM)	−2.81	3.369	0.83	0.42	−9.89 to 4.27
Mg (% of DM)	−4.15	8.712	0.48	0.64	−22.46 to 14.15
Actual milk yield (kg/cow per d)	−0.12	0.053	2.27	0.04*	−0.23 to −0.01
Milk ME (kg/cow per d)	−0.05	0.041	1.32	0.21	−0.14 to 0.03
Milk MP (kg/cow per d)	−0.01	0.044	0.23	0.82	−0.10 to 0.08
Actual milk true protein (% of milk)	2.54	1.989	−1.28	0.22*	−1.64 to 6.72
Actual milk fat (% of milk)	1.04	0.504	−2.05	0.02	−0.02 to 2.10

*Significant effects ($P < 0.2$) in the univariate meta-regression model are indicated by an asterisk. The effects $P < 0.2$ in the bivariate model were evaluated for fit in a mixed model meta-regression.

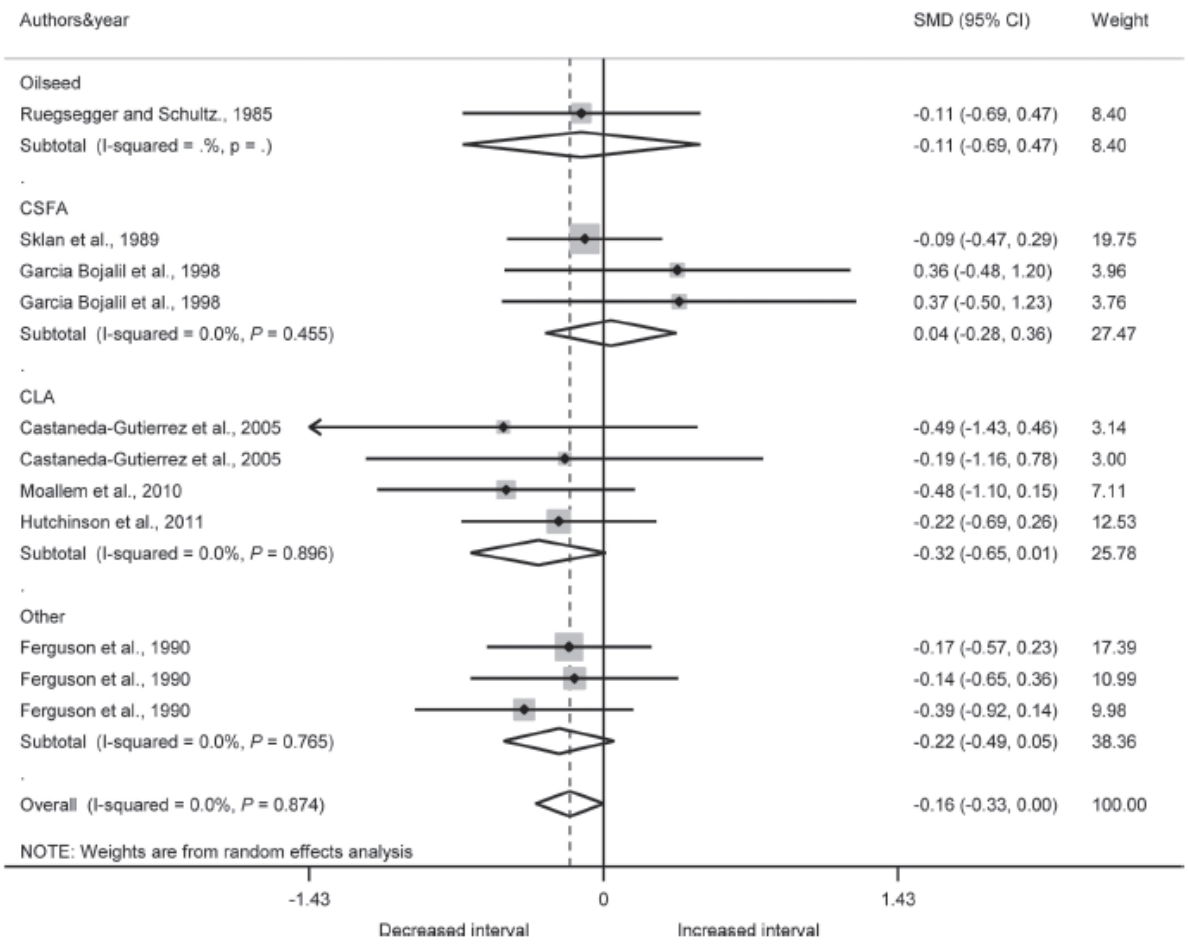

Figure 3. A forest plot of the effect size or standardized mean difference (SMD; standardized using the z-statistic) and 95% confidence interval for trials comparing the calving to pregnancy interval of cows supplemented with fats during the transition and early lactation period. Estimates were made of the SMD using a random effects method (DerSimonian and Laird, 1986). The weights that each study contributed are in the right hand column and are indicated by the size of the box. The larger the box, the greater the study contribution to the overall estimate. The solid vertical gray line represents a mean difference of zero or no effect. Points to the left of the line represent a reduction in days to pregnancy, whereas points to the right of the line indicate an increase. The upper and lower limit of the line connected to the square represents the upper and lower 95% confidence interval for the effect size. The overall pooled effects size and 95% confidence interval is indicated by the diamonds at the bottom of each fat group. This effect was homogeneous as indicated by the I^2 of 0%. An I^2 value for oilseeds is not available because this measure cannot be calculated for a single study, but this has been included in the overall pooled estimate.

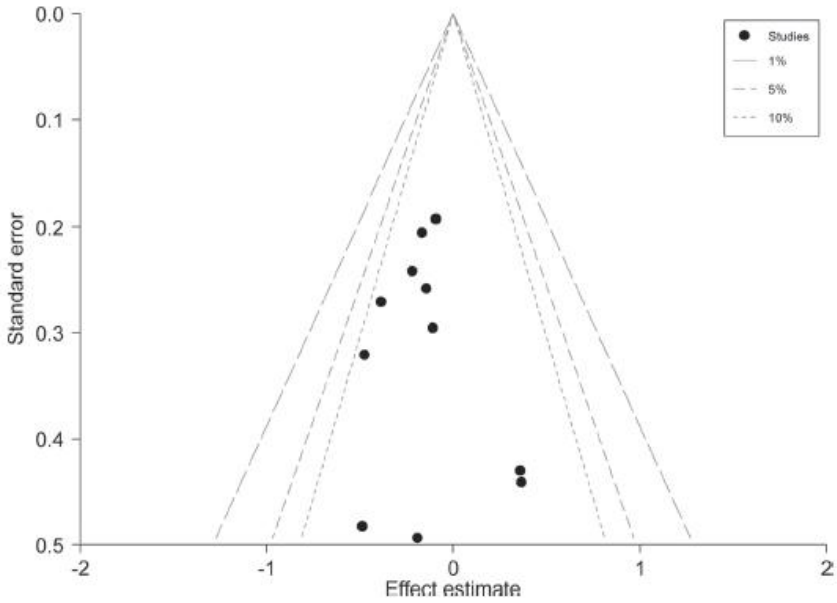

Figure 4. Contour-enhanced funnel plots for interval from calving to pregnancy for lactating dairy cows treated with different fats. Levels of significance for studies (●) within the gray broken lines are 0.01, 0.05, and 0.10.

Table 6. Meta-regression results: intervals from calving to pregnancy

Dietary variable	Coefficient	SE	t	$P > \|t\|$	95% CI
DMI (kg/cow per d)	0.13	0.275	−0.47	0.65	−0.49 to 0.75
Estimated ME balance (MJ/cow/d)	0.01	0.029	−0.02	0.98	−0.06 to 0.07
Estimated MP balance (g/cow per d)	0.01	0.001	−0.02	0.98	−0.01 to 0.01
Bacterial MP (% of MP intake)	−0.01	0.031	0.15	0.88	−0.07 to 0.06
CP (% of diet)	−0.06	0.151	0.37	0.72	−0.40 to 0.29
CP eaten (kg/cow per d)	−0.08	0.600	0.14	0.89	−1.44 to 1.27
RUP (% of CP)	0.01	0.023	−0.17	0.87	−0.05 to 0.06
RUP eaten (kg/cow per d)	0.03	0.440	−0.07	0.95	−0.97 to 1.03
RDP (% of CP)	−0.01	0.023	0.17	0.87	−0.06 to 0.05
RDP eaten (kg/cow per d)	−0.27	0.849	0.32	0.75	−2.20 to 1.65
Soluble protein (% of CP)	−0.01	0.038	0.08	0.94	−0.09 to 0.08
Soluble protein eaten (kg/cow per d)	−0.20	1.193	0.17	0.87	−2.90 to 2.50
Urea cost (MJ/cow/d)	−0.44	0.545	0.81	0.44	−1.67 to 0.79
Predicted PUN (mg/dL)	−0.02	0.045	0.37	0.72	−0.12 to 0.09
Long-chain FA (% of diet)	0.09	0.103	−0.90	0.39	−0.14 to 0.33
Ether extract (% of diet)	0.12	0.114	−1.08	0.31	−0.13 to 0.38
NDF (% of diet)	0.05	0.045	−1.10	0.30	−0.05 to 0.15
NFC (% of diet)	−0.02	0.065	0.30	0.77	−0.17 to 0.13
Sugar (% of diet)	0.02	0.220	−0.10	0.93	−0.48 to 0.52
Starch (% of diet)	−0.01	0.054	0.06	0.95	−0.13 to 0.12
Peptides (% of requirement)	−0.01	0.012	0.18	0.86	−0.03 to 0.02
Peptides and ammonia (% of requirement)	−0.01	0.011	0.13	0.90	−0.03 to 0.02
Methionine (% of requirement)	−0.01	0.014	0.15	0.89	−0.03 to 0.03
Lysine (% of requirement)	−0.01	0.014	0.20	0.85	−0.04 to 0.03
C12:0 intake (g/cow per d)	0.19	0.197	−0.99	0.35	−0.25 to 0.64
C14:0 intake (g/cow per d)	0.02	0.023	−0.83	0.43	−0.03 to 0.07
C16:0 intake (g/cow per d)	0.01	0.001	−0.72	0.49	−0.01 to 0.01
C16:1 intake (g/cow per d)	0.05	0.379	−0.12	0.91	−0.81 to 0.90
C18:0 intake (g/cow per d)	−0.01	0.001	0.36	0.73	−0.01 to 0.01
C18:1trans intake (g/cow per d)	−0.01	0.077	0.17	0.87	−0.19 to 0.16
C18:1cis intake (g/cow per d)	0.01	0.002	−1.46	0.18	−0.01 to 0.01
C18:2 intake (g/cow per d)	0.01	0.006	−0.79	0.45	−0.01 to 0.02
C18:3 intake (g/cow per d)	−0.002	0.022	0.08	0.94	−0.05 to 0.05
Other FA intake (g/cow per d)	−0.00	0.005	0.75	0.47	−0.01 to 0.01
C12:0 duodenal (g/cow per d)	0.19	0.197	−0.99	0.35	−0.25 to 0.64
C14:0 duodenal (g/cow per d)	0.02	0.023	−0.83	0.43	−0.03 to 0.07
C16:0 duodenal (g/cow per d)	0.01	0.001	−0.79	0.45	−0.01 to 0.01
C16:1 duodenal (g/cow per d)	0.21	0.785	−0.27	0.79	−1.56 to 1.99
C18:0 duodenal (g/cow per d)	0.01	0.001	−0.01	0.99	−0.01 to 0.01
C18:1*trans* duodenal (g/cow per d)	0.01	0.003	−0.12	0.91	−0.01 to 0.01
C18:1*cis* duodenal (g/cow per d)	0.01	0.003	−1.48	0.17	−0.01 to 0.01
C18:2 duodenal (g/cow per d)	0.01	0.004	−0.59	0.57	−0.01 to 0.01
C18:3 duodenal (g/cow per d)	0.01	0.031	−0.30	0.77	−0.06 to 0.08
Other FA duodenal (g/cow per d)	−0.01	0.006	0.69	0.51	−0.02 to 0.01
Total intake of fermented carbohydrate (kg/cow per d)	0.06	0.445	−0.13	0.90	−0.95 to 1.06
Carbohydrate fermented NDF intake (kg/cow per d)	0.181	1.393	−0.13	0.90	−2.98 to 3.33
Carbohydrate fermented starch intake (kg/cow per d)	0.02	0.287	−0.06	0.95	−0.63 to 0.67
Carbohydrate fermented soluble fiber intake (kg/cow per d)	−0.41	1.356	0.30	0.77	−0.47 to 2.66
Carbohydrate fermented sugar intake (kg/cow per d)	0.18	0.974	−0.18	0.86	−0.03 to 2.38
Ca (% of DM)	0.79	0.887	−0.89	0.40	−0.22 to 2.79
P (% of DM)	−8.09	9.564	0.85	0.42	−9.73 to 13.54
Mg (% of DM)	−55.40	44.954	1.23	0.25	−57.09 to 46.29
Actual milk yield (kg/cow per d)	0.05	0.125	−0.38	0.71	−0.23 to 0.33
Milk ME (kg/cow per d)	0.02	0.087	−0.27	0.79	−0.17 to 0.22
Milk MP (kg/cow per d)	0.01	0.022	−0.12	0.9	−0.05 to 0.05
Actual milk true protein (% of milk)	−0.19	1.731	0.11	0.92	−4.10 to 3.73
Actual milk fat (% of milk)	0.27	0.335	−0.81	0.44	−0.49 to 1.03

Production Outcomes and Body Weight

Overall, milk yield tended to increase with feeding fats during the transition period (pooled SMD = 0.33, 95% CI = -0.01 to 0.67) (Figure 5). Meta-analysis results for production variables and body weight are outlined in Table 4. Pooled estimates showed that feeding fats tended to have little effect on milk fat % (SMD = -0.03, 95% CI = -0.32 to 0.26), except where CLA were fed and a significant decrease was observed (SMD = -1.39, 95% CI = -2.04 to -0.74). Similarly, milk fat yield increased with Oilseed and CSFA feeding (SMD = 0.29, 95% CI = 0.03 to 0.55) and SMD = 0.64, 95% CI = 0.05 to 1.23, respectively), but decreased when CLA were fed (SMD =

-1.00, 95% CI = -1.55 to -0.44). No tallow studies reported this variable. The difference in fat yield among groups for the overall pooled estimate was neutral (SMD = 0.04, 95% CI -0.39 to 0.47). Feeding fats during transition tended to decrease milk protein percentage, overall. Feeding CLA significantly reduced the percentage of protein in milk (SMD = -0.45, 95% CI -0.87 to -0.03). The CSFA and Tallow groups tended to decrease milk protein percentage, however oilseeds tended to increase protein percentage. Overall, feeding fats tended to increase protein yield, however this was not statistically significant (SMD = 0.34, 95% CI -0.07 to 0.75). For all milk yield and composition variables there was a high level of heterogeneity among studies (I^2 = > 80%) and funnel plots were asymmetrical suggesting a potential for publication bias.

There was no effect of feeding fats on body weight (SMD = - 0.15, 95%, CI -0.69 to 0.40). Of these groups, Tallow and CLA could not be explored individually as all comparisons within each group were from a single paper. Again, heterogeneity was high (I^2 = 90.1%).

DISCUSSION

Despite more than 5,000 papers being initially identified in a systematic literature search on this topic, only 17 of these, providing 26 comparisons, were suitable for inclusion; a lower number than had been expected. The limited number of studies available for the current analyses highlights a need for more controlled studies to be conducted, containing sufficient information on exposure variables, in this case diet, examining reproductive outcomes such as pregnancy risk and interval to pregnancy.

One of the strengths of meta-analysis is that similar metrics, such as those used to measure proportion of cows pregnant to service, and interval to pregnancy, can be pooled using effect size measures such as those used in this study. There was also substantial variability in the fat content and type in control diets, a finding that is ***c***onsistent with a meta-analysis by Rabiee et al. (2012) that explored the effects of fat nutrition on milk yield and composition. Meta-analysis and meta-regression methods allow these sources of variation to be explored as a single data set and can help overcome these limitations by evaluating differences in treatment amounts of fat or differences in diet structure resulting from fat inclusion.

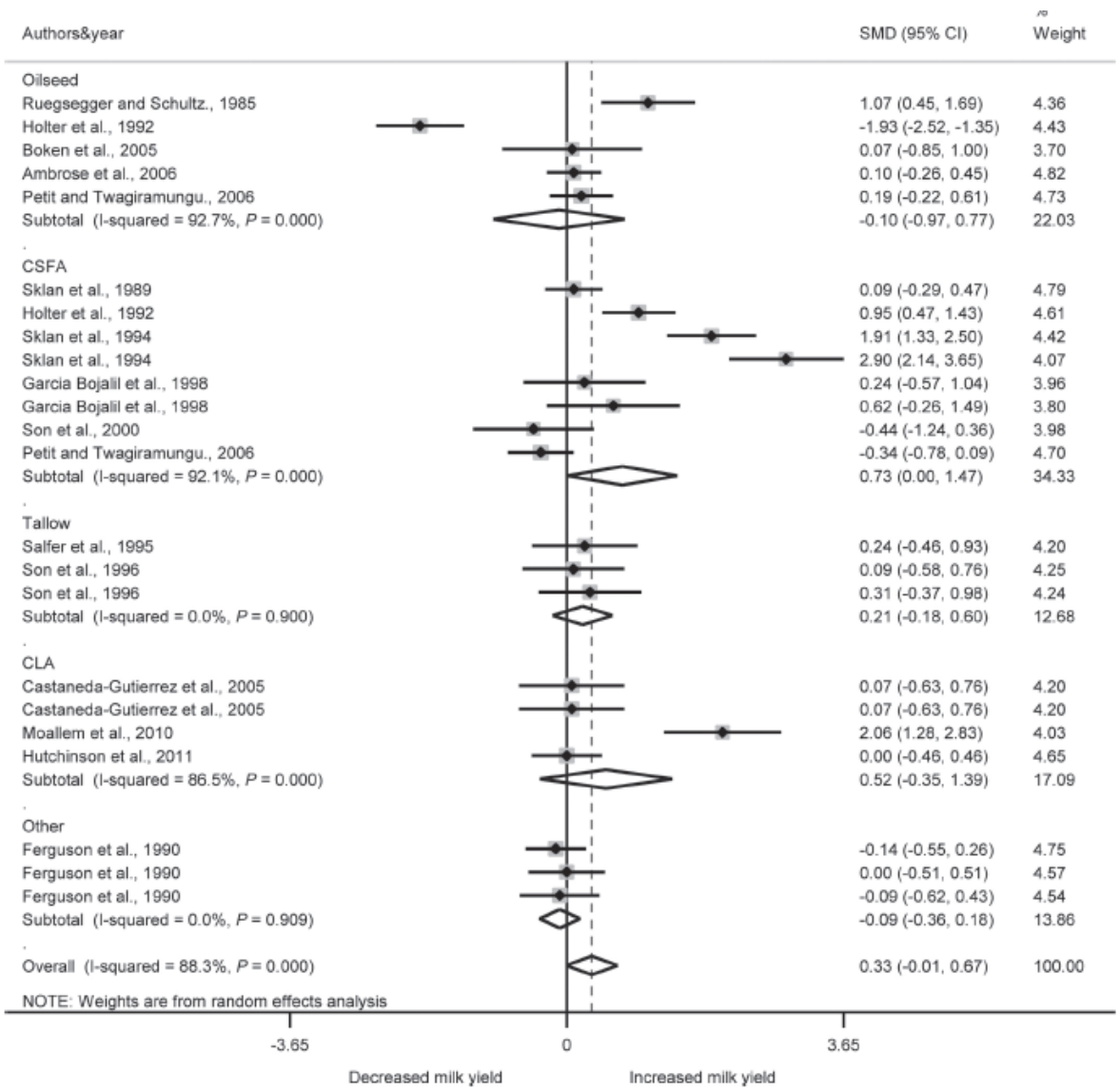

Figure 5. Forest plot of individual standardized mean difference (SMD), 95% CI, and weights for trials comparing the milk yield of cows supplemented with fats during the transition and early lactation period. Estimates were made of the SMD using a random effects method (DerSimonian and Laird, 1986). The weights that each study contributed are in the right hand column and are indicated by the size of the box. The larger the box, the greater the study contribution to the overall estimate. The solid vertical gray line represents a mean difference of zero or no effect. Points to the left of the line represent a reduction in days to pregnancy, whereas points to the right of the line indicate an increase. The upper and lower limit of the line connected to the square represents the upper and lower 95% confidence interval for the effect size. The overall pooled effects size and 95% confidence interval is indicated by the diamonds at the bottom of each fat group. This effect was heterogeneous, as indicated by the I^2 of 88.3%.

Fat feeding during transition has a variable impact on reproductive performance of lactating dairy cows with studies reporting mixed results(Grummer and Carroll, 1991). While the feeding of fats before and immediately after calving has the potential to reduce DMI, particularly in heifers, there are now many studies where beneficial effects of dietary fat have been observed on production and reproduction (Doepel et al., 2002, McNamara et al., 2003, Petit and Benchaar, 2007). McNamara et al. (2003) found that feeding fats increased first service pregnancy, but did not change overall percentage of cows pregnant. In the current study, the overall effects of fat feeding increased the proportion of cows pregnant to service and tended to reduce the interval from calving to pregnancy in treated cattle (Figures 1 and 2).When explored individually, the

results show that each fat group tended to improve fertility, however the limited number of studies available for analysis, and small size of many of these studies, prevented clear effects being identified. For the studies suitable for inclusion in this meta-analysis, reproductive responses were consistent with an I^2 of 19.9% and 0%, indicating low heterogeneity for the proportion pregnant to service and interval from calving to pregnancy, respectively. Many of the studies rejected for inclusion in the meta-analysis that still presented valid interventions (Table 2) also had positive responses to fats.

Increased milk yield (kg/d) of the treatment groups decreased the proportion pregnant in both univariate and multivariate meta-regression models (P = 0.02 and P = 0.04 respectively (unpublished and Table 5)). Milk production demands of the freshly lactating cow exceed the capacity of DMI to deliver key nutrients including amino acids and energy precursors, ensuring most cows are in a state of negative nutrient balance in early lactation. Substantial energy deficits contribute to incidence of metabolic disease, decreased production (persistence and volume) and poor reproductive efficiency (Butler, 2000). Including fat can increase energy density of the diet, without increased dependence on rapidly fermentable carbohydrates which, when fed at high levels, can compromise rumen and metabolic health. Inclusion of fats in the diet may also reduce liver triglyceride accumulation (Selberg et al., 2002) and concentrations of NEFA in blood (Doepel et al., 2002) immediately after calving and increase serum cholesterol concentrations (Rafalowski and Park, 1982, Carroll et al., 1990), a factor associated with better fertility. Westwood et al. (2000) found that higher concentrations of plasma cholesterol were associated with a shorter interval from calving to pregnancy, with greater probabilities of conception and successful pregnancy by day 150 of lactation. This finding is consistent with those of Kappel et al. (1984) and Ruegg et al. (1992), who found positive associations between cholesterol concentrations and fertility measures. Similarly, Moss (2001) found that low blood cholesterol concentrations at mating were strongly associated with pregnancy failure. Fatty acids are essential precursors for reproductive hormones and Grummer and Carroll (1991) speculated that the presence of cholesterol-enriched lipoproteins could enhance progesterone production. This was supported by detection of increased levels of PGF2α after feeding prilled long chain fatty acids (Carroll et al., 1990). Lipogenic precursors are also required for efficient milk production, and the optimal requirement was estimated to be 15 to 25% of energy supplied as lipogenic precursors, or about 8% long chain fatty acids in the diet (Kronfeld, 1976). Additionally, the tendency for pregnancy to be increased with higher milk fat percentages (P =

0.055) suggest that the ability of animals to spare fat for milk production is an indication of good metabolic status supporting reproduction.

While no fat type individually increased fertility in this meta-analysis, feeding CLA has been an area of investigation previously showing positive results, although the number of high quality studies is limited. De Veth et al. (2009) combined 5 studies and observed a marked improvement in median time to pregnancy (reduced from 151 to 117) days in cows fed a ruminally protected CLA compared to unsupplemented cows. Thatcher et al. (2006), also found positive effects of supplementation with ruminally protected CLA and palm fatty acids on reproduction and health. Von Soosten et al. (2012) identified a trend towards lower body mass mobilization in cattle fed protected CLA, when compared to a stearic acid based fat supplement, suggesting a protective effect of CLA supplementation on use of body reserves in early lactation, possibly through more efficient utilization of metabolizable energy. The current meta-analysis did not show a significant impact of fat feeding on body weight, but was not able to explore CLA feeding individually as only one of the papers that reported CLA responses provided details on body weight.

Fats are also important sources of essential fatty acids. Linoleic (C18:2) and linolenic fatty acids (C18:3) are classified as essential fatty acids and must be supplied in the diet (Mattos et al., 2000). Unsaturated fatty acids (especially linoleic acid, linolenic acid, eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6)) may target reproductive tissues when supplied in a form absorbed in the lower gut (Thatcher et al., 2006). Lean and Rabiee (2006) estimated that availability of essential fatty acids at the duodenum is approximately half that for cattle fed in total mixed rations based on maize and alfalfa silage compared to cows fed pasture based diets. Linolenic acid (C18:3) predominates in forage lipids (Palmquist and Jenkins, 1980) and concentrations of linoleic acid (C18:2) are high in some pastures. This difference, combined with high digesta flow rates of lush pastures, may in part explain the differences in reproductive performance seen between pasture based herds and those maintained in total mixed ration systems, such as those seen in North America. Hutchinson et al. (2011) found a trend towards lower services per pregnancy, but little overall effect of supplementation, with feeding protected CLA on fertility of cows on pasture, a finding consistent with the suggestions of Lean and Rabiee (2006) that at least some of the difference in fertility of cows on pasture based diets and those on TMR diets may reflect the CLA generated from pasture.

In this meta-analysis, the potentially confounding effects of diet formulation to include fats in the diet were controlled by using meta-regression. Differences were identified in intakes and duodenal concentration of fatty acids among the different groups of fats fed (Table 3), however these differences did not influence outcomes when evaluated by meta-regression. Increasing dietary intake of slower fermenting carbohydrates (NDF and soluble fiber) favored proportion pregnant, possibly because slower fermentation results in more stable rumen conditions and promotes microbial growth. Chalupa et al (1986) found that including high levels of fat in the diet affected microbial metabolism, as indicated by a decrease in the ratio of acetate to propionate concentrations in the rumen. This response in acetate: propionate ratio varied with the type of fat used, as the depression was greater in response to oleic acids and animal tallows than CSFA and stearic acid. The positive association between the energetic cost of urea synthesis and pregnancy was unexpected, but may reflect a need for soluble protein intake to increase in high fat diets to maintain microbial protein synthesis and highlights the multivariable responses to nutritional intervention.

While milk yield was not significantly increased by feeding fats during the transition period, other meta-analyses that included a greater number of comparisons and found significant increases (Rabiee et al., 2012, Boerman, 2014). These studies (Rabiee et al., 2012, Boerman, 2014) both found an overall milk yield response of 1.05 kg/cow/d from fat feeding. A meta-analysis (Onetti and Grummer, 2004) found no significant change in milk yield when tallow of selected hydrolyzed fatty acids were fed, whereas including CSFA in the diet increased yield. In the current study, CSFA interventions increased yield and milk fat yield, but did not affect milk composition as there were no differences in milk protein, but the study power was low compared to other meta-analyses.

There was more consistency in reproductive responses to fats than for milk and milk components where marked differences in responses to different fats were observed. This greater variability is consistent with Rabiee et al. (2012), who included studies in which fat was fed any time during lactation, whereas this paper has a focus only on fats fed during transition. This distinction is important as there is increasing evidence that nutrition during the transition period has a pivotal role on performance, especially reproduction in the following lactation (Thatcher et al., 2011).

CONCLUSIONS

Feeding fats has a positive effect on fertility and a tendency to increase production when fed during the transition period. Feeding fats during transition may be an essential component of an integrated response to the challenges of controlling tissue mobilization in early lactation and limiting the amount of fermentable carbohydrate fed. However, meta-regression of the difference in diets between treatment and control groups did not identify the reasons for these improvements in regard to the fatty acid composition of the diet. The limited number of papers identified from the literature search and the positive results of this study, support the need for further work exploring the effects of including fat in the diet of the transition cow on fertility and the development of guidelines to assist study design in this area of research.

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CHAPTER FOUR: PRE-CALVING AND EARLY LACTATION FACTORS THAT PREDICT MILK CASEIN AND FERTILITY IN THE TRANSITION DAIRY COW

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OVERVIEW OF CHAPTER FOUR

The meta-analysis presented in Chapter 2 demonstrated clear effects of peri-parturient nutrition on reproductive outcomes, including, positive and negative associations of protein nutrition with reproductive outcomes. This chapter utilises and builds on existing data from a study of the influence of genetic merit and dietary protein degradability, to explore the influence of pre-partum nutrient intakes on reproductive performance as this had not previously been examined using these data. This Chapter also examines rarely reported milk casein and casein variants, and identifies pre-calving and early lactation variables that predict production outcomes.

ABSTRACT

Multiparous Holstein cows (n = 82) of either high or low genetic merit (**GM**) (for milk fat + protein yield) were allocated to one of two diets in a 2 X 2 factorial design. Diets differed in the ratio of rumen-undegradable protein (**RUP**) to rumen-degradable protein (37% RUP vs. 15% RUP) and were fed from 21 d pre-calving to 150 DIM. This study evaluated the effects of these diets and GM on concentrations of milk casein (**CN**) variants and aimed to identify pre-calving and early lactation variables that predict milk, CN and protein yield and composition, and fertility of dairy cows. It explored the hypothesis that low milk protein content is associated with lower fertility, extending this to also evaluate the association of CN contents with fertility.

Yields (kg/d) for CN variants were 0.49 and 0.45 of alpha CN, 0.38 and 0.34 of beta CN, 0.07 and 0.06 for kappa CN, and 0.10 and 0.09 of gamma CN for high and low RUP diets, respectively. Increased RUP increased milk, CN and milk protein yields. Increased GM increased milk protein and gamma CN yields and tended to increase milk CN yield. The effects of indicator variables on CN variant yields and concentrations were largely consistent, with higher body weight and alpha amino nitrogen resulting in higher yields, but lower concentrations. An increase in cholesterol was associated with decreased CN variant concentrations, while disease lowered CN variant yield. A diet high in RUP increased proportion of first services that resulted in pregnancy from 41 to 58%. Increased pre-calving metabolizable protein (**MP**) balance decreased the proportion of first services that resulted in pregnancy when evaluated in a model containing CN %, milk protein yield, diet, and GM. This suggests that the positive effects of increasing dietary RUP on fertility may be curvilinear as cows with a very positive MP balance before calving were less fertile that those with a lower, but positive, MP balance. Prepartum MP balance was important to production and reproductive outcomes, while surprisingly, metabolizable energy balance was not. The hazard of pregnancy in the first 150 d of lactation was 28% lower in cows producing milk with the lowest quartile of protein percentage when compared with cows with milk in upper three quartiles. Milk CN % was positively associated with improved pregnancy at first service. This study demonstrates the importance of protein metabolism to reproductive performance of the dairy cow.

Keywords: fertility, protein degradability, casein

INTRODUCTION

Good nutritional management of dairy cattle during the transition period can improve their responses to the metabolic challenges posed by late pregnancy and early lactation (Bell, 1995, Lean et al., 2014). Hence, metabolic status and dietary interventions before calving are valuable to study as predictors of future productivity and reproductive performance (Bell, 1995). While much of the research in this area focused on nutritional interventions after calving, or around the time of conception (Patton et al., 2007), these metabolic processes can be influenced before calving and in early lactation, as the body tissue reserves the cow has available affects subsequent productivity and health (LeBlanc, 2010, Lean et al., 2013, Bradford et al., 2015). Identifying key indicators that are associated with better productive and reproductive outcomes may allow cattle to be better fed and managed to achieve maximum productivity. There is evidence that higher producing cows have lower fertility (Lean 1989, Spalding 1975), and that genetic selection for increased production can reduce fertility (Hageman et al., 1991, Buckley et al., 2000, Horan et al., 2005, Pollott and Coffey, 2008). These observations suggest the need to investigate differences in productive and reproductive performance and metabolism in cows of differing genetic merit (GM).

Bovine milk contains 2.5-3.5% true protein, approximately 80% of which is casein (CN) (Coulton et al., 1998). There are four main variants of CN (alpha-$_{s1}$, alpha-$_{s2}$, beta, and kappa) with an additional class, gamma CN, which is a product of post-secretion proteolytic breakdown of beta CN (Fox and Mulvihill, 1982). Each CN variant may have distinct properties but it is not known if different CN variants are associated with better production or reproductive outcomes, or what factors affect production of specific variants. While it is well recognized that increased dietary RUP and metabolizable AA flux can increase milk yield when fed during early lactation (Carroll et al., 1994, Cunningham et al., 1996, McCormick et al., 1999, Doepel et al., 2004), knowledge of the effects of protein nutrition during the pre-calving period is more limited and two meta-analyses failed to identify optimal estimated MP (Lean et al., 2003) or dietary CP concentrations (Lean et al., 2012). The role of specific AA nutrition pre-calving on production and reproductive performance remains to be determined, but nutritional principles dictate that AA nutrition must be important.

Cattle can use nutrients from the diet and from endogenous body reserves, hence the diet both before calving when body reserves are being accreted, and during lactation and the breeding

period is important. Dietary protein intake is of particular interest as improved protein nutrition, in transition and early lactation can improve production, fertility and health in the dairy cow (Van Saun et al., 1993). Milk yield was unchanged when RUP concentration in the diet was increased before calving (Van Saun et al., 1993), but milk protein percentage increased from 2.96% to 3.18%. Increasing the amount or degradability of dietary protein lowers the proportion of cows pregnant to service (Butler, 2000, Lean et al., 2012), however, decreasing the degradability of the dietary protein can improve reproductive outcomes (Folman et al., 1981, Carroll et al., 1994). Further, large observational studies suggest that reproductive performance is improved in cows with high milk protein concentration (Moss, 2001, Buckley et al., 2003, Morton, 2004, Madouasse et al., 2010). Given that the CN represent 80% of milk proteins, it can be hypothesized that evaluating the pre-calving factors that influence CN variants and milk protein concentration may provide insight into the metabolic pathways that are associated with both lower milk protein and lower fertility. Further, the effects of genetic merit on CN, milk, milk protein production and content, and fertility should be examined in the context these studies. Given that there are very few detailed studies of milk CN variants and no others that provide reproductive data, this study provides a unique opportunity to examine relationships among these and diet, GM, and metabolic states as reflected by pre-calving indicators and immediate post-calving measures, albeit with limited numbers of cattle.

The indicator variables that most reliably predict future reproductive and productive performance are yet to be defined. Body weight and BCS are often used as easily measured proxies for energy or more critically, nutrient reserves (Edmondson et al., 1989) and excessive loss or low levels of these are associated with lower fertility (Garnsworthy and Topps, 1982, Buckley et al., 2003, Lopez-Gatius et al., 2003). Similarly, changes in, or absolute values for, blood metabolites including glucose, NEFA, BHB, insulin, and IGF-1, have been associated with changes in reproductive performance (Leroy et al., 2005, Leroy et al., 2008, LeBlanc, 2010, Ospina et al., 2010, Chapinal et al., 2012), however, these indicators are not uniformly associated with adverse reproductive outcomes (Chapinal et al., 2012). The importance of metabolic factors during lactation that influence fertility was examined by Westwood et al. (1998, 2000, 2002), however, metabolite concentrations during the period before calving have not been as comprehensively evaluated as indicators of future performance. As such, one goal of this study was to screen factors during the prepartum period as potential indicators of future productive and reproductive performance.

We received grant support (Dairy Australia Project Number: C100000540) to revisit a high quality study (Garvin 1999, Westwood 2000, 2002) to i) describe the effects of protein nutrition and GM on CN variants; ii) to explore pre-calving and early lactation factors that may predict production, especially protein and CN production, and reproductive performance, iii) to evaluate an *a priori* hypothesis that low milk protein content is associated with poor fertility and to understand the factors, including CN variant yield and composition, that contribute to this.

MATERIALS AND METHODS

Cows and Feeding

This study is a re-examination and extension of data obtained from studies by Garvin (1999) and Westwood et al. (2000, 2002). Multiparous Holstein-Friesian cows (n = 82) were blocked by GM (high or low Australian Breeding Value (**ABV**) of milk fat + milk protein yield) and maintained on one of two diets differing in protein degradability from 21 d pre-calving to 150 d postpartum in a 2 X 2 factorial arrangement. Within GM groups, cows were paired according to calving date, age and lactation number before random allocation to dietary group. Diets were isonitrogenous (dry cow 10.5% CP; lactating cow 19.3% CP) and isoenergetic (dry cow 10.0 MJ per kg of ME; lactating cow 11.3 MJ per kg of ME) and differed in the ratio of RUP to RDP protein (37% RUP : 63% RDP vs. 15% RUP : 85% RDP). There were 19 cows of high GM and 21 cows of low GM, receiving the diet high in RUP and 21 cows in each of the high and low genetic groups that received the diet low in RUP. Cows were housed in an outdoor, shaded corral with a dirt floor and feed was individually delivered, removed and measured in a TMR via a Calan feeding system (American Calan Inc., Northwood, NH, USA) located under shade, on a concrete base, which was scraped daily. Before entry into the experiment cows were trained in use of this system. Total mixed rations consisted of chaffed alfalfa and oaten hays and a concentrate pellet. Full details of feed composition and analysis are available in Westwood et al. (2000) and in Supplementary Table 1.

Data Collection

Daily DMI was calculated individually for each cow, and averaged weekly. Weekly BW and BCS (1-5 scale (Edmondson et al., 1989)) were recorded every 7 ± 3.5 d from entry into trial until week 10 of lactation.

Sample Collection and Analysis

Coccygeal blood samples were collected weekly, commencing three weeks prior to parturition until week 10 of lactation (n = 14/cow). Samples were analysed for plasma BHB, cholesterol, glucose, urea, alpha amino nitrogen (**AAN**) and serum NEFA concentrations as described in Westwood et al. (2000).

Cows were milked twice daily with milk yields recorded manually at each milking and an average daily production calculated weekly for the first 10 weeks of lactation. Milk fat and protein concentrations were determined weekly for the first 10 weeks of lactation using infra-red spectrometry (Milkoscan 605 analyser, Foss Electric, 3400 Hillerød, Denmark). Skim milk was retained for CN precipitation and analysis at weeks 1 to 10 of lactation for casein yield and percentage, and weeks 2, 6 and 10 of lactation for casein variant yields and percentages. This was multiplied by the amount of skim milk as a percentage of whole milk to obtain the CN concentration in whole milk (mg/mL). Individual CN variant yields were determined by Fast Protein Liquid Chromatography, using SDS-polyacrylamide gel electrophoresis. The method used was described by Davies and Law (1987) with minor modifications as described by Garvin (1999). Alpha CN concentration was a combination of both alpha-$_{s1}$ and alpha-$_{s2}$ CN. For this study, average concentrations of blood metabolites and estimated ME and MP balance taken in the 3 weeks before calving and milk yield and composition in the first 3 weeks after calving, were evaluated as indicator variables for inclusion in statistical models evaluating production and reproduction (Table 1).

Table 1. Description of variables included in models

Variable[1]	Description[2]	Unit
AAN	Plasma AAN, average for wk –3 to –1	mmol/L
Genetic merit	Australian Breeding Value (ABV) for milk fat + protein yield	
BW	Body live weight, average of wk –3 to –1	kg
BCS	BCS, average for wk –3 to –1	1–5 scale, assessed to quarter points
Calcium	Plasma calcium, average for wk –3 to –1	mmol/L
Cholesterol	Plasma cholesterol, average for wk –3 to –1	mmol/L
BHB	Plasma BHB, average for wk –3 to –1	mmol/L
Glucose	Plasma glucose, average for wk –3 to –1	mmol/L
ME balance	ME balance, average for wk –3 to –1	MJ of ME/d
MP balance	MP balance, average for wk –3 to –1	g/d
Milk yield	Unless otherwise stated, average for milk yield for wk 1–3 of lactation	kg/d
Milk protein yield	Milk protein yield, average for wk 1–3 of lactation	kg/d
Milk protein percentage	Milk protein percentage, average for wk 1–3 of lactation	% whole milk
Milk CN yield	Milk CN yield, average for wk 1–3 of lactation	kg/d
Milk CN percentage	Milk CN percentage, average for wk 1–3 of lactation	% whole milk
CN variant yield	Milk α-, β-, γ-, or κ-CN yield, average for wk 2, 7, and 10 of lactation	kg/d
CN variant percentage	Milk α-, β-, γ-, or κ-CN percentage, average for wk 2, 7, and 10 of lactation	% whole milk
Free fatty acids	Serum free fatty acids, average for wk –3 to –1	µmol/L
Urea	Plasma urea, average for wk –3 to –1	mmol/L

[1]AAN = α-amino nitrogen.
[2]Week in relation to parturition.

Reproduction

Commencing 21 ± 3.5 d after calving, all cows were monitored by rectal palpation of the reproductive tract to identify any condition that may compromise reproductive performance. This was repeated every three weeks for cows that had not been inseminated during the previous 42 d. A voluntary waiting period of 45 d was observed after which cows were bred 6-18 hours after the observation of primary and/or secondary heat signs. Estrus detection, starting immediately after calving, was aided by visual observation for at least 14h/d and by placing KaMaR Heatmount detectors (Steamboat Springs, CO) on the tail-head of each cow. Cows were paired (one from each dietary group) and artificially inseminated with semen from the same ejaculate. All cows were inseminated by the same technician. Successful pregnancy was identified by a positive pregnancy test via palpation 42 d after a mating had occurred. Palpation was repeated 7 d later to confirm pregnancy, and once confirmed, routine palpation was not continued. The interval from calving to pregnancy was defined as number of days between calving and mating that resulted in a positive pregnancy test by palpation 42 d after mating. Full details of reproductive management and measurement are available in Westwood et al. (2000).

Disease

Treatment records were used to identify incidence of disease. Cows were classified as either being affected or not affected by 'severe disease' between 6 weeks pre-calving to 150 DIM. Severe disease was classified as having one or more of the following conditions; acute mastitis, retained foetal membrane/retained placenta, metritis/peritonitis, hypocalcaemia, ketosis, or displaced abomasum. The workers coding the diseases were not aware of the cows' production or reproductive outcomes.

Nitrogen and Energy Balance Modelling

Weekly MP balance was estimated for each cow from 3 weeks prepartum until week 10 of lactation using CPM-Dairy (version 3.08; Cornell-Penn-Miner, http://cahpwww.vet.upenn.edu/node/77). Calculations were based on dry cow rations in weeks -3 to 0 (weeks numbered in relation to parturition at week 0), early lactation ration in estimates for week 1, and lactation ration for weeks 2 to 10 inclusive. Calculations included individual weekly DMI, BW, BCS, milk yield, milk fat percentage, milk protein percentage, parity, lactation status, age, and days pregnant. Dietary evaluations were based on the feed analysis results for the individual components of the diet. Feed evaluations were conducted on change of pellet or

forage. Weekly ME balances were calculated based on formulae described by AFRC (1993) as detailed by Westwood et al. (2000).

Statistical Analysis

All data analysis was performed using Stata (Intercooled Stata v. 13, StataCorp, College Station, TX).

***Production variables*.** Descriptive analysis of variables was undertaken and effects of diet and GM, for each variable are provided in Table 2. Diet and GM were included in all models as categorical variables. Mixed models (XTMIXED, Stata version 13.1) were used to explore the significance of the pre-calving indicator variables on milk yield and milk protein and CN yield and composition. Pre-calving indicator variables refer to serum NEFA, plasma AAN, calcium, cholesterol, BHB, glucose, and urea, ME balance, MP balance, BW, BCS and "severe disease" variable (Table 1). For all factors other than disease, an average value for the three weeks before calving was used. The following linear mixed model was used:

$$Y_{ijkl} = \mu + \beta_i + \gamma_j + \theta_k + (\beta\gamma)_{ij} + (\beta\gamma\theta)_{ijk} + X_l + \varepsilon_{ijkl}$$

Where Y_{ijkl} = response to diet i(i = high RUP or low RUP) and GM j (j = high or low ABV for milk fat + protein) at the kth time (k = week 1 to 10 of lactation) for cow number l (l = 1 to 82); μ = overall mean; β_i = fixed effect of diet; γ_j = fixed effect of GM; θ_k = fixed effect of week (time); $(\beta\gamma)_{ij}$ = effect of diet by GM interaction; $(\beta\gamma\theta)_{ijk}$ = effect of diet by GM by week interaction; X_l = random effect of animal; ε_{ijkl} = random residual error within animal l, on diet i, with GM j at week k. Indicator variables were included in models containing diet, GM and week of lactation as a priori fixed effects. An autoregressive (AR1) covariance structure was used based on superior fit to other covariance structures. Variables that had a univariable P-value of less than 0.2 were included for evaluation in multivariable models using stepwise modelling with backward elimination. Once removed, variables were not eligible for re-entry into the model. Final models were assessed based on Wald tests and Akaike's information criteria (AIC) to evaluate model fit and confounding was assessed by evaluating changes in coefficients as new terms were added into the models. A similar mixed model with an independent covariance structure (XTMIXED, Stata version 13.1) was used to explore the significance of the pre-calving indicator variables on 8 factors of milk CN variant composition and yield (yield and percentage

for each of alpha, beta, gamma and kappa CN). Multivariable models were determined in the same way as for milk yield and protein variables.

Table 2. Descriptive analysis of BW, BCS, and predictor variables [mean (SD) averaged for the 3 wk before calving][1]

	Diet		Genetic merit	
Variable	High RUP	Low RUP	Low ABV	High ABV
BW (kg)	665.73 (74.06)	655.41 (65.73)	650.65 (68.65)	669.77 (70.21)
BCS (1–5)	3.39 (0.32)	3.44 (0.23)	3.42 (0.30)	3.41 (0.25)
MP balance (g/d)	511.38 (183.34)	356.18 (128.14)	444.83 (168.89)	426.95 (183.58)
ME balance (MJ of ME/d)	27.44 (33.34)	57.14 (30.85)	44.99 (23.77)	39.00 (43.52)
Free fatty acids (μmol/L)	264.57 (149.63)	298.95 (243.40)	258.90 (145.37)	305.33 (246.76)
Urea (mmol/L)	5.66 (1.34)	5.87 (1.20)	5.72 (1.22)	5.81 (1.33)
α-Amino nitrogen (mg/100 mL)	3.73 (0.47)	3.69 (0.47)	3.83 (0.50)	3.59 (0.41)
Glucose (mmol/L)	3.63 (0.52)	3.62 (0.40)	3.71 (0.43)	3.55 (0.49)
Calcium (mmol/L)	2.29 (0.27)	2.26 (0.25)	2.30 (0.26)	2.24 (0.25)
Cholesterol (mmol/L)	2.58 (0.81)	2.50 (0.58)	2.58 (0.67)	2.50 (0.73)
BHB (mmol/L)	1.82 (1.51)	1.34 (0.36)	1.36 (0.31)	1.30 (0.31)

[1]ABV = Australian Breeding Value for milk fat + protein yield.

Reproductive outcomes. The proportion of first services that resulted in pregnancy was assessed using logistic regression models (LOGISTIC, Stata version 13.1) with diet and GM included as *a priori* fixed effects. Indicator variables and early lactation production factors were included as individual covariables. Early lactation production factors included milk yield and protein and CN yield and percentage and an average of the first 3 weeks of lactation was used. Those with P-values less than 0.2 were evaluated in multivariable backward stepwise modelling. A logistic normal non-parametric accelerated time failure survival analysis (STREG, Stata version 13.1) was used to further explore the effect of these pre-calving indicator variables and early lactation production factors on the risk of pregnancy up to 150 DIM. Cows that were not pregnant at 150 DIM were considered non-pregnant, even if they became pregnant after 150 DIM, resulting in right censoring of the data. Cows were then separated into quartiles based on their milk protein percentage (average of first three weeks after calving) using the lowest quartile as reference group. A further analysis was then performed to assess the following time-varying covariables using a Cox's proportional hazards regression model (STCOX, Stata version 13.1) for calving to pregnancy interval; ME balance, MP balance, milk yield, milk protein percentage, milk protein yield, CN percentage, CN yield, serum NEFA, and plasma urea, AAN, glucose, calcium, cholesterol and BHB.

Milk Protein Percentage. Cows were again separated into quartiles based on their milk protein percentage and cows with lowest milk protein percentage were compared with those of the upper three quartiles using canonical linear discriminant analysis (CANDISC, Stata version 13.1). Significant differences that existed between the two groups in the pre-calving indicator variables

and early lactation production traits were identified. A backward stepwise logistic regression (LOGISTIC, Stata version 13.1) was also performed to assess the results of the discriminant analysis and validated that these variables did differ (data not shown) between the groups with the lowest and higher milk protein content.

RESULTS

Effect of Dietary Protein Degradability and GM on Indicator Variables Including ME and MP Balance

Descriptive analyses of the average blood concentrations of indicator variables in the three weeks prepartum are included in Table 2. Cows receiving more RUP in the diet, or of higher GM had numerically higher BW and lower BCS. An increased proportion of RUP in the diet increased prepartum MP balance (Table 3 and Figure 1), while cows of high GM tended to have a lower prepartum MP balance (Table 3). Figure 1 shows this pattern continued into early lactation.

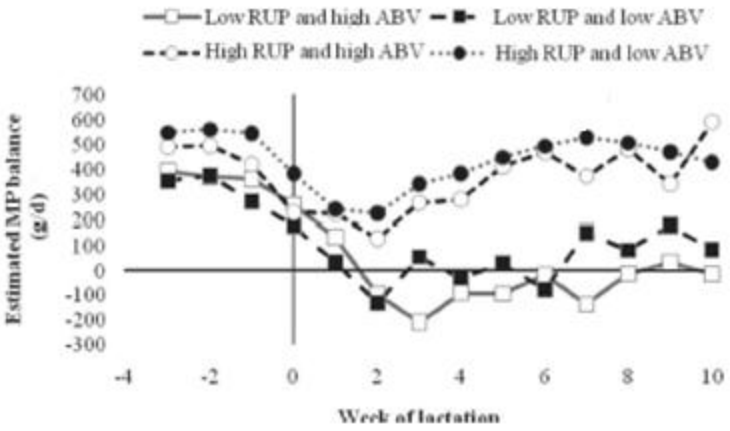

Figure 1. Effect of diet and genetic merit on estimated MP balance. ABV = Australian Breeding Value for milk fat + protein yield.

Table 3. Effects of diet and genetic merit (GM) on MP balance; model includes effects of diet, GM, and diet × GM interaction[1]

Evaluation	Predicted mean (SE) (g/d)	95% CI	Significance (*P*-value)
Diet			0.001
Low RUP	87.59 (24.08)	40.40–134.78	
High RUP	405.77 (23.50)	359.72–451.82	
Genetic merit			0.096
High ABV[2]	223.15 (23.50)	177.10–269.20	
Low ABV	279.34 (24.08)	232.15–326.53	
Diet × GM[3]			0.794

[1]Week $P = 0.0001$; diet × GM × week $P = 0.6295$.

[2]ABV = Australian Breeding Value for milk fat + protein yield.

[3]Predicted means for each of the 4 diet × GM interactions did not differ significantly and are not shown.

Production Responses

Univariable and multivariable relationships between indicator and production variables, in which diet, GM and week were included as *a priori* factors, are provided in Supplementary Table 2 and Table 4, respectively.

Milk protein yield and percentage. The increase in milk protein production with the higher RUP iet was marked (0.13kg/cow/d) (Table 4). Milk protein yield increased by approximately 0.08 kg/cow/d in the group with higher GM for milk solids production (Table 4). Protein yield was associated with higher pre-calving plasma AAN concentrations, BW and MP balance (Table 4, Supplementary Table 2). Milk protein percentage was unaffected by diet or GM (Table 4). Increased prepartum blood cholesterol, BW and plasma AAN were associated with lower milk protein percentage (Table 4, Supplementary Table 2).

Milk casein yield and percentage. Decreasing the degradability of the protein in the diet increased milk CN yield by 0.09kg/d (Table 4, Figure 2). Body weight, MP balance, glucose and disease all had significant associations with CN yield (Supplementary Table 2). Higher CN yield was observed in cows with higher pre-calving BW, consistent with the positive influence of BW on total milk protein yield. Incidence of severe disease lowered CN yield, when included in the model with BW. Increased prepartum plasma glucose concentrations were associated with lower CN concentration, as was disease, plasma calcium and cholesterol when explored individually (Supplementary Table 2), but these latter three factors were confounded by glucose concentrations and became non-significant in multivariable models containing plasma glucose (Table 4). Milk CN concentration was lower, consequently, with higher glucose, BW and AAN (Table 4). Diet and GM did not influence milk CN percentage (Table 4, Figure 3).

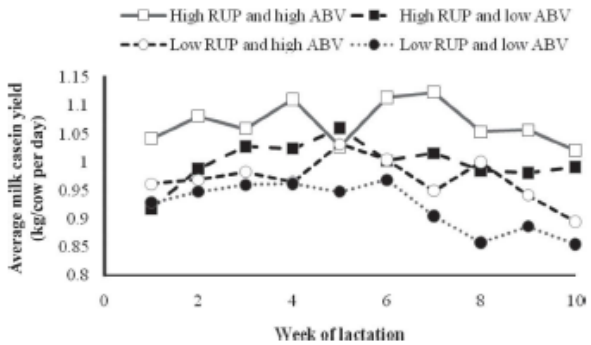

Figure 2. Average milk casein yield by week for diet and genetic merit groups. ABV = Australian Breeding Value for milk fat + protein yield.

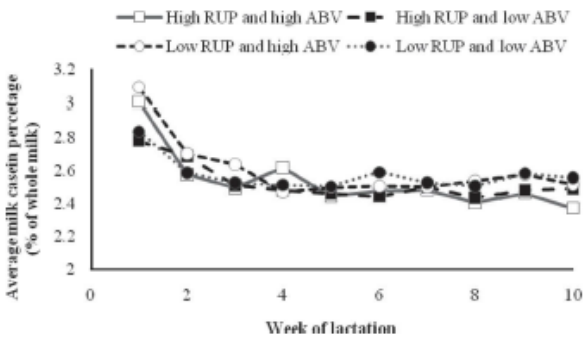

Figure 3. Average milk casein percentage by week for diet and genetic merit groups. ABV = Australian Breeding Value for milk fat + protein yield.

Casein variant yields and percentages. Yields (kg/d) for CN variants were 0.49 and 0.45 of alpha CN, 0.38 and 0.34 of beta CN, and 0.07 and 0.06 of kappa CN, for high and low RUP diets, respectively. Gamma CN had yields of 0.10 and 0.09 for high and low RUP diets, respectively. Reducing the degradability of protein in the diet increased yields of alpha and beta CN, but did not significantly influence concentrations of any CN variants (Table 4). Largely consistent associations were found in the models evaluating the effects of individual indicator variables on CN variant yields and concentrations, with higher BW and AAN resulting in higher yield, but lower concentrations; decreased protein yield was associated with disease and an increase in cholesterol was associated with decreased CN concentration. Alpha and beta CN yields were affected by the same variables as CN yield; increased by BW and decreased by disease (Table 4). Increased BW lowered alpha and beta CN percentage, but increased kappa CN yield; while increased BCS increased only gamma CN yield. Increased cholesterol concentrations were associated with lowered alpha, gamma and kappa CN percentages and higher pre-calving plasma calcium concentrations lowered kappa CN yield. Increased AAN concentrations pre-calving increased kappa CN yield and decreased alpha and beta CN percentages.

Table 5. Summary of reproductive variables; mean (no of cows) for pregnancy to first service, mean (SD) for interval from calving to pregnancy

	Diet		Genetic merit[2]	
Reproductive variable[1]	High RUP	Low RUP	Low ABV	High ABV
Pregnancy to first service (%)	58 (23/40)	41 (17/41)	46 (18/39)	52 (22/42)
Interval from calving to pregnancy (d)	94.75 (32.47)	104.05 (36.28)	102.90 (5.33)	96.26 (33.92)

[1]Cows that were not pregnant at 150 DIM were considered nonpregnant, even if they became pregnant after 150 DIM, resulting in right censoring of the data.
[2]ABV = Australian Breeding Value for milk fat + protein yield.

Table 4. Multivariable models for milk yield and composition including indicator variables, and diet and genetic merit as a priori factors

Dependent variable	Covariable	Regression coefficient (SE)	Significance	Diet [Mean (SE)]			Genetic merit [Mean (SE)]		
				High RUP	Low RUP	*P*-value	High ABV[1]	Low ABV	*P*-value
Milk yield (kg/d)	BW	0.043 (0.008)	0.001	39.66 (0.85)	36.30 (0.82)	0.004	39.07 (0.84)	36.74 (0.85)	0.053
	AAN[2]	4.042 (1.270)	0.001						
Milk protein yield (kg/d)	BW	0.001 (0.001)	0.001	1.26 (0.02)	1.13 (0.02)	0.001	1.23 (0.02)	1.15 (0.02)	0.014
	MP balance	0.001 (0.001)	0.002						
	AAN	0.101 (0.019)	0.001						
Milk protein %	Cholesterol	−0.079 (0.029)	0.007	3.07 (0.03)	3.09 (0.03)	0.664	3.07 (0.03)	3.09 (0.03)	0.689
	AAN	−0.096 (0.046)	0.035						
	BW	−0.001 (0.001)	0.038						
Milk CN yield (kg/d)	BW	0.001 (0.001)	0.001	1.04 (0.02)	0.95 (0.02)	0.002	1.01 (0.02)	0.97 (0.02)	0.097
	Disease	−0.077 (0.029)	0.009						
Milk CN %	BW	−0.001 (0.001)	0.001	2.54 (0.03)	2.58 (0.03)	0.353	2.54 (0.03)	2.58 (0.03)	0.408
	Glucose	−0.134 (0.047)	0.005						
	AAN	−0.214 (0.048)	0.001						
Milk α-CN yield (kg/d)	BW	0.001 (0.001)	0.001	0.49 (0.01)	0.45 (0.01)	0.002	0.48 (0.01)	0.46 (0.01)	0.248
	Disease	−0.033 (0.016)	0.034						
Milk α-CN %	BW	−0.001 (0.001)	0.005	1.19 (0.02)	1.22 (0.02)	0.039	1.19 (0.02)	1.22 (0.02)	0.126
	AAN	−0.076 (0.028)	0.007						
	Cholesterol	−0.045 (0.019)	0.015						
Milk β-CN yield (kg/d)	Disease	−0.036 (0.013)	0.006	0.38 (0.01)	0.34 (0.01)	0.001	0.36 (0.01)	0.36 (0.01)	0.555
	BW	0.001 (0.001)	0.003						
Milk β-CN %	BW	−0.001 (0.001)	0.001	0.93 (0.02)	0.92 (0.02)	0.685	0.91 (0.02)	0.95 (0.02)	0.119
	AAN	−0.078 (0.025)	0.002						
Milk γ-CN yield (kg/d)	BCS	0.026 (0.010)	0.009	0.10 (<0.01)	0.09 (0.004)	0.149	0.10 (<0.01)	0.09 (<0.01)	0.015
Milk γ-CN %	Cholesterol	−0.029 (0.012)	0.011	0.24 (0.011)	0.25 (0.011)	0.539	0.25 (0.011)	0.24 (0.011)	0.341
Milk κ-CN yield (kg/d)	BW	0.001 (0.001)	0.001	0.07 (<0.01)	0.06 (<0.01)	0.442	0.07 (<0.01)	0.07 (<0.01)	0.944
	AAN	0.014 (0.003)	0.001						
	Ca	−0.014 (0.006)	0.020						
Milk κ-CN %	Cholesterol	−0.019 (0.006)	0.001	0.16 (0.01)	0.18 (0.01)	0.063	0.16 (0.01)	0.18 (0.01)	0.174
	AAN	0.019 (0.009)	0.028						

[1]ABV = Australian Breeding Value for milk fat + protein yield.
[2]AAN = α-amino nitrogen.

Reproductive Responses

Increasing the percentage of RUP in the diet increased the percentage of pregnancies to first service from 41% to 58% and tended to decrease the calving to pregnancy interval by 9 d (Table 5), although diet and GM were not significant when assessed in the multivariable model (Table 6). In the multivariable model, higher early lactation CN % and milk protein yield (kg/d), and decreased prepartum MP balance (g/d) improved the proportion of first services that resulted in pregnancy when diet and GM, and their interaction, were included in the statistical model (Table 6). Individually, improved CN yield tended to be associated with increased pregnancy to first service (Supplementary Table 3), a result consistent with the positive effect of the higher RUP diet on CN yield and pregnancy to first service. None of the other factors tested univariably or as time-varying covariables influenced calving to pregnancy interval (data not shown).

Table 6. Logistic regression model of factors affecting the proportion of first services that resulted in pregnancy

Variable	Odds ratio (SE)	95% CI	Significance (*P*-value)
Diet[1]	0.86 (0.622)	0.210–3.545	0.839
Genetic merit (GM)[2]	0.86 (0.603)	0.217–3.401	0.828
Diet × genetic merit	1.10 (1.090)	0.156–7.697	0.926
CN %	9.86 (10.261)	1.283–75.782	0.028
Milk protein yield (kg/d)	20.09 (27.721)	1.344–300.264	0.030
MP balance (g/d)	1.00 (0.002)	0.993–0.999	0.044

[1]The diet higher in RUP was used as the reference group. The effect of diet is at the reference category of GM.
[2]The higher ABV was used at the reference group; the effect of GM is at the reference category of diet.

Low Milk Protein and Casein

To explore the hypothesis that low milk protein can predict poor fertility, cows were divided into quartiles and the group producing the lowest milk protein percentage was compared with the cows in the upper three quartiles. The hazard of pregnancy was 28% lower in cows producing the lowest quartile of milk protein percentage during the first 150 DIM when compared with the upper three quartiles (Table 7, Figure 4). Differences in the pre-calving indicator variables between the two groups were explored using discriminant analysis and logistic regression. Both methods identified the same factors associated with lowest milk protein percentage (Table 8). Cows producing a lower milk protein percentage produced, on average, 3.87L/cow/d more milk than cows in the other three quartiles (Table 9) and had higher pre-partum plasma calcium and glucose, and estimated MP balance compared to the higher milk protein percentage producing group (Tables 7 and 8). The low milk protein producing group had on average, 0.17mmol/L less urea in the blood prepartum, than the group producing a higher milk protein percentage.

Table 7. Multivariable model of factors predicting time to pregnancy within 150 d of calving using a logistic normal nonparametric accelerated time failure survival analysis, with milk protein percentage as a binary variable (lowest quartile as reference group)

Variable	Hazard ratio (SE)	95% CI for hazard ratio	Significance (*P*-value)
Diet[1]	1.05 (0.115)	0.850–1.303	0.640
Genetic merit[2]	1.01 (0.110)	0.812–1.245	0.962
Milk protein %	0.72 (0.096)	0.556–0.936	0.014

[1]The diet higher in RUP was used as the reference group.

[2]Genetic merit is defined as Australian Breeding Value (ABV) for milk fat + protein yield. The higher ABV was used as the reference group.

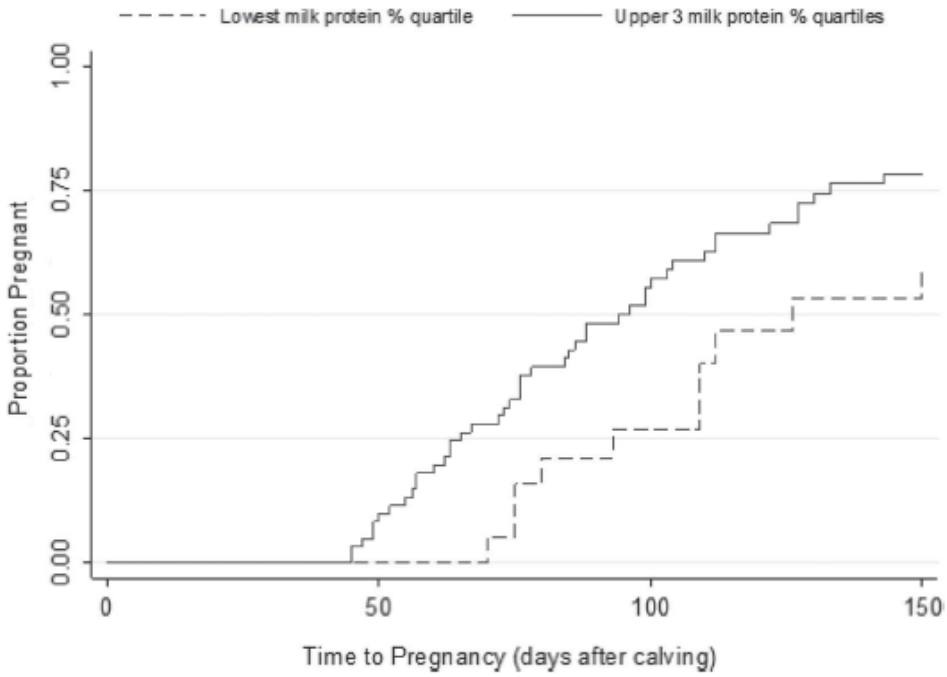

Figure 4. Kaplan-Meier survival plot of the time to pregnancy within 150 d, separated for cows producing the lowest quartile of milk protein percentage and the upper 3 quartiles of milk protein percentage.

DISCUSSION

This study re-examines and builds on the original work of Garvin (1999) and Westwood (1998, 2000, 2002), using their milk composition and reproductive data sets respectively. The detail and high quality of this data set provided the opportunity to explore new hypotheses and improve metabolic understanding of milk protein and CN variants. This study had three main aims; to i) describe the effects of protein nutrition and GM on CN variants; ii) to explore pre-calving and early lactation factors that may predict production, especially protein and CN production, and reproductive performance, iii) to evaluate an *a priori* hypothesis that low milk protein content is associated with poor fertility and to understand the factors, including CN variant yield and composition, that contribution to this. Factors after calving and around breeding that can be used as predictors of fertility and production were well-explored by Westwood et al. (2000, 2002). This study explores factors before calving and in very early lactation that influence fertility and

production. Due to the large amount of analyses, we have not sought to address all of these in the discussion but have focused on the most important results, which are summarised in the conclusion, and focused on consistently identified metabolic influences on production and reproduction in the study.

Table 8. Multivariable model for factors affecting low milk protein percentage, created using backward stepwise logistic regression

Variable	Odds ratio	95% CI	Significance (*P*-value)
Average milk yield (kg/d)	0.91 (0.041)	0.831–0.993	0.034
Average urea (mmol/L)	1.97 (0.624)	1.063–3.666	0.031
Average glucose (mmol/L)	0.24 (0.170)	0.058–0.971	0.045
Average calcium (mmol/L)	0.04 (0.055)	0.002–0.653	0.024
Average MP balance (g/d)	1.00 (0.002)	0.992–1.000	0.029

Table 9. Group means (SD) of indicator variables differing between cows of lowest quartile for milk protein percentage or in the 3 higher quartiles identified using canonical linear discriminant analysis

Variable	Low milk protein	Average-high milk protein
Average milk yield (L/d)	40.70 (6.901)	36.83 (6.844)
Average urea (mmol/L)	5.64 (1.247)	5.81 (1.302)
Average glucose (mmol/L)	3.77 (0.380)	3.57 (0.486)
Average calcium (mmol/L)	2.37 (0.295)	2.24 (0.235)
Average MP balance (g/d)	497.38 (174.013)	414.43 (175.375)

Individual CN composition may affect milk processing outcomes, however; there is little information on yields of CN variants (Barry and Donnelly, 1980, Kroeker et al., 1985, Davies and Law, 1987) or nutritional and metabolic factors that may determine individual CN yields or percentages. Average CN content varied considerably between individual cows (range 1.3 - 4.5% total milk, average 2.6%) (Garvin, 1999). In this study, when determined as a percentage of total CN, alpha, beta, kappa and gamma represented approximately 47, 37, 7 and 9% respectively (Garvin, 1999). Alpha CN concentrations were similar to those presented by Davies and Law (1980), Barry and Donnelly (1980) and Kroeker et al. (1985) (48.3, 48.7 and 59.9 % respectively). Beta CN percentage was similar to that of Davies and Law (1980) and Barry and Donnelly (1980) (35.7 and 33.8%, respectively) although kappa CN was lower and gamma CN was higher than previously reported (12.8 and 3.2% for kappa and gamma CN, respectively) (Davies and Law, 1980). Different milk protein polymorphisms may also be associated with changes in milk, milk fat and milk protein yields (Ng-Kwai-Hang et al., 1984), but these were not explored in this study. It is unsurprising that the factors affecting alpha and beta CN yields were the same those identified for total CN yield because, when combined, these accounted for more than 80% of total CN yield.

Milk yield and milk protein responses to changes in dietary protein and AA supply are variable (Doepel et al., 2004). In this study, increasing the ratio of RUP : RDP in the diet by using a high quality protein source increased early lactation milk, milk protein and milk CN yield. The milk yield response is similar to linear increases in production in response to increased RUP supplementation (Wright et al., 1998). However, in a review of 127 trials that replaced soybean meal with sources higher in RUP, only 17% of comparisons identified significantly higher milk yield in groups fed higher RUP (Santos et al., 1998). This effect varied, based on the type of RUP source used, possibly due to changes in profile of absorbed AA. Sannes et al. (2002) did not find an effect on milk yield of feeding increased RDP, nor did Reynal and Broderick (2005). These varying responses reflect the complexity of assessing and improving MP status and a limitation in study design of nutritional studies, in which the substitution of one feed for another creates a complex series of changes that may not simply increase the MP supply of the most rate limiting AA, nor improve the overall nutrient balance. Importantly, in the current study, lower protein degradability significantly increased estimated MP balance (Table 3) and increased milk, protein, and CN yields all indicating a positive effect of the diet higher in RUP (Table 2, Table 4). Further, milk alpha, beta and gamma CN yields were significantly increased by feeding the diet higher in RUP (Table 2, Table 4) with only the percentage of alpha CN declining significantly with diet or GM. In general, increased yield of milk proteins is associated with lower concentrations, often as a result of increased milk production.

The tendency for increased early lactation milk yield for cows with higher ABV (Table 4) has been identified in other studies (Flux et al., 1984). Increased prepartum MP balance increased milk protein yield (Table 4). Cows in both dietary groups had excellent weight gain before calving; suggesting neither group was limited by ME or MP availability prepartum (Table 2). The cows with higher ABV tended to have lower prepartum estimated ME and MP balances (Westwood et al., 2000) (Table 2, Table 4). There is abundant literature examining the detrimental effects of a negative energy balance on fertility (Wathes et al., 2007, Butler, 2012). However, in this study, ME balance before calving did not contribute to any production or fertility model (Table 4). Milk production and DMI are key determinants of ME and MP balance and there should be further consideration of the importance of MP balance in fertility studies, given the current results.

Further insight into the factors present before calving that influence production traits, is provided in Table 4 and Supplementary Table 2. Prepartum BW and AAN, increased production for most

production variables studied (Table 4, Supplementary Table 2). Body weight reflects the endogenous nutrient reserves available to the cow and it is unsurprising that this factor increased milk, protein, CN, alpha CN, beta CN, and kappa CN yields, as it reflects an improved energy and nutrient status of cows. However, percentages of CN and beta CN were lower with higher BW pre-calving. Free circulating AA, as indicated by a higher AAN concentration, are the main precursors for milk protein and perhaps reflect labile protein stores, and were associated with higher milk, protein, and kappa CN yields. Again lower milk CN concentration, including alpha and beta CN, and consequently lowered milk protein percentage were associated with higher prepartum AAN (Table 4). It is possible that the lower percentages of proteins may have resulted from the positive effect of BW and AAN on milk yield. A negative effect of cholesterol on milk protein, alpha, gamma and kappa CN percentages (Table 4) was unexpected and a causal link is not immediately obvious. However, increased cholesterol reflects a more positive energy balance (Lean et al., 1992) and may indicate a more anabolic state in which milk production is increased, decreasing milk protein and CN content. Therefore, the association of cholesterol itself with milk protein and CN content may not be causal but reflect this relationship with yield. Higher blood glucose, calcium and cholesterol concentrations were associated with lower CN concentration, but glucose statistically confounded the other variables which became non-significant in multivariable models. Prepartum plasma calcium was a significant factor in several models (Supplementary Table 2), but only increased kappa CN yield in the multivariable model. This may reflect the metabolic interactions between calcium and other indicator variables. For example, bone calcium and glucose are causally linked through osteocalcin and insulin metabolism, as was shown in murine studies (Lee et al., 2007), and reviewed in a bovine context (Lean et al., 2014). Elegant intervention studies by Martinez et al. (2014) demonstrated the central role of hypocalcaemia in influencing insulin and glucose concentrations. Further work is required to confirm the importance of these observations, but suggests that relationships among bone and energy metabolism may be reflected in production and reproductive outcomes.

Westwood et al. (2002) identified a positive effect of increased RUP intake on fertility. However, in a multivariable model containing diet, GM, diet x GM interaction, CN %, and milk protein yield, higher prepartum MP balance lowered the risk of pregnancy to first service (Table 6). Other factors in this model, namely, CN % and milk protein yield, increased the proportion pregnant to first service, suggesting that cows receiving a diet higher in RUP that resulted in higher milk CN %, were less successful reproductively if they retained more MP pre-calving or were less efficient with MP utilization. On average, cows in both dietary groups had substantial

positive MP balances before calving. These findings, in conjunction with the results in Table 4 and Supplementary Table 2, suggest the possibility that the reproductive response to anabolic stimuli pre-calving such BW, MP balance, and AAN that produced more milk, protein and CN yields, may be curvilinear. Diets after calving may not be able to provide the protein, energy, fats and other nutrients required to produce the extra proteins, fat, lactose and other components of increased milk production stimulated by improved protein status pre-partum.

Support for this concept comes from the observation that cows in the lowest quartile of milk protein percentage had slower times to pregnancy within 150 d of calving compared with cows in the other three quartiles. Further, a numerically lower proportion of the cows in this group were pregnant by 150 DIM (Table 7, Figure 4). Cows with higher milk CN percentage had greater odds of conception to first service (Table 6). The multivariable model that described the linear milk protein percentage response (Table 4) differs from the factors that were associated with the lowest milk protein percentage quartile group (Table 9), however there is considerable consistency as both models indicate that factors that reflect an increased stimulus to milk reduced milk protein percentage. Further, the cows in these groups differed between the analyses, as the later compared one quartile of cows with the other three. Cows in the lowest protein group had on average, higher milk yield than those producing milk of a higher protein percentage. Plasma calcium and glucose, and estimated MP balance were all higher before calving in the group producing low milk protein percentage. The latter measures reflect anabolic processes, and support the observation that these cows had increased early lactation milk yield. The lowest milk protein group would, therefore, have had a greater irreversible loss of nutrients in milk, in particular thorough increased lactose production, and possibly an increase in protein and glucose used for mammogenesis in the early lactation period. Bickerstaffe et al. (1974) estimated that 69-98% of circulating glucose was used by the mammary gland. Most of this glucose is used as a precursor for lactose synthesis, but it is also essential as the main source of energy for the mammary gland (Wood et al., 1965) and ovary (Rabiee et al., 1997). Consequently, low milk protein and CN content may reflect disordered metabolic control in cows stimulated to produce more lactose and protein. Indicator variables before calving and a diet higher in RUP that had positive effects on reproduction and production reflected anabolic processes; however, it appears that not all cows are capable of integrating the nutrient demands for lactation with those required for reproductive outcomes. It is possible that this population may be identified through a low milk protein or CN concentration.

CONCLUSIONS

The objectives of this study were: i) to describe the effects of protein nutrition and GM on CN variants; ii) to explore pre-calving and early lactation factors that may predict production, especially protein and CN production, and reproductive performance; and iii) to evaluate an a priori hypothesis that low milk protein content is associated with poor fertility and to understand the factors, including CN variant yield and composition, that contribution to this. In relation to each of these objectives, we were able to conclude that: i) Decreasing protein degradability in the diet increased milk, milk protein and CN yields. Increased GM increased milk protein and gamma CN yields and tended to increase milk CN yield ii) The effects of indicator variables that may predict productive and reproductive outcomes were largely consistent, confirming the importance of some well determined causal factors such as BW and disease, and also identifying some less well explored indicators including cholesterol and AAN that are worthy of further exploration. A diet high in RUP increased the proportion of first services that resulted in pregnancy, suggesting a positive effect of increased MP on fertility. However, when combined in a model containing CN yield, diet, and GM, and diet x GM interaction, higher prepartum MP balance lowered the risk of pregnancy to first service. This suggests that the effects of increasing dietary RUP on fertility may be curvilinear. Anabolic factors measured pre-partum, including BW, AAN, cholesterol and glucose, increase production of milk, protein, CN and CN variants, but tended to reduce the percentage of these in milk. iii) Low milk protein percentage in early lactation was associated with a lower risk of pregnancy within 150 DIM and a low milk CN percentage was associated a lower proportion of cows pregnant at first service. Early lactation milk yield, and pre-partum MP balance, and glucose and calcium concentrations were higher in the cows producing lower milk protein percentage while pre-partum urea was higher in this group. Metabolizable protein balance was significant and important to both production and reproduction, while surprisingly, ME balance was not. This study demonstrates the importance of protein metabolism to productive and reproductive performance.

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Supplementary Table 1. Ingredients and nutrient composition of the diets.

Ingredient (% of TMR)	TMR				
	High RUP Dry Cow	Low RUP Dry Cow	High RUP Lactating A	High RUP Lactating B	Low RUP Lactating
Forage (chopped)					
Alfalfa hay			35.0	35.0	35.0
Oaten hay	60.0	60.0	5.0	5.0	5.0
Pelleted concentrate	40.0	40.0	60.0	60.0	60.0
Components of pelleted concentrate (% of pellet) as formulated					
Sorghum, ground	10.6	5.3	24.8	24.5	27.8
Wheat middlings	22.4	23.9	7.3	10.5	4.3
Wheat, ground		8.0			12.0
Meatmeal	19.4		9.3	9.2	1.8
Cottonmeal	4.7		14.9		
Soymeal				3.8	
Protected canola				8.3	
Tallow		7.0	3.3	3.4	4.5
Limestone					0.8
Dicalcium phosphate	1.0	0.9			1.6
Salt				0.01	0.1
Sodium bicarbonate	0.2	0.2	0.3	0.3	0.3
Urea		0.9			2.4
Dairy premix	0.1	0.1	0.1	0.1	0.1
Composition of TMR from NLIS analyses (% of DM)					
DM%	89.4	89.4	89.5	NT	89.8
CP	10.4	10.9	19.1	NT	19.5
RUP, % of CP as formulated	44.7	14.2	34.8	NT	15.3
RUP, % of DM as formulated	4.6	1.5	6.6	NT	3.0
RUP, % of CP, actual	NT	NT	40.1	NT	25.4
RUP, % of DM, actual	NT	NT	7.7	NT	5.0
Crude fiber	21.7	21.5	16.8	NT	16.6
ADF	27.1	26.8	21.0	NT	20.8
ME (MJME/kg DM)	9.9	10.0	10.9	NT	10.9

High RUP lactating diet A was fed to cows in this group for the majority of the study. Diet B was fed to cows in this group for 12 weeks of the 2 year study, when a period of drought limited the availability of dietary ingredients.
NT = not tested

Supplementary Table 2. Significance (P-values) of univariable associations between disease, indicator variables (average non-esterified fatty acids, urea, alpha amino nitrogen, glucose, calcium, cholesterol, beta-hydroxybutyrate, cholesterol, body condition score and body weight) measured in the three weeks before calving, and production measures including milk yield, protein percentage and yield and protein variant percentages and yield. Variables were univariably tested in mixed models containing diet, genetic merit and week. Those with P<0.2 are highlighted in bold.

	P-value											
	BW (kg)	BCS (1-5 scale)	MP Balance (g/d)	ME Balance (MJME/d)	NEFA (µmol/L)	BHBA (mmol/L)	Urea (mmol/L)	AAN (mg/100mL)	Glucose (mmol/L)	Calcium (mmol/L)	Cholesterol (mmol/L)	Disease
Milk Yield (kg/d)	0.001 +	0.547	0.062 +	0.645	0.796	0.875	0.864	0.024 +	0.717	0.614	0.540	0.228
Milk Protein Yield (kg/d)	0.001 +	0.490	0.017 +	0.391	0.572	0.940	0.664	0.021 +	0.404	0.934	0.884	0.294
Milk Protein %	0.128 -	0.780	0.489	0.853	0.852	0.917	0.950	0.011 -	0.088 -	0.116 -	0.004 -	0.863
Milk Casein Yield (kg/d)	0.001 +	0.189 +	0.132 +	0.819	0.670	0.741	1.000	0.338	0.153 -	0.445	0.629	0.091 -
Milk Casein %	0.019 -	0.840	0.211	0.647	0.178 +	0.410	0.621	0.001 -	0.031 -	0.006 -	0.007 -	0.035 -
Milk Alpha Casein Yield (kg/d)	0.001 +	0.202	0.090 +	0.834	0.592	0.433	0.989	0.730	0.371	0.662	0.629	0.153 -
Milk Alpha Casein %	0.059 -	0.986	0.331	0.793	0.316	0.302	0.128 -	0.003 -	0.030 -	0.019 -	0.006 -	0.077 -
Milk Beta Casein yield (kg/d)	0.012 +	0.328	0.211	0.462	0.688	0.371	0.548	0.826	0.764	0.936	0.543	0.023 -
Milk Beta Casein %	0.002 -	0.792	0.298	0.200	0.493	0.281	0.967	0.010 -	0.288	0.234	0.279	0.027 -
Milk Gamma Casein yield (kg/d)	0.060 +	0.009 +	0.860	0.749	0.127 +	0.664	0.348	0.776	0.336	0.055 -	0.046 -	0.405
Milk Gamma casein %	0.446	0.061 +	0.424	0.686	0.095 +	0.583	0.258	0.154 -	0.354	0.026 -	0.011 -	0.689
Milk Kappa Casein yield (kg/d)	0.001 +	0.350	0.432	0.538	0.365	0.686	0.090 -	0.004 +	0.123 -	0.104 -	0.174 -	0.797
Milk Kappa Casein %	0.923	0.598	0.311	0.680	0.040 +	0.847	0.006 -	0.165+	0.049 -	0.010 -	0.005 -	0.616

+ or – following the value indicates the point direction, increased + or decreased -, provided for variables where P <0.2

Supplementary Table 3. Individual logistic regression models of the effects of milk and casein variants, and metabolic indicators, on proportion of first services that resulted in pregnancy including diet and genetic merit as *a priori* factors. Metabolic indicators with P>0.2 have been removed.

Variable	Coefficient (SE)	95% Confidence Interval	Significance (P-value)
Milk Yield (kg/d)	1.02 (0.035)	0.958 to 1.094	0.494
Milk Protein Yield (kg/d)	6.39 (7.757)	0.591 to 69.027	0.127
Milk Protein %	8.69 (10.129)	0.883 to 85.402	0.064
Milk Casein Yield (kg/d)	16.59 (24.454)	0.923 to 298.198	0.057
Milk Casein %	7.07 (6.788)	1.075 to 46.438	0.042
Milk Alpha Casein Yield (kg/d)	6.94 (18.787)	0.034 to 1401.413	0.475
Milk Alpha Casein %	1.49 (2.296)	0.072 to 30.649	0.797
Milk Beta Casein Yield (kg/d)	915.12 (3352.429)	0.697 to 1201568	0.063
Milk Beta Casein %	29.04 (57.480)	0.600 to 1405.096	0.089
Milk Gamma Casein yield (kg/d)	322337.3 (2840369)	0.010 to $1.02e^{+13}$	0.150
Milk Gamma Casein %	78.91 (278.115)	0.079 to 78922.54	0.215
Milk Kappa Casein Yield (kg/d)	294.62 (4412.143)	$5.27e^{-11}$ to $1.65e^{+15}$	0.704
Milk Kappa Casein %	1.29 (7.893)	$7.65e^{-06}$ to 216016.1	0.967
MP Balance (g/d)	1.00 (0.002)	0.995 to 1.000	0.094
ME Balance (MJME/d)	1.00 (0.007)	0.973 to 1.002	0.088
Urea (mmol/L)	1.28 (0.238)	0.892 to 1.846	0.179

CHAPTER FIVE: METABOLIC AND PRODUCTION RESPONSES TO CALCIDIOL TREATMENT IN MID-LACTATION DAIRY COWS

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OVERVIEW OF CHAPTER FIVE

The study of vitamin D in cattle has addressed the important roles of vitamin D in mineral, and particularly, calcium metabolism. There is, however, increasing evidence of a wider role of vitamin D in integrated metabolism, through linkages with bone and energy. This Chapter presents the results of two randomised controlled experiments conducted to evaluate the usefulness of calcidiol supplementation to alter mineral, bone, and energy metabolism in mid-lactation cows. Mid-lactation cows were used in this study to allow effects to be assessed without the metabolic complications brought about by the transition to lactation. The first experiment examined responses of cattle to increasing calcidiol doses, and the second used time-series analysis to explore relationships between metabolites associated with vitamin D, mineral, and energy metabolism over time.

ABSTRACT

The study of vitamin D in cattle has often focused on its role in calcium and mineral metabolism. However, there is evidence of a wider role for vitamin D in bone and energy metabolism. Two studies were conducted to explore relationships between calcidiol supplementation, blood minerals and metabolites in mid-lactation dairy cows. In experiment one, a dose response study was conducted in which 25 mid-lactation cows were fed one of five supplementary calcidiol doses (0, 0.5, 1, 2 or 4 mg calcidiol/day) for 30 days, with blood samples taken every 10 days. Increasing calcidiol dose increased plasma calcidiol ($P = 0.001$), 24,25-$(OH)_2$-D_3 ($P = 0.001$) and serum phosphate ($P = 0.003$) in a curvilinear manner, increased and then decreased plasma 25-OH-D_2 ($P = 0.004$) and linearly increased 3-epi 25-OH-D_3 ($P = 0.001$) and milk calcidiol concentrations ($P = 0.001$). Calcidiol supplementation did not affect milk yield or composition, body weight or condition score. In experiment two, relationships between blood calcidiol and mineral and metabolite concentrations over time were explored using time series analysis. Ten mid-lactation cows were fed either 0 or 0.5 mg calcidiol/day for 27 days with blood samples taken every 3 days. Feeding calcidiol increased plasma calcidiol ($P = 0.001$), 24,25-OH-D_3 ($P = 0.038$), and insulin ($P = 0.046$), but decreased 25-OH-D_2 ($P = 0.008$) concentrations. Positive associations were identified between blood calcidiol and concentrations of other metabolites including cholecalciferol, calcium, osteocalcin, glucose, insulin, non-esterified fatty acids, beta-hydroxybutyrate, cholesterol, magnesium, phosphorus and total protein at varying lags (± 0, 3 or 6 d), while negative relationships were identified between calcidiol and 24,25-$(OH)_2$-D_3, and phosphorus 3 d later. Importantly, strong positive associations between calcidiol and indicators of energy metabolism were identified. Overall, these experiments provide support for a positive effect of calcidiol treatment on dairy cow metabolism.

Keywords: calcidiol, osteocalcin, vitamin D

INTRODUCTION

The study of vitamin D in cattle has traditionally focused on its role in calcium (**Ca**) and mineral metabolism in response to hypocalcaemia and parturient paresis (Olson et al., 1973, Julien et al., 1977, Horst et al., 2003). Oral calcidiol (25-OH-D_3) supplementation can increase both blood calcidiol and calcitriol (1,25-$(OH)_2$-D_3) concentrations in cattle (Wilkens et al., 2012). In combination with a negative dietary cation-anion difference (DCAD) during the periparturient period, calcidiol increased plasma concentrations of ionized Ca when fed at doses of 3 mg calcidiol/day (Wilkens et al., 2012). Hence, strategic feeding of calcidiol may reduce the interval that occurs between incidence of low blood Ca and hormonally controlled increases in Ca replenishment, allowing the body to respond to metabolic stressors more rapidly.

Vitamin D is also essential for correct bone function, regulating the activity of osteoblasts and osteoclasts that remodel and regulate bone mass (Tanaka and DeLuca, 1971). Increased serum calcitriol, in combination with parathyroid hormone (**PTH**), stimulates bone resorption when Ca concentrations are low (Goff et al., 1991). However, evidence suggests that calcidiol and calcitriol will not mobilize Ca from bone without the presence of increased PTH, and may directly increase bone deposition by increasing Ca and phosphate (**P**) available for bone mineralization (McGrath et al., 2015). In contrast to calcitriol, 24,25-$(OH)_2$-D_3 may have a role in inhibiting bone mobilization, resulting in impaired mineral homeostasis (Smith et al., 1982). Vitamin D also stimulates the production of osteocalcin (**OC**) from mature osteoblasts. There are 2 forms of OC, a carboxylated form (**cOC**) which has high affinity for bone and is considered biologically inactive, and, following a vitamin K dependant decarboxylation, an undercarboxylated, biologically active, form (**uOC**) which influences the regulation of energy metabolism by increasing insulin release and tissue sensitivity of insulin (Lee et al., 2007).

The integrated metabolic response, including the timing of response, between mineral balance, skeletal function, and energy metabolism is not well understood. Lean et al. (1992) used time series cross-correlation analysis to identify temporal relationships between metabolites of energy balance (beta-hydroxybutyrate (**BHB**), glucose, cholesterol, and non-esterified fatty acids (**NEFA**)) over a period as long as 9 days, that were consistent with homeorhetic changes in metabolism in response to increasing milk production and decreasing estimated net energy balance. The links between vitamin D and bone metabolism require examination in a homeorhetic context. Despite evidence for the positive metabolic effects of supplementary

calcidiol, the optimum dose required to improve dairy cattle performance and health is unknown. Excessive doses of calcidiol may have negative effects on mineral homeostasis including phosphorus metabolism (McGrath et al., 2012) or hypocalcaemia (Weiss et al., 2015).

Experiment one explored the response of mid-lactation cows to increasing doses of calcidiol and aimed to identify the dose that increased plasma calcidiol concentration to approximately double the normal concentration of the control cows. We hypothesized that plasma calcidiol concentrations would increase as supplementary calcidiol dose increased. Experiment two utilized time series analysis methods to explore the response of blood minerals and metabolites to calcidiol treatment and their interactions over time to better understand the underlying physiological processes and integration of vitamin D metabolism. We hypothesized that associations among calcidiol and glucose, cholesterol, NEFA and BHB would be observed.

MATERIALS AND METHODS

All practices were approved and reviewed by S*cibus* Animal Care and Ethics Committee (SBScibus 1213-1214), a committee accredited by the NSW Department of Primary Industries. Two studies were conducted to examine the effects of calcidiol feeding on blood mineral and metabolite concentrations and production. The first of these experiments examined the responses of mid-lactation cattle to increasing calcidiol dose and was intended to identify the dose required to double the normal plasma calcidiol concentration after 30 days of feeding, and identify any effects of calcidiol feeding on milk production and composition, body weight (**BW**) and body condition score (**BCS**). The second experiment utilized time series analysis techniques to examine the relationships between blood concentrations of calcidiol, Ca, P, Magnesium (**Mg**), bone hormones and metabolites over time.

Experiment One: Dose Response

Cows, treatments and environment. This study was conducted between August and September (Australian winter-spring) in Camden, New South Wales, Australia. Over this period, the average maximum daily temperature was 25◦C ± 5, average minimum temperature was 9◦C ± 3, total precipitation was 46.4 mm and daily sunshine hours were 7-8 hours/day. Twenty five mid-lactation Holstein dairy cows (206 ± 53 days in milk) of mixed ages were blocked by age (63 ± 17 mo), and milk production (20 ± 9 L) and randomly allocated to receive one of five doses of supplementary calcidiol (0, 0.5, 1, 2, or 4 mg of active calcidiol/day) (n = 5 cows/treatment).

Sample size was determined to be the number of cows among which we could expect to see differences, based on previous work by one of the authors (McGrath et al., 2012). Treatments were pre-mixed with wheat mill run and individually top dressed onto wheat mill run offered in the parlour once daily for 30 d. Feed bins were fitted with liners that were cleaned between each cow. Cows were milked twice daily, at approximately 05:30 and 14:00 in a 10 bale walkthrough parlour.

Diet. Cows were maintained on a diet representative of an extensive grazing dairy system in Australia. The diet consisted of a lucerne (*Medicago sativa*) based pasture grazing allocation each morning, supplemented with wheat mill run in the parlour during milking twice a day, and a mixture of greenchop (lucerne or oats (*Avena sativa)*) following the afternoon milking. From daily observations, the size of grazing allocations varied each day. Samples of each feed were taken weekly and composited by feed type into early (weeks 1-2 of trial) or late (weeks 3-4) diets for analysis. Pasture samples were collected by taking representative quadrats of pasture to the height to which the cows grazed the same pasture the previous day. The daily supplement allocation was weighed in a mixer wagon. Samples were dried and the dry matter availability was calculated for the daily grazing area or total amount of supplement on offer and divided by the number of cows in the herd. Cows were allowed to graze on kikuyu (*Pennesetum clandestinum*) based pasture between evening and morning milkings.

Feed analysis. Samples were dried in an oven (45˚C) for at least 48 h prior to analysis. The predicted chemical composition of the diet was calculated using the CPM-Dairy Ration Analyzer (version 3.1 ; Cornell-Penn-Miner, Cornell University, Ithaca, NY,USA) from ration components analysed by wet chemistry (Dairy One Cooperative Inc., Forage Testing Laboratory, Ithaca, NY) as per the methods described in Golder et al. (2012). Details of the predicted diet are provided in Table 1.

Sample collection. Milk volume was measured and samples were taken every 2 weeks, starting in the week prior to commencement of treatment. Samples were tested for milk protein yield and percentage, fat yield and percentage, and somatic cell count (**SCC**) by Dairy Express (Armidale, New South Wales Australia), and calcidiol concentrations were measured by DSM Nutritional Products (Basel, Switzerland). Blood serum and plasma (preserved with lithium heparin) samples were taken from the coccygeal vein or artery every 10 days, commencing the day before the start of treatment (n = 4/cow). Plasma samples were placed on ice immediately after

collection; while, serum samples were maintained at room temperature. Samples were centrifuged (Allegra X-12R; Beckman Coulter Australia Pty. Ltd., Gladesville, NSW, Australia) at 1,512 × g for 20 minutes at 4°C to separate plasma or serum. Plasma or serum was pipetted into 1.5 mL aliquots and frozen at -20°C for future analysis. Body weight and BCS (1-5 scale, (Edmondson et al., 1989)) were recorded after the morning milking every 2 weeks.

Sample processing and analysis. Serum concentrations of Ca, Mg, P, NEFA, BHB, cholesterol, glucose, and plasma calcidiol, cholecalciferol (vitamin D_3), 24,25-$(OH)_2$-D_3, 3-epi 25-OH-D_3, and 25-OH-D_2 concentrations were determined for each of the 4 samples taken from each cow. The following metabolites and minerals were measured using commercial assay kits according to manufacturer's protocols; P (981891/0, Thermo Fisher Scientific Oy, Vantaa, Finland); Ca (981367/981772, Thermo Fisher Scientific Oy, Vantaa, Finland), Mg (981884/5, Thermo Fisher Scientific Oy, Vantaa, Finland); Glucose (981340/981779, Thermo Fisher Scientific Oy, Vantaa, Finland); Cholesterol (TR13421, Fisher Diagnostics, Middletown, USA); Total Protein (Fisher Diagnostics, Middletown, USA); NEFA (FA115, Randox Laboratories Limited, Crumlin, UK); and BHB (RB1007, Randox Laboratories Limited, Crumlin, UK). Vitamin D metabolite concentrations were measured by DSM Nutritional Products (Basel, Switzerland) in which, after an addition of internal standard, a protein precipitation was performed with acetonitrile. After centrifugation, the supernatant was evaporated and the residue is reconstituted with methanol-acetonitrile-water solution. An aliquot was then injected in LC (Agilent 1290, PFP and C18 HPLC columns) MS/MS (ABSciex 4000) system and the detection of the specific fragment ions performed using multiple reaction monitoring mode (MRM). To assess the daily and long-term laboratory performance of the method, dedicated standard and quality control samples were analysed daily with unknown samples to ensure the accuracy and precision of the method. Data acquisition of extracted ion chromatograms, integration and quantification were performed by Analyst® software (ABSciex, Mt Waverley, Victoria, Australia).

Statistical analysis. All analysis was performed using Stata (Intercooled Stata v.13; StataCorp LP, College Station, Texas, USA). Autoregressive mixed models (STMIXED) were used to compare treatment and control groups over time for each blood metabolite. Somatic cell count concentrations were log transformed (log_e) before analysis. Pre-treatment metabolite concentrations taken at day 0 were included in each model as a covariate. The following linear mixed model was used:

$$Y_{ijl} = \mu + \beta_i + \gamma_j + (\beta\gamma)_{ij} + X_l + \varepsilon_{ijl}$$

Where Y_{ijl} = response to treatment I (i = 0, 0.5, 1, 2, or 4 mg calcidiol/day) at the j^{th} time (j = 1 to 4 sampling point) for cow number l (l = 1 to 25); μ = overall mean; β_i = fixed effect of treatment; γ_j = fixed effect of time period; $(\beta\gamma)_{ij}$ = effect of treatment by time interaction; X_l = random effect of cow; ε_{ijkl} = random residual error within cow l, on treatment i, at time j. An autoregressive (AR1) covariance structure was used after testing for covariance structures with best fit. As the treatment effects of calcidiol supplementation were expected to be cumulative, we also analysed the response at the final time point only (after 30 days of feeding) using a mixed model with a covariate, but no repeated measures. One cow from the group receiving 1 mg calcidiol/day was removed from Vitamin D analyses as this cow had no change in plasma calcidiol concentration over the treatment period and was several standard deviations below all others in the group after 20 and 30 days of treatment.

Experiment Two: Time Series Analysis

Cows, treatments, and environment. Ten mid-lactation, multiparous Holstein dairy cows (259 ± 61 days in milk) were blocked by age (83 ± 28 mo) and milk production (26 ± 5 L) and randomly allocated to receive one of two treatments (n = 5 cows / treatment); control diet (mill run only) or supplementary calcidiol (0.5 mg of active calcidiol/day combined with mill run). Treatment pre-mixes were individually top dressed onto mill mix in the parlour once daily for 27 days, following the same procedure as described for experiment one. This experiment occurred during January and February (summer) in Camden, NSW, Australia. During the experimental period, average daily maximum temperatures were 29°C ± 5, average daily minimum temperatures were 16°C ± 3, total precipitation was 56.8mm and daily sunshine hours were 6-7 hours/day.

Diet. As per experiment one, the daily ration consisted of a grazing allocation supplemented with wheat mill run and greenchop lucerne. Brewer's grain was also fed with the afternoon greenchop allocation. This diet was representative of a ration fed during drought conditions, as were occurring at the time the study. Samples of each feed were taken weekly and composited by feed type, and stage (early or late) of the experiment, and analysed by Dairy One Cooperative Inc. (Dairy One Cooperative Inc., Forage Testing Laboratory, Ithaca, NY). Details of the diet are provided in Table 1.

Table 1. Dietary ingredients and nutrient composition of diets fed in Experiment 1 and Experiment 2. These diets were for grazing cows and represent diets early (weeks 1 and 2 of feeding) and late (weeks 3 and 4 of feeding) in the study periods for each trial. Diets for grazing cattle vary daily with grass allocations and pasture composition.

Ingredient (% DM)	Experiment 1		Experiment 2	
	Early Weeks 1-2	Late Weeks 3-4	Early Weeks 1-2	Late Weeks 3-4
Mill run	13.8	13.8	21.0	20.5
Brewers grain	-	-	10.3	9.6
Lucerne Chop	18.6	-	30.9	30.6
Oaten Chop	-	28.9	-	-
Lucerne based pasture	19.2	9.1	10.1	13.6
Mixed kikuyu pasture	48.3	48.2	27.7	25.7
Metabolizable Energy Balance (MJ)	26.8	24.9	-33.9	-20.5
Metabolizable Protein balance (g)	432.0	244.8	-116.0	-53.4
CP (%)	18.4	15.7	18.3	17.8
Rumen Undegradable Protein (% Crude Protein)	36.8	36.6	38.8	37.8
Neutral Detergent Fibre (%)	45.9	52.3	51.9	54.1
Neutral Detergent Fibre from forage (% Dry Matter)	40.3	46.6	37.4	40.2
Non-Fiber Carbohydrate (%)	26.9	23.1	22.9	20.3
Starch (%)	3.7	3.5	5.6	4.9
Lignin (%)	5.5	4.6	7.5	6.2
Long-chain Fatty Acids (%)	2.2	2.2	2.8	2.9
Ca (%)	0.73	0.46	0.77	0.43
P (%)	0.44	0.39	0.41	0.44

Sample collection. Milk volume and composition, BW and BCS were recorded every 2 weeks, as per the method described for experiment one. Blood serum and plasma (preserved with lithium heparin) samples were taken from the coccygeal vein or artery every 3 days over a 27 day period, commencing the day before the start of treatment (n = 10/cow) and processed as described for experiment one.

Sample processing and analysis. As for experiment one, serum concentrations of Ca, Mg, P, NEFA, BHB, cholesterol, glucose, and total protein, and plasma calcidiol, cholecalciferol, 24,25-$(OH)_2$-D_3, 3-epi 25-OH-D_3, and 25-OH-D_2, as well as insulin, cOC, and uOC concentrations

were determined for all time points sampled. Concentrations of the following metabolites were measured using commercial assay kits according to manufacturers protocols; plasma insulin using a radio-immunoassay (RIA) kit (TKIN2, Coat-a-Count Insulin, Siemens Healthcare Diagnostics, Los Angeles, USA) and counted on a gamma counter (Perkin Elmer, Waltham, Massachusetts, USA). Carboxylated OC and uOC was determined by enzyme immunoassay in accordance with the manufacturer's protocols (Gla-type Osteocalcin EIA kit, MK111, Takara Bio, Otsu, Japan; Under-carboxylated Osteocalcin EIA kit, MK118, Takara Bio, Otsu, Japan, respectively). Absorbance was measured within 10 mins at 450 nm, with 650 nm as a reference wavelength. For the measurement of uOC, samples were mostly diluted 1:2 in sample diluent prior to assay, and for 2 cows further sample dilution was necessary. The limit of detection for both assays was 0.125 ng/mL.

Statistical analysis. As for experiment one, Stata (Intercooled Stata v.13) was used to perform all analyses. Autoregressive mixed models were used to compare treatment and control groups over time for each blood metabolite as well as the glucose: insulin ratio. Pre-treatment measures were included in each model as a covariate. One cow in the treatment group had abnormally high plasma concentrations (more than 16 times greater than any other cow) of uOC consistently both before and during treatment and was excluded from this measure.

A time series analysis was conducted to compare interactions between calcidiol and other metabolites over time. The data from each cow and metabolite were de-trended separately to produce approximately stationary series (Shumway, 1988). Cross-correlations were then performed between calcidiol and other metabolites. Finally, cross-correlation coefficients were transformed using Fisher's transformation and a random effects, pooled effect of estimate was produced using DerSimonian and Laird (DerSimonian and Laird, 1986) random effects meta-analytic methods where the transformed cross-correlations for each cow were treated as a separate study as described by Hedges and Vevea (1998) and Lean et al. (2014). Effect sizes were not transformed back to correlations. Heterogeneity of results among the trials was quantified using the I^2 statistic (Higgins and Thompson, 2002). This measure of the impact of heterogeneity in meta-analysis is independent of the number of studies and the treatment effect metric. I^2 is a transformation of the square root of the χ^2 heterogeneity statistic divided by its degrees of freedom and describes the proportion of total variation in study estimates that is due to heterogeneity. Negative values of I^2 were assigned a value of zero, consequently the value I^2

lies between 0 and 100%. An I^2 value greater than 50% indicates moderate heterogeneity (Higgins et al., 2003).

Table 2. Covariate adjusted mean plasma concentrations of vitamin D metabolites with increasing calcidiol treatment. Calculated using a mixed model with repeated measures and autoregressive covariance structure. Pre-treatment values were used as covariates for adjustment (Experiment one).

Vitamin D metabolite	Treatment (mg calcidiol/day) Mean (SE)					*P*-value		
	0	0.5	1	2	4	treatment	Time[a]	treatment x time
Calcidiol (ng/mL)	28.9 (9.52)	53.5 (10.47)	79.3 (11.38)	141.8 (9.59)	188.1 (9.55)	0.001[b]	0.001 +	0.001
Cholecalciferol (ng/mL)	2.64 (0.261)	2.05 (0.258)	2.41 (0.264)	2.77 (0.225)	2.34 (0.225)	0.262	0.001 +	0.038
3-epi 25-OH-D_3 (ng/mL)	5.9 (0.44)	7.7 (0.50)	8.4 (0.50)	11.4 (0.43)	13.3 (0.43)	0.001[c]	0.001 +	0.001
24,25$(OH)_2D_3$ (ng/mL)	1.76 (0.759)	2.87 (0.600)	4.93 (0.664)	9.53 (0.602)	21.50 (0.589)	0.001[b]	0.001 +	0.001
25-OH-D_2 (ng/mL)	10.3 (0.72)	10.6 (0.71)	10.4 (0.78)	12.0 (0.70)	8.1 (0.70)	0.004[b]	0.001 -	0.001

[a] Where there is a significant effect of time, the point direction has been specified; + = increases over time; - = decreases over time
[b] Curvilinear response to calcidiol treatment
[c] Linear response to calcidiol treatment

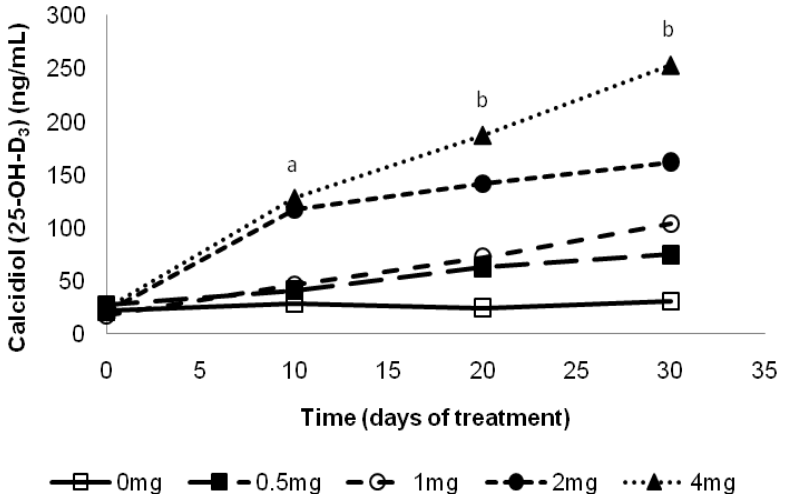

Figure 1. Plasma calcidiol concentration over time by calcidiol dose (Experiment one). [a] average calcidiol concentrations for the 2 mg and 4 mg calcidiol treatments vary from the control at this time point. [b] average calcidiol concentrations for the 1 mg, 2 mg and 4 mg calcidiol treatments vary from the control at this time point.

RESULTS

Experiment One: Dose Response

Vitamin D. Increased calcidiol dose increased both plasma calcidiol and 24,25-$(OH)_2$-D_3 in a curvilinear manner (Table 2, Fig. 1). All groups had similar calcidiol concentrations pre-treatment. Average plasma calcidiol concentrations for the 1 mg treatment varied from the control at 20 and 30 days of treatment, while 2 mg and 4 mg plasma calcidiol treatments varied from the control at 10, 20 and 30 days of treatment. Plasma calcidiol doubled in the group receiving 0.5 mg calcidiol/day after the 30 days of feeding when compared with the control group (67 vs. 32 ng/mL for 0.5 mg and control treatment groups respectively, Table 3). There was no response in plasma cholecalciferol in response to feeding calcidiol (Table 2). Increasing calcidiol, linearly increased 3-epi 25-OH-D_3 in the blood (Table 2). Calcidiol treatment also increased and then decreased plasma 25-OH-D_2 concentration with a curvilinear response, as cows receiving 4 mg/day had numerically lower blood concentration than other groups. Plasma concentrations of all Vitamin D metabolites changed over time, and a treatment by time interaction was evident for all metabolites. These relationships were very similar when the final time point only was analyzed, apart from the positive effect of increasing calcidiol dose on plasma 25-OH-D_2 that was not clearly curvilinear or linear (Table 3). There appeared to be a cumulative effect of calcidiol supplementation over time.

Table 3. Covariate adjusted mean plasma concentrations of vitamin D metabolites or serum mineral and metabolites concentrations, with calcidiol treatments after 30 days of feeding. Means are derived from a random effects mixed model analysis using pre-treatment concentrations as a covariate (Experiment one).

Metabolite or mineral	Treatment (mg calcidiol/day) Mean (SE)					*P*-value
	0	0.5	1	2	4	treatment
Calcidiol (ng/mL)	32.0 (14.92)	67.5 (16.43)	110.5 (17.86)	164.0 (15.03)	251.5 (14.96)	0.000 [a]
Cholecalciferol (ng/mL)	3.44 (0.352)	2.47 (0.348)	3.04 (0.355)	2.68 (0.303)	3.10 (0.303)	0.377
3-epi 25-OH-D_3 (ng/mL)	7.4 (0.75)	8.9 (0.85)	10.6 (0.87)	12.9 (0.74)	18.3 (0.73)	0.000 [a]
24,25$(OH)_2D_3$ (ng/mL)	1.99 (1.228)	3.73 (0.970)	7.23 (1.074)	13.25 (0.973)	34.14 (0.952)	0.000 [a]
25-OH-D_2 (ng/mL)	9.7 (0.69)	8.8 (0.68)	9.9 (0.75)	8.0 (0.67)	6.7 (0.67)	0.008
Calcium (nmol/L)	2.16 (0.190)	2.42 (0.186)	2.28 (0.191)	2.38 (0.189)	2.25 (0.187)	0.872
Glucose (nmol/L)	3.28 (0.127)	3.26 (0.129)	3.21 (0.125)	3.47 (0.125)	3.28 (0.126)	0.622
NEFA (nmol/L)	0.09 (0.040)	0.08 (0.040)	0.11 (0.041)	0.16 (0.040)	0.07 (0.040)	0.514
BHB (nmol/L)	0.82 (0.058)	0.79 (0.058)	0.78 (0.058)	0.73 (0.058)	0.76 (0.058)	0.874
Cholesterol (nmol/L)	2.84 (0.331)	3.30 (0.318)	2.83 (0.349)	3.52 (0.314)	3.21 (0.384)	0.531
Magnesium (nmol/L)	0.94 (0.063)	1.07 (0.059)	1.03 (0.059)	1.14 (0.059)	1.00 (0.064)	0.161
Phosphate (nmol/L)	2.05 (0.261)	1.92 (0.259)	1.99 (0.287)	1.87 (0.261)	2.57 (0.267)	0.333
Total protein (g/L)	77 (2.1)	76 (2.1)	75 (2.1)	80 (2.1)	75 (2.1)	0.580

[a] Curvilinear response to calcidiol treatment

Blood metabolites and minerals. The only effect of calcidiol treatment on metabolites or minerals was on serum P which had a curvilinear response to calcidiol treatment. Cows receiving 4 mg calcidiol/day had higher concentrations of P than other groups, apart from the group receiving 0.5 mg/day (Table 4). Glucose, BHB, and cholesterol concentrations all varied over time and there was no treatment by time interaction for any blood metabolite or mineral. When examined at the final time point only, no concentrations of blood metabolites or minerals were affected by increasing calcidiol dose (Table 3).

Table 4. Covariate adjusted mean serum concentrations of minerals and metabolites with increasing calcidiol dose. Calculated using a mixed model with repeated measures and autoregressive covariance structure. Pre-treatment values were used as covariates for adjustment. (Experiment one).

	Treatment (mg calcidiol/day) Mean (SE)					*P*-value		
Metabolite	0	0.5	1	2	4	Treatment	Time [a]	treatment x time
Calcium (nmol/L)	2.26 (0.070)	2.25 (0.071)	2.29 (0.069)	2.39 (0.070)	2.38 (0.069)	0.452	0.601	0.852
Glucose (nmol/L)	3.20 (0.064)	3.32 (0.063)	3.30 (0.065)	3.45 (0.063)	3.25 (0.063)	0.056	0.001 -	0.100
NEFA (nmol/L)	0.09 (0.015)	0.12 (0.016)	0.10 (0.015)	0.14 (0.016)	0.09 (0.015)	0.108	0.608	0.725
BHB (nmol/L)	0.81 (0.045)	0.84 (0.045)	0.83 (0.045)	0.79 (0.045)	0.83 (0.045)	0.963	0.001 +	0.838
Cholesterol (nmol/L)	3.55 (0.170)	3.42 (0.179)	3.73 (0.163)	3.99 (0.161)	4.08 (0.197)	0.083	0.001 -	0.655
Magnesium (nmol/L)	1.02 (0.031)	1.05 (0.030)	1.06 (0.030)	1.09 (0.030)	1.01 (0.032)	0.318	0.702	0.382
Phosphate (nmol/L)	1.93 (0.125)	2.14 (0.137)	1.82 (0.124)	1.86 (0.125)	2.43 (0.128)	0.003[a]	0.217	0.872
Total protein (g/L)	78 (1.5)	77 (1.5)	78 (1.5)	79 (1.5)	78 (1.5)	0.913	0.088	0.262

[a] Curvilinear response to calcidiol treatment
[b] Where there is a significant effect of time, the point direction has been specified; + = increases over time; - = decreases over time

Milk yield and composition. Milk yield, protein yield, protein percentage, fat percentage, and SCC were unaffected by treatment; however, milk yield, protein yield, and fat percentage varied over time (Table 5). Treated cows tended to have increased fat yield over time (Table 5). No treatment by time interaction was present for any milk yield or composition variable. Milk calcidiol concentration increased linearly with increasing calcidiol dose. There was a significant influence of time on milk calcidiol concentration; although no time by treatment interaction was observed. When the final time point was examined only, no influence of calcidiol dose on milk production, protein and fat yield or percentage, or SCC was observed.

Body weight and body condition score. Neither BW nor BCS were affected by treatment; while, both varied significantly over time (Table 5). Neither BW nor BCS differed between groups at the final time point (after 30 days of calcidiol supplementation).

Table 5. Covariate adjusted average milk yield, protein and fat yield and percentage, somatic cell count and vitamin D concentrations, body weight (BW), and body condition score (BCS), with increasing calcidiol treatment (Experiment one). Average values are calculated using a mixed model with repeated measures and autoregressive covariance structure. Pre-treatment values were used as covariates for adjustment.

Variable	Treatment (mg calcidiol/ day) Mean (SE)					*P*-value		
	0	0.5	1	2	4	treatment	Time[a]	Treatment x time
Milk Yield (kg/day)	16.9 (2.24)	18.5 (2.25)	20.8 (2.39)	22.4 (2.27)	21.3 (2.23)	0.400	0.001 -	0.905
Protein yield (kg/day)	0.55 (0.066)	0.60 (0.066)	0.66 (0.068)	0.69 (0.065)	0.68 (0.065)	0.552	0.048 -	0.929
Protein %	3.48 (0.235)	3.24 (0.230)	3.25 (0.236)	3.08 (0.261)	3.25 (0.229)	0.863	0.159	0.392
Fat yield (kg/day)	0.63 (0.075)	0.73 (0.075)	0.82 (0.080)	0.86 (0.076)	0.90 (0.076)	0.086	0.882	0.945
Fat %	3.95 (0.228)	3.96 (0.230)	3.96 (0.229)	3.81 (0.238)	4.19 (0.250)	0.897	0.001 +	0.063
SCC ($x10^3$)	1.93 (0.150)	1.99 (0.147)	2.06 (0.149)	2.25 (0.144)	2.41 (0.151)	0.156	0.051	0.470
Milk calcidiol (ng/kg)	163.8 (26.07)	250.1 (26.37)	250.5 (25.40)	414.5 (25.43)	522.9 (25.48)	0.001	0.001 +	0.290
BW (kg)	597 (13.4)	614 (13.5)	614 (13.4)	593 (13.4)	595 (13.8)	0.649	0.001 +	0.503
BCS[b]	2.60 (0.106)	2.46 (0.106)	2.59 (0.106)	2.66 (0.106)	2.55 (0.106)	0.739	0.005 -	0.003

[a] Where there is a significant effect of time, the point direction has been specified; + = increases over time; - = decreases over time

[b] BCS was measured on a 1-5 scale, as described by Edmondson et al. (1989).

Experiment Two: Time Series

Blood metabolites and minerals. Feeding calcidiol significantly increased plasma calcidiol and 24,25-$(OH)_2$-D_3, and decreased 25-OH-D_2 concentration (Table 6). All vitamin D metabolites varied over time. Plasma insulin increased when calcidiol was fed. Calcium, Mg, cOC, and glucose concentrations (Table 6) and glucose:insulin ratio (P = 0.002) varied over time, but were unaffected by calcidiol treatment. Blood P, uOC, NEFA, BHB, cholesterol, and total protein did not differ between treatment and control groups or over time.

Table 6. Mean metabolite and minerals concentrations in plasma or serum and significances for control and treatment (0.5 mg calcidiol/day) groups (Experiment two). Average concentrations were calculated using an autoregressive mixed model, corrected for the pre-treatment value.

Metabolite	Mean (SE)		*P*-value		
	control	0.5 mg calcidiol	treatment	time[a]	treatment x time
Calcidiol (ng/mL)	32.1 (1.87)	52.4 (1.87)	0.001	0.001 +	0.001
Cholecalciferol (ng/mL)	4.09 (0.540)	3.73 (0.540)	0.704	0.001 +	0.150
3-epi 25-OH-D_3 (ng/mL)	9.4 (1.14)	9.0 (1.14)	0.830	0.003 +	0.087
24,25-$(OH)_2$-D_3 (ng/mL)	5.14 (0.482)	6.61 (0.482)	0.038	0.001 +	0.001
25-OH-D_2 (ng/mL)	21.6 (0.6)	19.3 (0.6)	0.008	0.001 v	0.222
Calcium (nmol/L)	2.27 (0.025)	2.30 (0.025)	0.361	0.004 v	0.718
Uncarboxlated osteocalcin (ng/mL)	6.3 (0.39)	6.9 (0.35)	0.273	0.690	0.406
Carboxylated Osteocalcin (ng/mL)	36.90 (1.130)	37.00 (1.130)	0.950	0.002 +	0.067
Glucose (nmol/L)	2.79 (0.070)	2.94 (0.070)	0.133	0.003 -	0.433
Insulin (mIU/mL)	3.36 (0.177)	3.86 (0.177)	0.046	0.001 +	0.195
NEFA (nmol/L)	0.15 (0.018)	0.14 (0.018)	0.579	0.279	0.578
BHB (nmol/L)	0.69 (0.031)	0.61 (0.031)	0.075	0.135	0.105
Cholesterol (nmol/L)	5.02 (0.092)	4.92 (0.092)	0.470	0.994	0.837
Magnesium (nmol/L)	1.00 (0.010)	1.01 (0.010)	0.604	0.025 v	0.147
Phosphate (nmol/L)	2.00 (0.062)	2.09 (0.062)	0.359	0.109	0.840
Total protein (g/L)	87 (1.1)	87 (1.1)	0.526	0.998	0.999

[a] Where there is a significant effect of time, the point direction has been specified; + = increases over time; - = decreases over time; v = highly variable over time

Milk yield and composition. Milk yield, milk protein yield and percentage, milk fat yield and percentage and SCC did not differ (P > 0.1) between cows receiving no supplementary calcidiol and those receiving 0.5 mg/day. Average milk production was 17.8 (± 2.27) and 17.7 (± 2.27) kg/day, milk protein percentage was 3.05 (± 0.18) and 3.46 (± 0.18)%, milk fat percentage was 4.31 (± 0.18) and 4.48 (± 0.18)%, and SSC ($x10^3$) was 5.10 (± 0.35) and 5.37 (± 0.35) for control and calcidiol treated groups respectively.

Body weight and body condition score. Cows that received 0.5 mg/day of calcidiol had an average weight of 654 kg ± 7.4 and BCS of 2.26 ± 0.1 while cows that did not receive supplementary calcidiol weighed on average 634 kg ± 7.4 and had BCS of 2.22 ± 0.14 (P = 0.06 and 0.06 for BW and BSC respectively).

Correlations between calcidiol and other metabolites and minerals over time. Table 7 shows, for the relationships between calcidiol and other metabolites and minerals at lags -6, -3, 0, 3, and 6 d, the effect size (random effects average response), 95% confidence interval for this response, the heterogeneity of response (I^2) and significance of difference between treatment and control groups (*P*-value). In general, treatment with calcidiol at 0.5 mg/day did not alter correlations between metabolites as very few differences between groups were significant. There was a positive, homogenous correlation between cholecalciferol concentrations with calcidiol concentrations 6 d before (I^2 = 0.0%) and cholecalciferol and calcidiol were positively associated on the same day in the group receiving calcidiol only. Plasma calcidiol concentrations were positively associated on the same day with 24,25-$(OH)_2$-D_3, 3-epi-25-OH-D_3 and 25-OH-D_2 in both treatment and control groups (Table 7) and treatment and control groups did not significantly differ ($P > 0.3$). Plasma 3-epi 25-OH-D_3 concentration was positively correlated with calcidiol 6 d later in the control group, but control and treatment groups differed significantly in response at this lag. Plasma 24,25-$(OH)_2$-D_3 concentration, was negatively correlated with calcidiol 3 d later in the treatment group and positively correlated 6 d later in the control group. Control and treatment groups differed in 24,25-$(OH)_2$-D_3 association with calcidiol at a lag of 3 d later, but not at 6 d later. The response in the treatment group was strongly negative 3 d later.

Calcium was positively correlated with calcidiol 6 d later in the control group, but was not associated at any other time lag. Undercarboxylated and carboxylated OC were positively correlated with calcidiol 6 d before and 3 d later, respectively, in the control group only. Both glucose and insulin were positively correlated with calcidiol on the same day in the control group, as was NEFA in both control and treatment groups for which the control group was highly homogenous and the treatment group was moderately homogenous. Blood BHB was positively associated with calcidiol 6 d before and 3 d later in the control group only and both treatment and control group results were homogenous. Cholesterol was positively correlated with calcidiol 6 d later in the control group and the result was homogenous. Magnesium and P both had positive, homogenous, correlations with calcidiol 6 d before only in the control group. There

was also a negative correlation between P and calcidiol on the same day in the group receiving calcidiol. Total protein was positively correlated with calcidiol in the control groups at 3 d before and 6 d later.

Table 7. Time-series analysis of relationships between calcidiol (25-OH-$D_{3)}$ and other metabolites and minerals at 3 day lags. Negative lags indicate the relationship of a metabolite or mineral to calcidiol 3 or 6 days before, while those in positive lags are 3 or 6 days later. Lag 0 is the relationship between the two variables on the same day. The effects size (ES), 95% confidence interval, I^2 (which is a measure of heterogeneity in response), and *P*-value for differences between control and treatment groups in ES is displayed. Analysis is based on correlation coefficients, and hence units for minerals and metabolites are not required.

		Lag									
		-6 days		-3days		0 days		3 days		6 days	
Factor		0 mg	0.5 mg	0 mg	0.5 mg	0 mg	0.5 mg	0 mg	0.5 mg	0 mg	0.5 mg
Vitamin D_3	ES[a]	**0.42**	**0.36**	0.05	-0.32	0.58	**0.73**	0.20	-0.23	0.23	0.21
	95% CI[b]	0.091, 0.753	0.025, 0.688	-0.500, 0.596	-0.708, 0.077	-0.028, 1.178	0.141, 1.316	-0.243, 0.650	-0.676, 0.227	-0.099, 0.563	-0.125, 0.538
	I^{2c}	0.0%	0.0%	63.5%	28.8%	69.8%	68.2%	45.0%	46.3%	0.0%	0.0%
	P	0.71		0.32		0.73		0.22		0.89	
3-epi 25-OH-D_3	ES	0.29	0.06	0.15	-0.18	**0.83**	**0.78**	0.02	-0.24	**0.52**	0.09
	95% CI	-0.037, 0.625	-0.271, 0.392	-0.531, 0.836	-0.667, 0.305	0.502, 1.165	0.265, 1.288	-0.599, 0.629	-0.755, 0.278	0.187, 0.850	-0.239, 0.423
	I^2	0.0%	0.0%	76.5%	53.5%	0.0%	58.1%	70.9%	58.8%	0.0%	0.0%
	P	0.22		0.46		0.85		0.55		**0.05**	
24,25- $(OH)_2$-D_3	ES	0.19	-0.11	0.19	-0.18	**0.87**	**1.24**	0.09	**-0.71**	**0.42**	0.27
	95% CI	-0.231, 0.609	-0.758, 0.546	-0.312, 0.697	-0.868, 0.509	0.344, 1.394	0.746, 1.734	-0.237, 0.426	-1.191, -0.228	0.021, 0.818	-0.064, 0.598
	I^2	37.8%	74.2%	56.9%	76.8%	60.2%	55.0%	0.0%	52.6%	30.8%	0.0%
	P	0.48		0.42		0.34		**0.03**		0.53	
25-OH-D_2	ES	0.19	-0.32	0.20	-0.25	**0.90**	**0.75**	0.07	-0.13	0.44	0.22
	95% CI	-0.160, 0.535	-1.064, 0.417	-0.370, 0.773	-0.684, 0.192	0.573, 1.235	0.254, 1.244	-0.518, 0.656	-0.783, 0.515	0.108, 0.770	-0.116, 0.547
	I^2	9.0%	3.6%	66.4%	42.8%	0.0%	55.2%	68.1%	74.0%	0.0%	0.0%
	P	0.29		0.26		0.61		0.66		0.33	
Calcium	ES	0.24	-0.08	0.32	0.30	0.26	0.18	0.14	0.24	**0.35**	-0.18
	95% CI	-0.100, 0.586	-0.443, 0.285	-0.219, 0.862	-0.250, 0.844	-0.096, 0.616	-0.298, 0.661	-0.200, 0.477	-0.212, 0.696	0.017, 0.680	-0.567, 0.203
	I^2	6.6%	17.1%	62.4%	63.4%	13.3%	52.3%	4.0%	46.7%	0.0%	25.9%
	P	0.24		0.95		0.80		0.73		0.06	
Uncarboxylated	ES	**0.39**	0.21	0.25	0.01	0.60	-0.08	0.18	0.05	0.27	0.14
osteocalcin	95% CI	0.036, 0.742	-0.186, 0.614	-0.118, 0.613	-0.983, 1.002	-0.133, 1.339	-0.868, 0.709	-0.396, 0.761	-0.555, 0.662	-0.066, 0.596	-0.192, 0.470
	I^2	11.8%	31.2%	17.8%	88.9%	79.8%	82.3%	67.2%	70.4%	0.0%	0.0%
	P	0.54		0.67		0.25		0.77		0.54	
Carboxylated	ES	0.31	0.21	0.35	0.24	-0.12	0.24	**0.46**	-0.07	0.30	0.19
Osteocalcin	95% CI	-0.021, 0.642	-0.286, 0.702	-0.124, 0.822	-0.603, 1.080	-0.912, 0.680	-0.268, 0.742	0.070, 0.840	-0.400, 0.263	-0.159, 0.763	-0.143, 0.519
	I^2	0.0%	55.0%	50.9%	84.5%	82.7%	57.0%	25.9%	0.0%	48.4%	0.0%
	P	0.74		0.83		0.48		0.07		0.66	

Table 7 Continued.

		Lag									
		-6 days		-3days		0 days		3 days		6 days	
Factor		0 mg	0.5 mg	0 mg	0.5 mg	0 mg	0.5 mg	0 mg	0.5 mg	0 mg	0.5 mg
Glucose	ES	0.33	-0.11	0.07	0.20	**0.38**	0.10	0.22	0.24	0.11	-0.08
	95% CI	-0.004, 0.659	-0.439, 0.223	-0.328, 0.471	-0.161, 0.559	0.049, 0.712	-0.451, 0.655	-0.117, 0.546	-0.120, 0.590	-0.289, 0.499	-0.413, 0.249
	I^2	0.0%	0.0%	31.2%	15.4%	0.0%	64.2%	0.0%	13.0%	29.2%	0.0%
	P	0.06		0.66		0.38		0.93		0.47	
Insulin	ES	0.20	0.13	-0.02	0.21	**0.40**	0.08	0.17	0.13	0.20	0.03
	95% CI	-0.183, 0.579	-0.453, 0.706	-0.383, 0.343	-0.356, 0.783	0.015, 0.784	-0.385, 0.541	-0.267, 0.615	-0.201, 0.461	-0.299, 0.699	-0.298, 0.364
	I^2	24.4%	67.3%	16.5%	66.2%	25.7%	48.7%	43.6%	0.0%	55.9%	0.0%
	P	0.85		0.52		0.33		0.86		0.55	
NEFA	ES	0.14	0.08	0.03	-0.35	**0.53**	**0.60**	-0.02	-0.22	-0.01	0.07
	95% CI	-0.416, 0.699	-0.255, 0.407	-0.392, 0.446	-0.780, 0.088	0.197, 0.859	0.204, 1.002	-0.349, 0.314	-0.655, 0.207	-0.340, 0.323	-0.260, 0.403
	I^2	64.7%	0.0%	37.4%	41.8%	0.0%	31.1%	0.0%	40.9%	0.0%	0.0%
	P	0.83		0.26		0.76		0.43		0.69	
BHB	ES	**0.59**	0.18	0.36	-0.18	0.12	0.15	**0.72**	0.12	0.16	0.22
	95% CI	0.257, 0.919	-0.263, 0.622	-0.091, 0.816	-0.739, 0.381	-0.690, 0.938	-0.314, 0.619	0.392, 1.055	-0.415, 0.651	-0.171, 0.492	-0.111, 0.551
	I^2	0.0%	43.9%	46.6%	65.0%	83.4%	49.6%	0.0%	0.0%	0.0%	0.0%
	P	0.17		0.18		0.95		0.10		0.79	
Cholesterol	ES	0.11	-0.10	0.23	0.12	0.49	0.13	0.06	0.10	0.41	0.06
	95% CI	-0.222, 0.440	-0.510, 0.315	-0.101, 0.561	-0.222, 0.457	-0.090, 1.061	-0.308, 0.560	-0.274, 0.389	-0.348, 0.539	0.073, 0.736	-0.382, 0.492
	I^2	0.0%	35.4%	0.0%	4.9%	66.9%	41.7%	0.0%	44.1%	0.0%	42.5%
	P	0.41		0.62		0.36		0.89		0.20	
Mg	ES	**0.34**	-0.03	0.28	0.18	-0.08	0.17	0.38	0.16	0.19	-0.10
	95% CI	0.004, 0.667	-0.591, 0.534	-0.080, 0.634	-0.180, 0.537	-0.512, 0.349	-0.498, 0.844	-0.009, 0.759	-0.439, 0.753	-0.150, 0.530	-0.590, 0.381
	I^2	0.0%	65.3%	13.8%	14.7%	40.7%	75.6%	25.7%	69.1%	5.0%	53.4%
	P	0.24		0.71		0.55		0.56		0.36	
Phosphate	ES	**0.34**	0.13	0.22	0.46	0.16	**-0.39**	0.18	0.24	0.34	0.28
	95% CI	0.010, 0.673	-0.380, 0.640	-0.114, 0.549	-0.017, 0.943	-0.340, 0.656	-0.754,-0.020	-0.268, 0.629	-0.221, 0.695	-0.218, 0.893	-0.050, 0.612
	I^2	0.0%	57.8%	0.0%	52.4%	55.8%	18.5%	45.5%	47.6%	64.4%	0.0%
	P	0.48		0.42		0.12		0.87		0.86	
Total protein	ES	0.23	0.09	**0.55**	0.18	0.23	0.16	0.10	-0.04	**0.38**	0.06
	95% CI	-0.199, 0.651	-0.417, 0.593	0.044, 1.051	-0.178, 0.538	-0.205, 0.662	-0.207, 0.518	-0.280, 0.476	-0.581, 0.508	0.053, 0.715	-0.410, 0.526
	I^2	39.2%	57.0%	56.7%	14.4%	41.6%	16.4%	23.1%	63.0%	0.0%	49.8%
	P	0.69		0.28		0.81		0.70		0.24	

[a] ES = Effect size is a standardized z-value measuring correlation between metabolites at different lags for the cows in each group.
[b] 95% CI = 95% Confidence interval.
[c] I^2 describes the percentage of total variation across studies that is due to heterogeneity (Higgins et al., 2003)
Bold text indicates the value differs significantly from 0 based on 95% CI.

DISCUSSION

In both experiments, mid-lactation dairy cows receiving no supplementary calcidiol had plasma calcidiol concentrations within the normal range of 20-50 ng/mL, reported by Horst et al. (1994). All doses of calcidiol supplementation increased calcidiol concentrations above this range, hence increasing plasma calcidiol concentrations above normal ranges rather than being used as a therapeutic agent to correct a vitamin deficiency. Other studies have shown supplementary feeding increases blood calcidiol (Rivera et al., 2005, Taylor et al., 2008, Weiss et al., 2015); however the increases have differed from those seen in this study, possibly due to the variety of methods used to deliver the calcidiol (dietary, bolus, or buccal administration) and other dietary factors. For example, negative DCAD diets are known to affect PTH sensitivity and amplify the effects of calcidiol on Ca metabolism (Wilkens et al., 2012, Lean et al., 2014). These studies all had baseline calcidiol concentrations higher than those observed in the current study, but examined cows during the transition period where the increased demand for nutrients, especially Ca, may have influenced Vitamin D metabolism. It should be noted that the cows in our experiments were fed pasture and consequently there would be daily fluctuations in availability of energy and nutrients. Variation also existed in the metabolism of individual cows, and we identified 2 cows, 1 in each experiment, with aberrant responses. One of these showed no apparent response to calcidiol treatment, while the other had very high concentrations of uOC both before and during the treatment period, and as such, these cows were not included in vitamin D metabolite or uOC analyses, respectively.

Calcidiol supplementation increased plasma concentrations of vitamin D metabolites for which it is a precursor. In the dose response study, the control group had 1.76 ± 0.76 ng/mL of 24,25-$(OH)_2$-D_3 in plasma, which was similar to the concentrations identified by Smith et al. (1982) (2.03 ± 0.34 ng/mL), and less than that reported by Littledike and Horst (1982) (4.0 ±1.4 ng/mL). The curvilinear increase of 24,25-$(OH)_2$-D_3 in response to increased calcidiol dose suggests that a feedback mechanism exists between the 2 metabolites. This feedback mechanism may involve CYP24A1 (24-hydroxylase), a cytochrome enzyme that adds OH groups in the 24 position in calcidiol, calcitriol and vitamin D_2 metabolites, inactivating these and increasing their removal from circulation (Horst et al., 1994). The expression of CYP24A1 is tightly regulated, but it is released in response to increased circulating calcitriol, and may be part of the mechanism for removing excess calcidiol and calcitriol from cells (Nelson and Merriman, 2014).

Traditional methods of studying the relationships between metabolites do not allow longer term, homeorhetic responses to be examined. Time-series analysis, utilizing meta-analytic techniques, enables evaluation of correlations between metabolites over time that provide evidence of such homeorhetic relationships (Lean et al., 1992). When a significant relationship was identified between calcidiol and other metabolites, heterogeneity was usually low to moderate. Perturbing metabolism with calcidiol may have resulted in more variability in response among treated cows and less likelihood of consistent responses over time. The small study number (n = 5/treatment) may also limit the ability to identify responses, and it is likely only large and consistent responses among cows were identified. No change in cholecalciferol concentrations in response to increased calcidiol dose was identified in this study, possibly indicating a lack of feedback between these metabolites.

The biological significance of 3-epi 25-OH-D_3 is poorly understood, although the linear increase with increasing calcidiol dose shows a clear association between the epimers. Despite this increase, the ratio of 3-epi-25-OH-D_3 to calcidiol decreased with increasing calcidiol supplementation. Blood 3-epi-25-OH-D_3 concentration may be important in calcidiol measurement, as there is evidence that 3-epi 25-OH-D_3 can be incorrectly assayed as calcidiol and may, consequently, confound conclusions if not considered separately (Singh et al., 2006, Lensmeyer et al., 2011). Vitamin D_2 may have a similar biological role to D_3 in most mammals, although there is some variation in their metabolism, and some mammals may discriminate against D_2 (Horst et al., 1994), with D_2 metabolites having a more rapid clearance rate than D_3 metabolites (Armas et al., 2004). Cholecalciferol supplementation resulted in higher plasma concentrations of calcidiol when compared with the effect of an equal ergocalciferol supplementation on plasma 25-OH-D_2 concentrations, and supplementary ergocalciferol can inhibit the efficiency with which cholecalciferol is converted to calcidiol (Hymøller and Jensen, 2010, Hymøller and Jensen, 2011). In experiment one, 25-OH-D_2 concentration increased and then decreased in a curvilinear manner with increasing calcidiol fed. The vitamin D_2 side chains of 25-OH-D_2 and 1, 25-$(OH)_2$-D_2) are susceptible to hydroxylation by CYP24A1. Hence we may expect, if activated by increased levels of vitamin D_3 metabolites such as observed in the supplemented cows, this enzyme to lower blood concentrations of 25-OH-D_2. It was interesting that the negative feedback between calcidiol and 25-OH-D_2 was observed only when the highest calcidiol dose was fed and may indicate that negative feedback occurs between the metabolites only at higher concentrations.

Increased blood calcidiol increases Ca absorption, and hence plasma Ca concentrations (McGrath et al., 2012). Despite this, there was no effect of calcidiol treatment on serum Ca identified in experiment one, a relationship similar to that described by Taylor et al. (2008). Blood Ca concentration is maintained under tight homeostatic control. Studies that found increases in total plasma Ca in response to calcidiol used non lactating cattle (Carnagey et al., 2008, McGrath et al., 2012, Wilkens et al., 2012), suggesting that in lactation, absorption, accretion in bone or other Ca pools, or excretion of Ca may differ from non-lactating cattle. A benefit of using time series analysis is the ability to identify lagged associations between variables, such as the positive association between Ca and calcidiol in the control group 6 days later. Rivera et al. (2005) found that supplementary calcidiol increased plasma Ca, within 24 h of supplementation in beef heifers, but these were single buccal dosages of 100 and 1000 mg calcidiol that are much higher than those used in our experiments, and cattle were not lactating. When the effects of a single 10 mg dose of calcidiol, were examined in the same study (Rivera et al., 2005), there was no effect of calcidiol on plasma Ca.

Magnesium and P play an important role in Ca homeostasis, and are particularly important for tissue sensitivity to calcitrophic hormones and PTH, and production of active vitamin D metabolites (Zofková and Kancheva, 1995). Hypophosphatemia increases hydroxylation of calcidiol in the kidney, thereby increasing blood calcitriol concentrations (Tanaka and DeLuca, 1973). Changes in blood Mg concentrations inversely affect PTH release, although in more severe cases of Mg deficiency PTH secretion can be inhibited and PTH sensitivity reduced in target tissues (Rude et al., 1978, Rude, 1998). Intestinal absorption and renal excretion of Ca and Mg are interdependent. The positive association between Mg and calcidiol 6 days before identified in the time series analysis suggests that calcidiol may improve Mg absorption or retention, although this was seen only in the control group. A curvilinear increase in P concentration in response to increasing supplementary calcidiol was observed in the repeated measures analysis, but not in the analysis of day 30 results only, possibly reflecting the increased precision of the repeated measures analysis. This increase in serum P concentration may have been expected, as vitamin D increases both Ca and P retention (McGrath et al., 2012) and the combined action of calcitriol and PTH is to trigger osteoclasts to resorb bone, releasing Ca and P. In another study, feeding vitamin D to rats increased blood Ca and possibly P levels, the latter occurring in only one of the two populations studied, but decreased blood Mg and the concentration of Mg was inversely related to that of P (Harrison and Harrison, 1964).

Besides increasing Ca and P retention, calcidiol and calcitriol also regulate cells involved in bone synthesis and remodeling, increasing mineralization of bone and stimulating the production of bone deposition marker OC from mature osteoblasts. The effect of calcidiol on skeletal metabolism is of particular interest as murine studies identified metabolic effects orchestrated by uOC which could result in increased insulin release and sensitivity (Lee et al., 2007). An increase in plasma concentration of uOC, the biologically active form of OC, in the group fed calcidiol may have been expected, although no overall difference in uOC was observed as a result of calcidiol supplementation, a result similar to Taylor et al. (2008) who fed a single 15 mg dose of calcidiol and measured total OC. In another study (Kim et al., 2011), calcitriol elevated plasma Ca, total OC, and uOC, although such results were not observed following treatment with calcidiol or cholecalciferol (Taylor et al., 2008). The calcidiol dose used in this study (0.5 mg/day) may have been too low to stimulate the processes involved in bone formation and indicates the need to explore higher doses of calcidiol. Nevertheless, our time series analysis identified differences between calcidiol treated and untreated groups in the relationship between OC and calcidiol, with concentrations of uOC in the control group being positively associated with calcidiol concentrations 6 d before. Further, the control group had a positive association between cOC, the biologically inactive form of OC, and calcidiol 3 d later. Altogether, these findings support the notion that changes in OC may occur in response to calcidiol, similar to those previously reported (Kim et al., 2011), but suggests that these may be delayed.

In Experiment 2 of this study, cows receiving calcidiol had increased plasma concentrations of insulin. Increased concentrations of uOC in mice increased insulin production and secretion and tissue sensitivity to insulin (Lee et al., 2007), although we did not observe any clear changes on OC concentrations in treated cattle. Nevertheless, the positive insulin response to calcidiol treatment, combined with the other positive associations between calcidiol and NEFA and glucose on the same day, and the lagged positive association of BHB with calcidiol 3 d later and 6 d before, provide further evidence of the role of calcidiol in energy metabolism. Martinez et al. (2014) found higher glucose and NEFA and lower insulin concentrations in cows with induced sub-clinical hypocalcaemia, suggesting a role for Ca in glucose stimulation of insulin secretion. A sampling regime more frequent that the 3 day interval used here may have provided greater potential to clearly identify relationships between calcidiol and indicators as energy metabolism, as the circulating half lives of some of the metabolites, including insulin and glucose, are quite short (Trenkle, 1972, Chagas et al., 2009).

In this study, there was no increase in milk production, fat and protein percentage or yield. Other studies found a lack of milk yield response when transition cows were fed calcidiol and compared with those fed cholecalciferol, when both vitamin D treatments were combined with negative DCAD diets (Weiss et al., 2015). In contrast, when cows were fed approximately 3 mg/day calcidiol for 21 days prepartum, 3.5% fat- and energy-corrected early lactation milk yield increased by 3.7 ± 1.2 kg/day when compared with cows fed cholecalciferol (Martinez et al., 2015). Milk calcidiol concentration also increased linearly with increasing calcidiol dose. Cows that were fed 40 000 IU/day cholecalciferol had higher milk concentrations of cholecalciferol and calcidiol than those receiving only 4 000 IU/day (Hollis et al., 1981). The effects of dietary calcidiol and cholecalciferol supplementation to hens on vitamin D concentrations in their eggs was explored, examining the potential for dietary supplementation of animals to address vitamin D deficiencies in man (Browning and Cowieson, 2014). The results of the current study suggest that, similar to the poultry studies, dietary supplementation of dairy cows may provide an alternative method of vitamin D delivery in human nutrition.

This study provided a preliminary understanding of relationships between calcidiol treatment and metabolite concentrations over time in mid-lactation dairy cows. Feeding supplementary calcidiol during times of high Ca demand may limit the deficit, or lag time observed between decreased blood Ca or other signals, and simulation of calcidiol and calcitriol synthesis. This decreased lag would allow the body to respond to metabolic stressors more quickly, potentially reducing the incidence of metabolic disease and improving productivity. This study showed some positive relationships between calcidiol feeding and vitamin D, metabolite and mineral metabolism, but these require further investigation, and the use of higher doses of calcidiol should be explored. While the time series methods provided insights to the homeostatic and homeorhetic responses, more frequent samplings with greater numbers of cattle, under conditions of greater dietary control may have enhanced the precision of observations made. These relationships should now be explored in cattle during periods of higher metabolic stress, such as during transition, where treatment effects could be highly beneficial, particularly in relation to calcium and energy metabolism.

CONCLUSIONS

Feeding supplementary calcidiol curvilinearly increased plasma concentrations of calcidiol in mid-lactation cows. Despite substantial differences in the nutrient densities of the diets offered in

the 2 experiments, consistent responses to the vitamin D intervention were found. Plasma calcidiol concentration in the group receiving 0.5 mg calcidiol/day was increased from 32 ng/mL to 52 and 67 ng/mL in the 2 experiments. Time series analysis was an effective way to explore the relationships between blood calcidiol and other minerals and metabolites over time, and highlighted several relationships worthy of further exploration, including those between calcidiol and Ca, P, insulin and OC. Importantly, strong positive associations between calcidiol and indicators of energy metabolism were identified. Overall, these experiments provide support for a positive effect of calcidiol treatment on dairy cow metabolism, and this should be further explored.

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CHAPTER SIX: EFFECTS OF PRE-PARTUM DIETARY CATION-ANION DIFFERENCE AND SOURCE OF VITAMIN D ON DAIRY COWS: VITAMIN D, MINERAL AND BONE METABOLISM

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OVERVIEW OF CHAPTER SIX

In this Chapter, we build on the work of Chapter 5 to examine how manipulation of vitamin D supplementation and dietary cation-anion difference in the pre-partum period can have prolonged positive effect on cow production and health extending well into lactation. This study was designed to examine the integration of mineral, bone and energy metabolism in dairy cattle, and the effects of dietary interventions on health, fertility and production. This work is presented as a series of related Chapters (Chapters 6, 7 and 8), with this Chapter focusing on vitamin D, mineral and bone metabolism

ABSTRACT

Pregnant Holstein cows, 51 parous cows and 28 nulliparous, were blocked by parity and milk yield and randomly allocated to receive diets that differed in dietary cation-anion difference (**DCAD**), +130 or -130 mEq/kg, and supplemented at 3 mg/11 kg of DM with either calcidiol or cholecalciferol from 255 d of gestation until parturition. Blood was sampled thrice weekly prepartum, and on d 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 postpartum to evaluate effects of the diets on vitamin D, mineral and bone metabolism, and acid-base status. Blood pH, and concentrations of minerals, vitamin D metabolites, and bone-related hormones were determined as were mineral concentrations and losses in urine and colostrum. Supplementing calcidiol increased plasma concentrations of 25-hydroxyvitamin D_3, 3-epi 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, 1,25-dihydroxyvitamin D_3, and 24,25-dihydroxyvitamin D_3 compared with cholecalciferol. Cows fed the diet with negative DCAD had smaller concentrations of vitamin D metabolites before and after calving than cows fed the diet with positive DCAD, except for 25-hydroxyvitamin D_2. Feeding the diet with negative DCAD induced a compensated metabolic acidosis that attenuated the decline in blood ionized Ca (**iCa**) and serum total Ca (**tCa**) around calving, particularly in parous cows, whereas cows fed the diet with positive DCAD and supplemented with calcidiol had the greatest 1,25-dihydorxyvitamin D_3 concentrations and the smallest iCa and tCa concentrations on days 1 and 2 postpartum. The acidogenic diet or calcidiol markedly increased urinary losses of tCa and total magnesium (**tMg**), and feeding calcidiol tended to increase colostrum yield and increased losses of tCa and tMg in colostrum. Cows fed the diet with negative DCAD had increased concentrations of serotonin and C-terminal telopeptide of type 1 collagen prepartum compared with cows fed the diet with positive DCAD. Concentrations of undercarboxylated and carboxylated osteocalcin and those of adiponectin did not differ with treatments. These results provide evidence that dietary manipulations can induce metabolic adaptations that improve mineral homeostasis with the onset of lactation that might explain some of the improvements observed in health and production when cows are fed diets with negative DCAD or supplemented with calcidiol.

Keywords: calcidiol, calcium, DCAD, vitamin D

INTRODUCTION

Nutritional interventions applied in the pre-calving period can have a prolonged positive effect on dairy cow production and health well into lactation, in particular prepartum dietary interventions that prevent mineral-related disorders in early lactation (Block, 1994; Lean et al., 2006). Calcium (**Ca**) metabolism is critical during the transition period because of the increased demands for fetal skeletal growth (House and Bell, 1993) and irreversible loss of Ca in milk at the onset of lactation (Ramberg et al., 1970), which explain the high incidence of transitory and prolonged hypocalcemia, especially in multiparous cows. In some cases, cows with hypocalcemia develop clinical signs of disease, also called milk fever, which markedly increases the risk of other diseases (Curtis et al., 1983; Martinez et al., 2012). Bone is a reservoir of Ca and phosphorus (**P**), but bone Ca reserves are limited, and cows must have effective gastrointestinal absorption of Ca to meet lactational demands. The skeleton also plays other roles in adaptation to lactation, and is the source of osteocalcin (**OC**), produced by mature osteoblasts. Osteocalcin concentrations are directly proportional to Ca and P concentrations in dairy cows at the onset of lactation (Naito et al., 1990).

Vitamin D plays a central role in mineral metabolism, regulating the absorption of Ca and P from the gut, mobilizing mineral from bone, and stimulating renal Ca reabsorption (Bronner, 1987). Reductions of blood ionized Ca (**iCa**) triggers the synthesis and release of parathyroid hormone (**PTH**), which regulates a cascade of metabolic responses including the hydroxylation and consequent activation of 25-hydroxyvitamin D_3 to 1,25-dihydroxyvitamin D_3 (Fraser and Kodicek, 1973) by directly acting on the 1α-hydroxylase gene *CYP27B1* (Brenza and DeLuca, 2000). Supplementation with 1,25-dihydoxyvitamin D_3, but not vitamin D_3 or 25-hydroxyvitamin D_3 (Taylor et al., 2008), increased blood concentrations of OC and Ca in non-lactating dairy cows (Kim et al., 2011). Furthermore, iCa concentrations increased in transition dairy cows receiving a combination of 25-hydroxyvitamin D_3 and a diet with negative DCAD (Wilkens et al., 2012). Rodney et al. (2017 – Chapter 5) identified weak positive correlations between plasma calcidiol and OC concentrations over time in mid-lactation dairy cows, although further work is required to better understand if similar relationships exist during the transition period.

Bone and Ca metabolism are highly integrated with energy metabolism and a major role for skeleton and Ca metabolism in integrating metabolic adaptations to lactation has been proposed

(Lee et al., 2007, Lean et al., 2014). Elegant murine studies demonstrated the integration of OC and energy metabolism through effects on insulin and glucose (Lee et al., 2007). In dairy cattle, hypocalcemia greatly increases the risk of other diseases that affect metabolism, and below normal concentrations of ionized (**iCa**) and total Ca (**tCa**) impair insulin release and result in increased lipolysis (Martinez et al., 2014), which has implications to energy metabolism during early lactation when adipose tissue insulin sensitivity is depressed and response to lipolytic signals enhanced (McNamara, 1991).

Dietary interventions that influence Ca and mineral metabolism include, but are not limited to altering the DCAD of prepartum diets (Block, 1984) and manipulating the source of vitamin D supplied in the diet (Wilkens et al., 2012). We hypothesized that 25-hydroxyvitamin D_3 would be more effective than vitamin D_3 in improving Ca homeostasis in transition dairy cows, particularly when fed with an acidogenic diet, which would reflect on a more integrated bone and mineral metabolism. Therefore, the objectives of the present experiment were to explore the individual and combined effects of diets with differing DCAD, alkalogenic or acidogenic, and vitamin D treatment, either vitamin D_3 or 25-hydoxyvitamin D_3 during the prepartum period on vitamin D, mineral and bone metabolism. This paper is from a series of companion papers (Martinez et al., 2017a; 2017b – Chapters 7 and 8) from an experiment designed to examine the effects of level of DCAD and source of dietary vitamin D on vitamin D, mineral, and energy metabolism and implications to postpartum performance and health.

MATERIALS AND METHODS

The University of Florida Institutional Animal Care and Use Committee approved all procedures involving cows in the experiment under the protocol number 201408331. Throughout the manuscript, the vitamins fed will be referred to as cholecalciferol (**CH**) and calcidiol (**CA**), whereas the same vitamins measured in blood plasma will be referred as vitamin D_3 and 25-dihydroxyvitamin D_3.

Cows and Housing

Eighty pregnant dry Holstein cows, 52 parous cows and 28 nulliparous, were enrolled in the experiment at the University of Florida Dairy Unit conducted between February and August 2014. Cows were moved into the into the experimental pen to acclimate to the facilities and fed a common diet for the first few days. Cows were assigned to individual feeding gates (Calan

Broadbent feeding system, American Calan Inc., Northwood, NH) based on sequence of enrollment. One parous cow was removed from the data analyses because of diagnosis of lymphosarcoma during late gestation. Therefore, 79 cows were included in all statistical analyses. Details of enrolment criteria and housing can be found elsewhere (Martinez et al., 2017a – Chapter 7). For consistency of terminology throughout the manuscript, cows are referred to as either nulliparous, those that were nulliparous prepartum and primiparous postpartum, or parous, those that had previously calved, throughout the manuscript.

Treatment Diets and Feeding

The experiment followed a randomized complete block design with cow as the experimental unit. Weekly cohorts of prepartum cows at 252 d of gestation were blocked by parity as nulliparous or parous, with parous cows also blocked by previous lactation 305 d milk yield and, within each block, assigned randomly to one of the four treatments. Treatments were arranged as a factorial with two levels of DCAD, positive (+130 mEq/kg) or negative (-130 mEq/kg), and two sources of vitamin D, cholecalciferol or calcidiol fed at 3 mg for each 11 kg of diet DM. The vitamin D products were provided by DSM as cholecalciferol (Rovimix D_3, 300 mg of CH per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ) or calcidiol (Hy-D, a product containing 153 mg of CA per kg; DSM Nutritional Products, LCC). Prepartum cows were expected to consume 11 kg of DM/d in the last 21 d of gestation, which would result in intake of 3 mg of either cholecalciferol or calcidiol. Therefore, the four treatments were positive DCAD with cholecalciferol (**PCH**), positive DCAD with calcidiol (**PCA**), negative DCAD with cholecalciferol (**NCH**), and negative DCAD with calcidiol (**NCA**). Treatment diets were fed from 252 d of gestation to calving. Upon calving, cows were fed the same lactation ration for the first 49 DIM. All diets were fed as TMR as depicted in Table 1. Details of feed sampling and analytical methods are presented in Martinez et al. (2017a – Chapter 7) and chemical analyses of diets are depicted in Table 1.

Table 1. Dietary ingredients and nutrient composition of diets fed pre- and postpartum

	Prepartum diets[1]				
	Positive DCAD		Negative DCAD		
Item	Cholecalciferol	Calcidiol	Cholecalciferol	Calcidiol	Postpartum diet
Ingredients, % of DM					
Corn silage	61.80	61.80	61.80	61.80	25.8
Bermuda hay	9.10	9.10	9.10	9.10	7.5
Brewer's grains, wet	---	---	---	---	8.6
Corn grain, finely ground	---	---	---	---	25.9
Citrus pulp	9.10	9.10	9.10	9.10	5.2
Soybean hulls	---	---	---	---	8.6
Whole cottonseed	6.40	6.40	6.40	6.40	3.4
Soybean meal, solvent extract	---	---	4.50	4.40	8.2
Soybean meal, cooker-processing[2]	11.18	11.08	---	---	3.3
Acidogenic supplement[3]	---	---	7.25	7.25	--
Cholecalciferol mixture[4]	0.08	---	0.08	---	--
Calcidiol mixture[5]	---	0.18	---	0.18	--
MgO + NaCl	0.54	0.54	---	---	---
Prepartum mineral[6]	1.80	1.80	1.80	1.80	--
Postpartum protein and mineral[7]	---	---	---	---	3.5
DM, %	55.4 ± 1.0	55.6 ± 1.0	55.4 ± 1.0	55.4 ± 1.0	69.5 ± 0.6
Nutrients, DM basis (± SD)[8]					
Net energy,[9] Mcal/kg	1.65	1.65	1.65	1.65	1.67
OM, %	94.0 ± 0.4	93.9 ± 0.4	94.2 ± 0.4	94.1 ± 0.4	94.0 ± 0.1
CP, %	13.5 ± 0.3	12.9 ± 0.3	13.5 ± 0.3	13.4 ± 0.3	15.7 ± 0.6
Starch, %	20.2 ± 0.2	20.1 ± 0.2	20.8 ± 0.2	20.9 ± 0.2	27.6 ± 1.0
Non-fibrous carbohydrates,[10] %	38.7 ± 1.1	38.1 ± 1.1	38.3 ± 1.1	38.5 ± 1.1	40.8 ± 1.2
NDF, %	37.8 ± 0.6	39.0 ± 0.6	38.3 ± 0.6	38.2 ± 0.6	33.3 ± 0.5
NDF from forage, %	30.8 ± 0.7	30.8 ± 0.7	30.8 ± 0.7	30.8 ± 0.7	15.8 ± 0.4
Fatty acids, %	3.28 ± 0.03	3.33 ± 0.03	3.45 ± 0.03	3.37 ± 0.03	3.93 ± 0.22
Ca, %	0.61 ± 0.08	0.62 ± 0.08	0.54 ± 0.08	0.55 ± 0.08	0.59 ± 0.03
P, %	0.32 ± 0.01	0.31 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	0.36 ± 0.01
Mg, %	0.39 ± 0.02	0.37 ± 0.02	0.38 ± 0.02	0.39 ± 0.02	0.27 ± 0.01
K, %	1.22 ± 0.08	1.19 ± 0.08	1.15 ± 0.08	1.15 ± 0.08	1.15 ± 0.06
Na, %	0.20 ± 0.01	0.20 ±0.01	0.16 ± 0.01	0.16 ± 0.01	0.46 ± 0.04
Cl, %	0.54 ± 0.04	0.55 ± 0.04	0.94 ± 0.04	0.90 ± 0.04	0.30 ± 0.01
S, %	0.17 ± 0.004	0.16 ± 0.004	0.37 ± 0.004	0.36 ± 0.004	0.18 ± 0.01
DCAD,[11] mEq/kg	145 ± 11	130 ± 119	-129 ± 11	-124 ± 11	293 ± 28

[1] Prepartum cows starting at 252 d of gestation were fed diets with either a positive (+130 mEq/kg) or a negative (-130 mEq/kg) dietary cation-anion difference (DCAD). Within each DCAD diet, cows were fed either 3 mg of cholecalciferol or 3 mg of calcidiol.
[2] Amino Plus (cooker-processing soybean meal; Ag Processing Inc., Emmetsburg, IA).
[3] Bio-Chlor (a fermentation product containing dried condensed extracted glutamic acid fermentation product, dried condensed corn fermentation solubles, processed grain by-products, and magnesium chloride; Arm & Hammer Animal Nutrition, Princeton, NJ).
[4] Rovimix D3 (a product containing 300 mg of cholecalciferol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ).
[5] Hy-D (a product containing 153 mg of calcidiol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ).
[6] Each kg contains (DM basis) 10.3% Ca, 0.7% P, 4.0% Mg, 0.9% K, 0.25% S, 1.8% Na, 2.7% Cl, 1,750 mg Zn, 600 mg Cu, 1,090 mg Mn, 21 mg Se, 75 mg Co, 21 mg I, 260,000 IU of vitamin A, and 7,500 IU of vitamin E.
[7] A supplement containing 30% blood meal enriched with rumen-protected lysine and methionine (LysAAMet, Perdue Ag Solutions, LLC, Salisbury, MD). Each kg contains (DM basis) 26.4% CP, 5.1% Ca, 1.6% P, 4.1% Mg, 6.8% K, 0.3% S, 10.7% Na, 2.5% Cl, 665 mg Zn, 230 mg Cu, 416 mg Mn, 7.2 mg Se, 24 mg Co, 13.6 mg I, 110,000 IU of vitamin A, 33,000 IU of cholecalciferol (0.825 mg), 1,100 IU of vitamin E, and 460 mg of monensin (Rumensin 90, Elanco Animal Health, Eli Lilly and Co, Indianapolis, IN).
[8] Samples collected weekly and composited monthly for chemical analyses.
[9] Calculated based on the chemical analysis of dietary ingredients and using the NRC (2001) for a DM intake of 12.0 kg/d prepartum and 18 kg/d postpartum.
[10] Calculated using the equation DM – [(CP + NDF + fat + ash – (NDF insoluble protein)].
[11] Calculated using the equation [(mEq of Na + mEq of K) - (mEq of Cl + mEq of S)].

Prepartum cows were fed the respective treatment diets as TMR once daily at approximately 0730 h, whereas postpartum cows were fed twice daily, at 0730 and 1230 h. Refusals were weighed once daily, before the morning feeding, and feed allowances were calculated daily with the goal of 5% refusals.

Measurements of Colostrum Yield and Mineral Content

Cows were milked within the first 6 h after calving and colostrum yield was measured and duplicate samples were collected, frozen at -20^{o}C, and later analyzed for concentrations of tCa and total Mg (**tMg**) to calculate loss of mineral as colostrum. Throughout the experiment, cows were milked twice daily at 0700 h and 1900 h, and yields of milk and milk components are reported in Martinez et al., (2017a – Chapter 7).

Samples of colostrum were thawed, thoroughly homogenized, and triplicated aliquots of 2.5 mL pipetted into 50 mL tubes. Twenty-five mL of trichloroacetic acid 24% and 22.5 mL of deionized water were added to each tube. Samples were agitated and homogenized every 5 min for 30 min. The solution was filtrated and a 5-mL aliquot of the filtrate was transferred to a 50-mL tube. One mL of lanthanum chloride 5% and 44 mL of deionized water were added to each tube to result in a 50-mL solution. Samples were then analyzed by atomic absorption using a spectrophotometer (AAnalyst 200, Perkin-Elmer Inc., Waltham, MA) equipped with Ca and Mg specific hollow cathode lamps. The intra-assay CV averaged 4.6 and 5.2% for concentrations of tCa and tMg in colostrum, respectively. The amounts of tCa and tMg secreted in colostrum were calculated based on yields of colostrum and the respective concentrations of tCa and tMg.

Blood Sampling and Processing

Blood was collected 3 times per week from 265 days of gestation until calving, and on days 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 postpartum, by puncture of the coccygeal vein or artery into evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Tubes contained no anticoagulant agents for serum separation or K_2 EDTA or lithium heparin for plasma separation. For the prepartum period, only the last 4 samples relative to calving were used for analysis. Serum samples were allowed to clot at room temperature and then placed in ice until processing. Samples with anticoagulant were placed in ice until processing within 4 h of collection. Tubes were centrifuged for 15 min at 2,500 x *g* for plasma or serum separation. Plasma and serum samples were transferred into multiple aliquots of 0.5, 1.0 or 2.0 mL and stored frozen at -20 or -

80°C until analyses. All assays performed followed the initial randomization with blocks such that samples from each block were analyzed in the same assay.

Sampling of Whole Blood and Measurements of Ionized Ca and Acid-Base Measures

Whole blood was sampled by puncture of the jugular vein on days -9, -6, -3, -1, 0, 1, 2, 3, and 6 relative to calving and analyzed within 1 to 3 min for concentrations of iCa, pH, HCO_3, base excess, and partial pressure of O_2 (**pO_2**) and CO_2 (**pCO_2**) using a handheld biochemical analyzer (VetScan i-STAT, Abaxis, Union City, CA).

Plasma Vitamin D Metabolites

Plasma sampled on days -6, -3, -1, 0, 1, 2, 3, 6, 9, and 12 relative to calving was analyzed for concentrations of vitamin D_3, 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, 3-epi-25-hydroxyvitamin D_3, 1,25-dihydroxyvitamin D_3, and 24,25-dihydroxivitamin D_3 using HPLC coupled with MS detection by the Analytical Research Center of DSM Nutritional Products (Kaiseraugst, Switzerland). Personnel running the assays were blind to treatments. The lower limit of quantification for the assays were 0.5 ng/mL for vitamin D_3, 25-hydroxyvitamin D_2, 3-epi-25-hydroxyvitamin D_3 and 24,25-hydroxyvitamin D_3, 1.0 ng/mL for 25-hydroxyvitamin D_3, and 10 pg/mL for 1,25-hydroxyvitamin D_3.

Measurements of Serum Concentrations of tCa, tMg, and total P

Concentrations of tCa, tMg, and total P (**tP**) were analyzed in serum samples collected on days -9, -6, -3, -1, 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 relative to calving in duplicates. Samples were analyzed for tCa and tMg by atomic absorption (AAnalyst 200, Perkin-Elmer Inc., Waltham, MA) as previously described by Martinez et al. (2012). Intra- and inter-assays CV were, respectively, 1.6 and 3.3% for tCa, and 2.0 and 2.8% for tMg. Concentrations of tP were quantified in serum using the molybdenum blue method (Quinlan and DeSesa, 1955). The intra- and inter-assay CV were, respectively, 6.0 and 8.0%.

Measurements of Concentrations of PTH, Serotonin, C-Terminal Telopeptide of Type 1 Collagen, Undercarboxylated and Carboxylated OC, and Adiponectin

Personnel performing the assays were blind to treatments. Plasma sampled on days -6, -3, -1, 0, 1, 2, 3, 6, and 9 relative to calving was analyzed for concentrations of PTH by ELISA (Catalog # 60-3500; Immutopics, Athens, OH) at the Heartland Assays laboratory (Heartland Assays, LLC, Ames, IA). The Intra- and inter-assays CV were, respectively, 5.5 and 5.6%.

Serum samples collected on days -9, -6, -3, -1, 0, 1, 2, 3, and 6 relative to calving were analyzed for concentrations of serotonin using an enzyme immunoassay (Serotonin EIA Kit, Beckman Coulter, Inc., Brea, CA). Intra- and inter-assays CV were, respectively, 11.2 and 13.2%.

Serum samples collected on days -1, 0, 1, 2, and 3 postpartum were analyzed for the bone resorption marker C-terminal telopeptide of type 1 collagen (**CTX-1**) by ELISA (Catalog # AC-02F1; The Boldons, UK), and the intra- and inter-assays CV were, respectively, 2.2 and 7.7%.

Plasma samples collected on days -1, 0, 1, 2, and 3 relative to calving were analyzed for undercarboxylated (**uOC**) and carboxylated (**cOC**) osteocalcin by ELISA (catalog # MK111 and MK118; Clontech Labs, Takara Bio Inc., Mountain View, CA). The intra- and inter-assays CV were, respectively, 5.2 and 8.3% for uOC, and 3.7 and 1.4% for cOC.

Plasma samples collected on days -9, -6, -3, -1, 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 were analyzed for concentrations of adiponectin by ELISA conducted by CSIRO Agriculture (St Lucia, Queensland, Australia). The intra and inter-assays CV were, respectively, 11.9 and 10.2%.

Urine Collection and Analysis

Urine samples were collected twice weekly prepartum and on days 3 and 7 postpartum by massaging the perineal area until a clean and copious stream of urine was obtained. Samples were placed on plastic tubes and placed in ice and pH was measured within 10 minutes of collection using a portable pH meter (B-712 LAQUA twin, Horiba Scientific, Edison, NJ). Samples were then frozen at -20°C for later analyses. Prepartum samples collected closest to days -7 and -3, and those collected on days 3, and 7 relative to calving were used for statistical analyses of effects of treatments on pH.

Prepartum samples collected closest to day -5 and those collected on day 3 relative to calving were used for determinations of creatinine, tCa, and tMg concentrations. Samples were analyzed in triplicates for creatinine using a commercial colorimetric method (Creatinine Urinary Detection Kit, Arbor Assays, Ann Arbor, MI). The intra- and inter-assay CV for creatinine were 2.4 and 7.6%, respectively. Triplicate urine samples were diluted 1 to 400 with 0.5% lanthanum chloride and concentrations of tCa and tMg were analyzed by atomic absorption using a

spectrophotometer equipped with Ca and Mg specific hollow cathode lamps (AAnalyst 200, Perkin-Elmer Inc., Waltham, MA). Urinary tCa and tMg assays were performed as a single run and the intra-assay CV were 4.4 and 2.1%, respectively.

Creatinine was used as a marker to estimate daily urinary volume based on the constant excretion of 29 mg of creatinine per kg of BW per day (Valadares et al., 1999). The estimate of daily urinary volume was calculated for the pre- and postpartum periods using the mean BW of each cow in the last week of gestation and first week postpartum. The estimate of daily urinary volume was calculated as: BW kg x 29/urinary concentrations of creatinine in mg/L. Daily urinary excretions of tCa and tMg were calculated as the product of urinary volume and the respective concentrations of those minerals in the urine samples.

Estimated Mineral Balance

Estimated Ca and Mg balances were calculated in the last week of gestation and first 3 DIM based on measurements of DMI, concentrations of Ca and Mg in diets, urinary Ca and Mg losses, and Ca and Mg losses in colostrum and milk. Estimated absorptions of Ca and Mg and fetal tissues accretion were computed using the NRC (2001) according to diet composition and calf BW at birth. It was assumed no accretion of minerals in the last week of gestation by the dams other than accretion into fetal tissues. Also, it was assumed no sequestration of Ca by the mammary gland before secretion of colostrum because of lack of accurate data, although there is indication that Ca secreted in colostrum is sequestered in the mammary gland days before calving (Visek et al., 1953), although it has been calculated as 0 in Ca balance studies in prepartum cows (Ramberg et al., 1970).

Statistical Analysis

The experiment followed a randomized complete block design with cow as the experimental unit. All data were analyzed by ANOVA and results for the pre- and postpartum periods were analyzed separately. Normality of residuals and homogeneity of variance were examined for each continuous dependent variable analyzed after fitting the statistical model. Responses that violated the assumptions of normality were subjected to power transformation according to the Box-Cox procedure (Box and Cox, 1964) using the PROC TRANSREG in SAS (SAS ver. 9.4, SAS/STAT, SAS Institute Inc., Cary, NC). The LSM and SEM were back transformed for presentation according to Jørgensen and Pedersen (1998). Concentrations of vitamin D_3, 1,25-dihydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3, PTH, serotonin, uOC, and cOC had to be log-

transformed before analyses either because of heteroscedasticity or because residuals were not normally distributed.

Data were analyzed by mixed models using the MIXED procedure of SAS (SAS/STAT). Responses with a single measurement per cow were analyzed with the fixed effects of level of DCAD (positive vs. negative), source of vitamin D (cholecalciferol vs. calcidiol), interaction between DCAD and vitamin D, parity (nulliparous vs. parous), and the interactions between DCAD and parity, vitamin D and parity, and DCAD and vitamin D and parity, and the random effect of block. Data with repeated measures within experimental units were analyzed with the same mixed model described above, but also included the fixed effects of day, and the interactions between DCAD and day, vitamin D and day, parity and day, DCAD and vitamin D and day, DCAD and parity and day, vitamin D and parity and day, and DCAD and vitamin D and parity and day. Cow nested within DCAD and vitamin D was a random effect in the model. The Repeated statement was included in all mixed models with repeated measurements and day the specified repeated effect. The covariance structure was modeled based on spacing between measurements and selection was based on model fit that resulted in the smallest corrected Akaike's information criterion. The Kenward-Roger method was used to compute the approximate denominator degrees of freedom for the F tests in the statistical models. When an interaction was significant, pairwise comparisons were performed with the adjustment of Tukey.

Statistical significance was considered at $P \leq 0.05$, and tendency was considered at $0.05 < P \leq 0.10$.

RESULTS

Of the 79 cows included in the statistical analyses, one PCA cow developed pneumonia because of aspiration of an oral Ca drench used to treat clinical hypocalcemia and she had to be euthanized and was removed prematurely from the experiment and contributed with data from enrollment to 2 DIM. Therefore, analyses of data until 2 DIM included 79 cows, whereas data collected after 2 DIM included 78 cows.

Table 2. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on concentrations of vitamin D metabolites in plasma of Holstein cows postpartum[1]

	Positive		Negative			Parity			*P*-value[2]			
Item	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Vitamin D_3, ng/mL	17.70	1.20	12.18	0.98	0.80	4.45	3.57	0.35	0.005	< 0.001	0.40	0.03
25-OH D_3, ng/mL	62.1	261.2	57.4	212.7	9.1	146.3	150.4	8.5	0.003	< 0.001	0.01	0.71
25-OH D_2, ng/mL[¥]	9.35	9.42	9.21	11.57	0.59	11.10	8.67	0.55	0.08	0.03	0.04	0.002
3-Epi 25-OH D_3, ng/mL	11.9	19.1	10.5	15.4	0.8	13.6	14.8	0.8	< 0.001	< 0.001	0.11	0.27
1,25-$(OH)_2$ D_3, pg/mL	42.8	51.3	55.0	58.4	2.3	50.8	52.3	1.9	< 0.001	0.004	0.14	0.60
24,25-$(OH)_2$ D_3, ng/mL[§]	1.53	23.97	1.09	9.92	1.10	3.45	5.78	0.42	< 0.001	< 0.001	0.02	0.001
Ratio, ng/ng[3]	0.0296	0.0956	0.0231	0.0590	0.0051	0.0459	0.0577	0.0041	< 0.001	< 0.001	0.004	0.02

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Blood samples collected on d -9, -6, -3, and -1 relative to calving.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[3] Ratio in plasma of concentrations of 24,25-$(OH)_2$ D_3 to 25-OH D_3.

[*] Interaction between DCAD and parity ($P < 0.05$).

[§] Interaction between source of vitamin D and parity ($P < 0.05$).

[¥] Interaction between DCAD, vitamin D and parity ($P < 0.05$).

Table 3. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on concentrations of vitamin D metabolites in plasma of Holstein cows postpartum[1]

	Positive		Negative			Parity			*P*-value[2]			
Item	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Vitamin D_3, ng/mL[¥]	6.27	1.53	4.95	1.21	0.35	2.84	2.67	0.22	0.02	< 0.001	0.97	0.57
25-OH D_3, ng/mL	59.8	233.7	57.3	202.9	7.5	134.8	142.0	5.6	0.03	< 0.001	0.06	0.36
25-OH D_2, ng/mL	8.4	8.6	8.5	10.9	0.6	10.0	8.2	0.5	0.02	0.01	0.04	0.02
3-Epi 25-OH D_3, ng/mL[¥]	10.8	17.8	9.7	14.9	0.7	12.8	13.9	0.6	0.004	< 0.001	0.17	0.23
1,25-$(OH)_2$ D_3, pg/mL[*]	89.8	88.2	81.8	86.1	4.2	65.8	113.6	3.0	0.22	0.73	0.48	< 0.001
24,25-$(OH)_2$ D_3, ng/mL[§]	1.83	24.69	1.36	14.12	2.45	4.42	6.66	0.38	< 0.001	< 0.001	0.20	< 0.001
Ratio,[3] ng/ng	0.0362	0.1216	0.0283	0.0807	0.0052	0.0621	0.0713	0.0045	< 0.001	< 0.001	0.002	0.12

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Blood sampled on d 0, 1, 2, 3, 6, 9, and 12 postpartum.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[3] Ratio in plasma of concentrations of 24,25-$(OH)_2$ D_3 to 25-OH D_3.

[*] Interaction between DCAD and parity ($P < 0.05$).

[§] Interaction between source of vitamin D and parity ($P < 0.05$).

[¥] Interaction between DCAD, vitamin D and parity ($P = 0.07$).

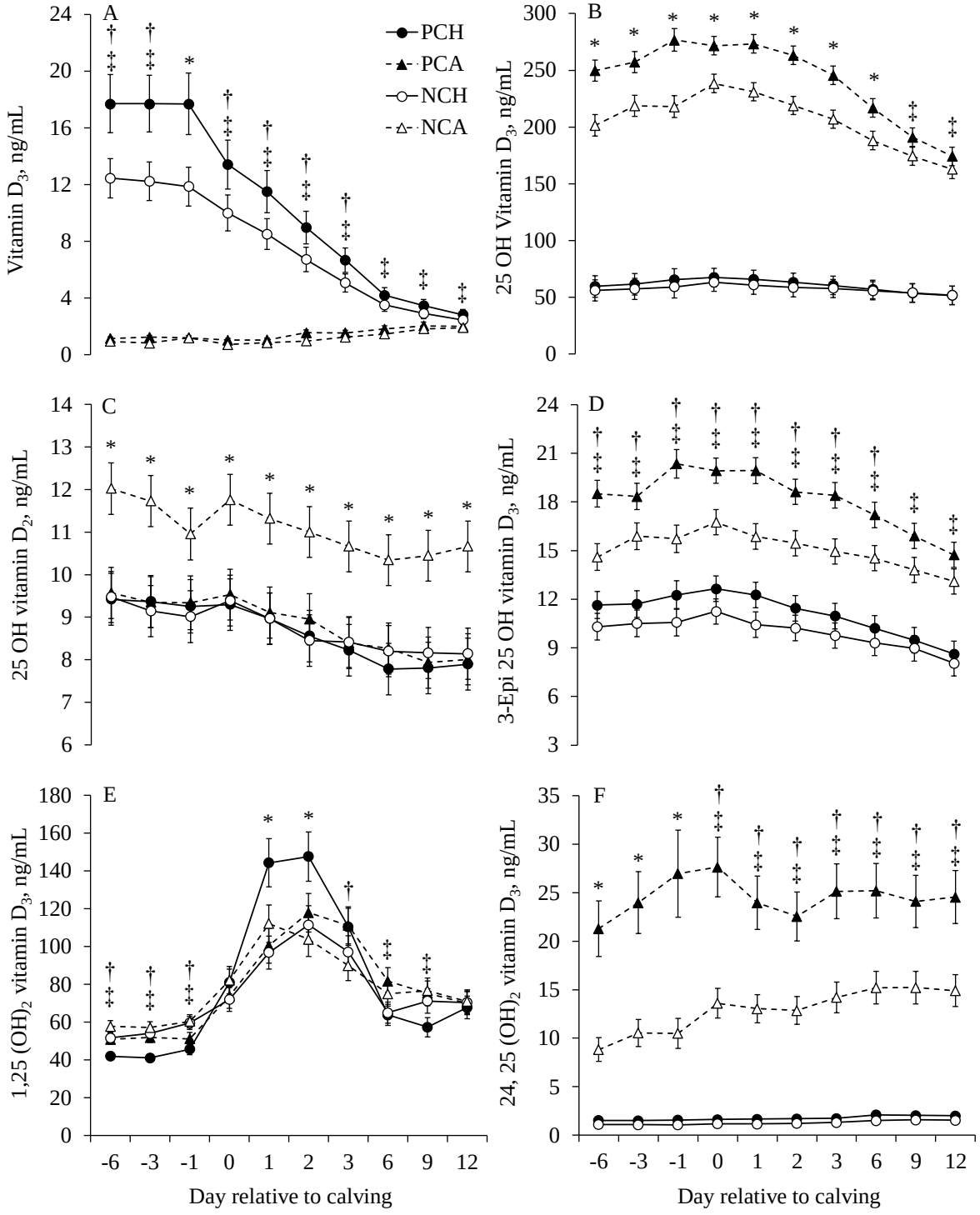

Figure 1. Concentrations of vitamin D_3 (A), 25-hydroxyvitamin D_3 (B), 25-hydroxyvitamin D_2 (C), 3-epi 25-hydroxyvitamin D_3 (D) 1,25-dihydroxyvitamin D_3 (E), and 24,25-dihydroxyvitamin D_3 (F) in plasma of cows fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Within a day, † denotes effect of DCAD ($P < 0.05$), ‡ effect of vitamin D ($P < 0.05$), and * interaction between DCAD and vitamin D ($P < 0.05$).

Vitamin D Metabolites

Cows fed the positive DCAD had greater (P = 0.005) vitamin D_3 concentrations in plasma prepartum than those fed the negative DCAD (positive = 4.60 vs. negative = 3.45 ng/mL), and feeding cholecalciferol markedly increased (P < 0.001) plasma concentrations of vitamin D_3 prepartum compared with cows fed calcidiol (CH = 14.68 vs. CA = 1.08 ng/mL; Table 2). The differences in concentrations of vitamin D_3 prepartum with treatments were equally observed in in both nulliparous and parous cows, although nulliparous had greater (P = 0.03) vitamin D_3 concentration in plasma prepartum than parous cows. The differences in concentrations of vitamin D_3 in plasma with treatments extended during the postpartum period (Figure 1A; Table 3), although they markedly declined in cows fed cholecalciferol, whereas a slight increase was observed in cows fed calcidiol.

Similar to vitamin D_3, concentrations of 25-hydroxyvitamin D_3 were greater (P = 0.003) throughout the prepartum period in cows fed the positive than those fed the negative DCAD, which averaged 161.6 and 135.1 ng/mL, respectively (Table 1, Figure 1B). As anticipated, feeding calcidiol increased (P < 0.001) plasma concentrations of 25-hydroxyvitamin D_3 4-fold (CH = 59.7 vs. CA = 237.0 ng/mL), but the increment was greater in cows fed positive than negative DCAD. No differences between parity groups or interactions between treatment and parity were observed for prepartum concentrations of 25-hydroxyvitamin D_3. Concentrations of 25-hydroxyvitamin D_3 during the postpartum period remained elevated in cows fed calcidiol compared with those fed cholecalciferol (CH = 58.5 vs. CA = 218.1 ng/mL; Figure 1B); however, a tendency (P = 0.06) for interaction between DCAD and vitamin D was observed because the increase in 25-hydroxyvitamin D_3 was greater in cows fed positive than negative DCAD (Table 3). The concentrations of 25-hydroxyvitamin D_3 in plasma of individual cows fed 3 mg of cholecalciferol for the last 3 wk of gestation did not surpass 90 ng/mL either in the pre- or postpartum period (Figure 2A and 2B), whereas cows fed calcidiol maintained concentrations of cholecalciferol always below 4 ng/mL.

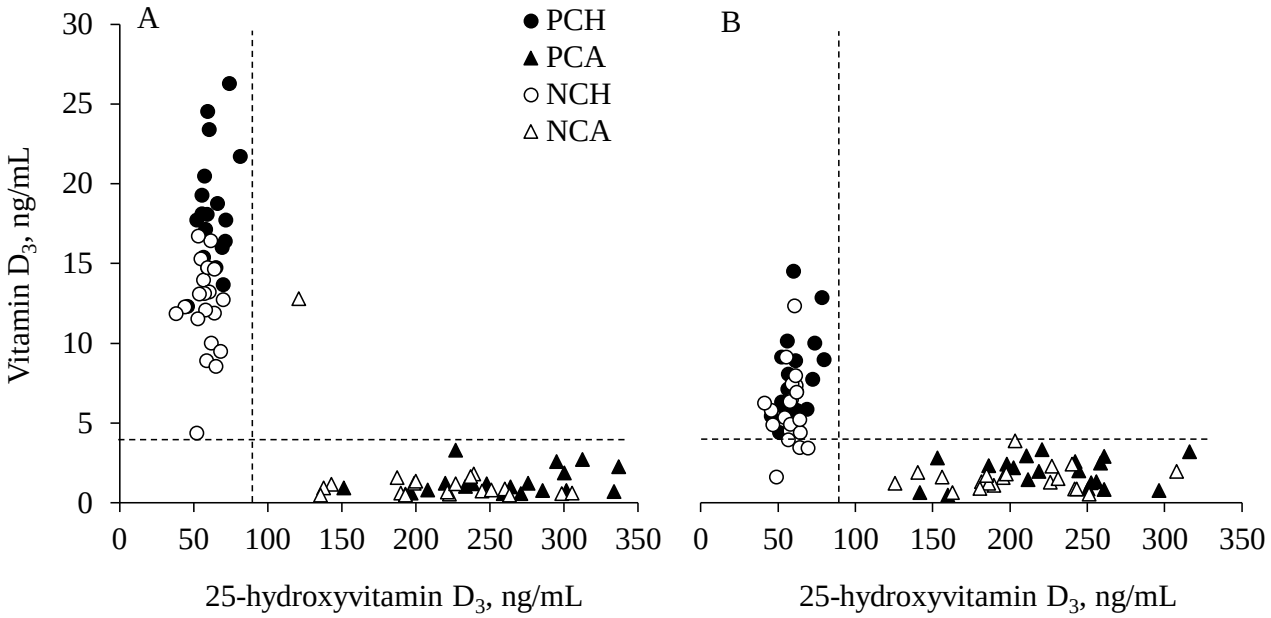

Figure 2. Scatter graphs of concentrations of vitamin D_3 and 25-hydroxyvitamin D_3 prepartum (A) and postpartum (B) in plasma of cows fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Values for a cow were averaged into a single mean for the pre- and postpartum periods. Dashed lines intersect at 4 ng/mL of vitamin D_3 and 90 ng/mL of 25-hydroxyvitamin D_3.

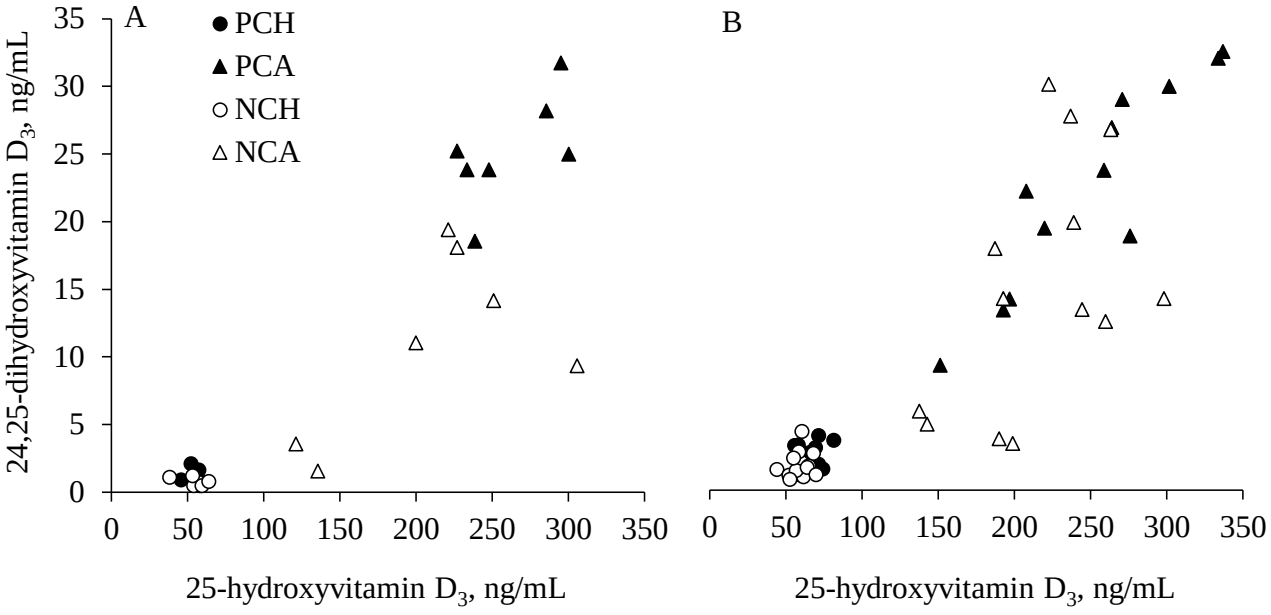

Figure 3. Scatter graphs of concentrations of 24,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 prepartum in nulliparous (A) and parous cows (B) fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Values for a cow were averaged into a single mean for the pre- and postpartum periods. A linear relationship ($P < 0.001$; $r^2 = 0.81$) was observed between 24,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 prepartum.

Prepartum concentrations of 25-hydroxyvitamin D_2 in plasma were affected by the interaction ($P = 0.04$) between DCAD and vitamin D source because cows fed NCA had greater concentrations than those fed the other three treatment diets (Table 2; Figure 1C). The differences observed prepartum extended to the postpartum period (Table 3), when all cows were fed the same diet. Parity group affected ($P < 0.02$) concentrations of 25-hydroxyvitamin D_2 during the pre- and postpartum periods (Tables 2 and 3). Nevertheless, an interaction ($P = 0.04$) among DCAD, vitamin D and parity was detected for concentrations of 25-hydroxyvitamin D_2 prepartum because the increment in plasma concentrations with feeding NCA was greater in nulliparous than in parous cows.

Feeding a diet with positive DCAD increased ($P < 0.001$) prepartum concentrations of 3-epi 25-hydroxyvitamin D_3 compared with cows fed the diet with negative DCAD (positive = 15.5 vs. negative = 12.9 ng/mL; Table 2), and the differences were extended to the postpartum period (positive = 14.3 vs. negative = 12.3 ng/mL; Figure 1D and Table 3). Similarly, feeding calcidiol compared with cholecalciferol increased ($P < 0.001$) concentrations of 3-epi 25-hydroxyvitamin D_3 prepartum (CH = 11.2 vs. CA = 17.2 ng/mL; Table 2) and postpartum (CH = 10.3 vs. CA = 16.4 ng/mL; Table 3). No differences were observed between parity groups or interactions between treatment and parity group for concentrations of 3-epi 25-hydroxyvitamin D_3.

Prepartum concentrations of 1,25-dihydroxyvitamin D_3 in plasma increased ($P < 0.01$) in cows fed negative compared with positive (positive = 46.9 vs. negative = 56.7 pg/mL) and in cows fed calcidiol compared with cholecalciferol (CH = 48.5 vs. CA = 54.7 pg/mL; Table 2). Postpartum, concentrations peaked at 2 DIM and an interaction ($P < 0.05$) between DCAD and vitamin D were observed on days 1 and 2 postpartum (Figure 1E) because those fed PCA had greater concentrations than cows fed the other treatments. A tendency ($P = 0.06$) for interaction between vitamin D and day was detected postpartum and cows fed calcidiol had greater concentrations of 1,25-dihydroxyvitamin D_3 in plasma on days 6 and 9 postpartum than cows fed cholecalciferol (Figure 1E).

Cows supplemented with calcidiol had a marked increase ($P < 0.001$) in concentrations of 24,25-dihydroxyvitamin D_3 in plasma prepartum (CH = 1.3 vs. CA = 15.4 ng/mL), and an interaction ($P = 0.02$) between DCAD and vitamin D was detected because the increase was more accentuated when fed the diet with positive compared with the negative DCAD (Table 2; Figure

1F). An interaction ($P < 0.001$) between source of vitamin D and parity was observed for concentrations of 24,25-dihydroxyvitamin D_3 in plasma prepartum because within those fed cholecalciferol, nulliparous cows had almost 2.5-fold smaller ($P < 0.001$) concentration than parous cows (nulliparous = 0.82 vs. parous = 2.04 ng/mL), whereas within cows fed calcidiol, no difference ($P = 0.66$) between nulliparous and parous cows was detected and concentrations averaged 14.5 and 16.4 ng/mL, respectively. Figure 3 depicts a scatter plot of 24,25-dihydroxyvitamin D_3 according to concentrations of 25-hydroxyvitamin D_3 in plasma of nulliparous and parous cows prepartum and the differences can be visualized when they were fed cholecalciferol. When we calculated the ratio of plasma concentrations of 24,25-dihydroxyvitamin D_3 to 25-hydroxyvitamin D_3 (ng/ng), it was smaller ($P = 0.02$) in nulliparous than parous cows when fed cholecalciferol (nulliparous = 0.0171 vs. parous = 0.0358 ± 0.005) or calcidiol (nulliparous = 0.073 vs. parous = 0.079 ± 0.005). Postpartum concentrations of 24,25-dihydroxyvitamin D_3 in plasma of dairy cows followed the same pattern as those observed prepartum (Figure 1F), and both DCAD (positive = 6.73 vs. negative = 4.38 ng/mL) as well as vitamin D (CH = 1.58 vs. CA = 18.67) affected ($P < 0.001$) concentrations of 24,25-dihydroxyvitamin D_3 (Table 3). Similar to the prepartum period, an interaction ($P < 0.001$) between vitamin D and parity was observed postpartum because within those fed cholecalciferol, nulliparous cows had the same 2.5-fold smaller ($P < 0.001$) concentration than parous cows (nulliparous = 1.02 vs. parous = 2.45 ng/mL), whereas within cows fed calcidiol, parity did not affect ($P = 0.50$) concentrations and they averaged 19.3 and 18.1 ng/mL in nulliparous and parous cows, respectively.

Whole Blood and Serum Concentrations of Minerals

Feeding a diet with negative DCAD prepartum increased ($P = 0.03$) whole blood concentrations of iCa prepartum (positive = 1.217 vs. negative = 1.240 m*M*), but the opposite response was observed for serum tCa (positive = 2.446 vs. negative = 2.379 m*M*; Table 4). Supplementing cows with calcidiol increased ($P < 0.001$) prepartum concentrations of blood iCa (CH = 1.200 vs. CA = 1.257 m*M*) and serum tCa (CH = 2.355 vs. CA = 2.470 m*M*). Nevertheless, interactions ($P < 0.05$) among DCAD and vitamin D and parity were observed for iCa and tCa prepartum. For iCa, nulliparous cows fed NCA had the greatest concentrations prepartum, particularly on days -3 and -1, whereas for parous cows, those fed PCA had the greatest concentrations prepartum (Figure 5A and 4B). On the other hand, for tCa, both nulliparous and parous cows fed PCA had the greatest concentrations prepartum (Figure 5C and 5D). Feeding a diet with negative DCAD attenuated ($P < 0.001$) the decline in blood iCa and serum tCa on days 0 and 1

postpartum (Figure 4A and 4B) and the benefits were observed in both nulliparous (Figure 5A and 5C) and parous cows (Figure 5B and 5D). Concentrations of iCa and tCa increased (P < 0.001) with day postpartum, and those of tCa reached a plateau earlier in nulliparous than parous cows based on the interaction (P < 0.001) between parity and day, at approximately 6 and 15 DIM, respectively (Figure 5C and 5D).

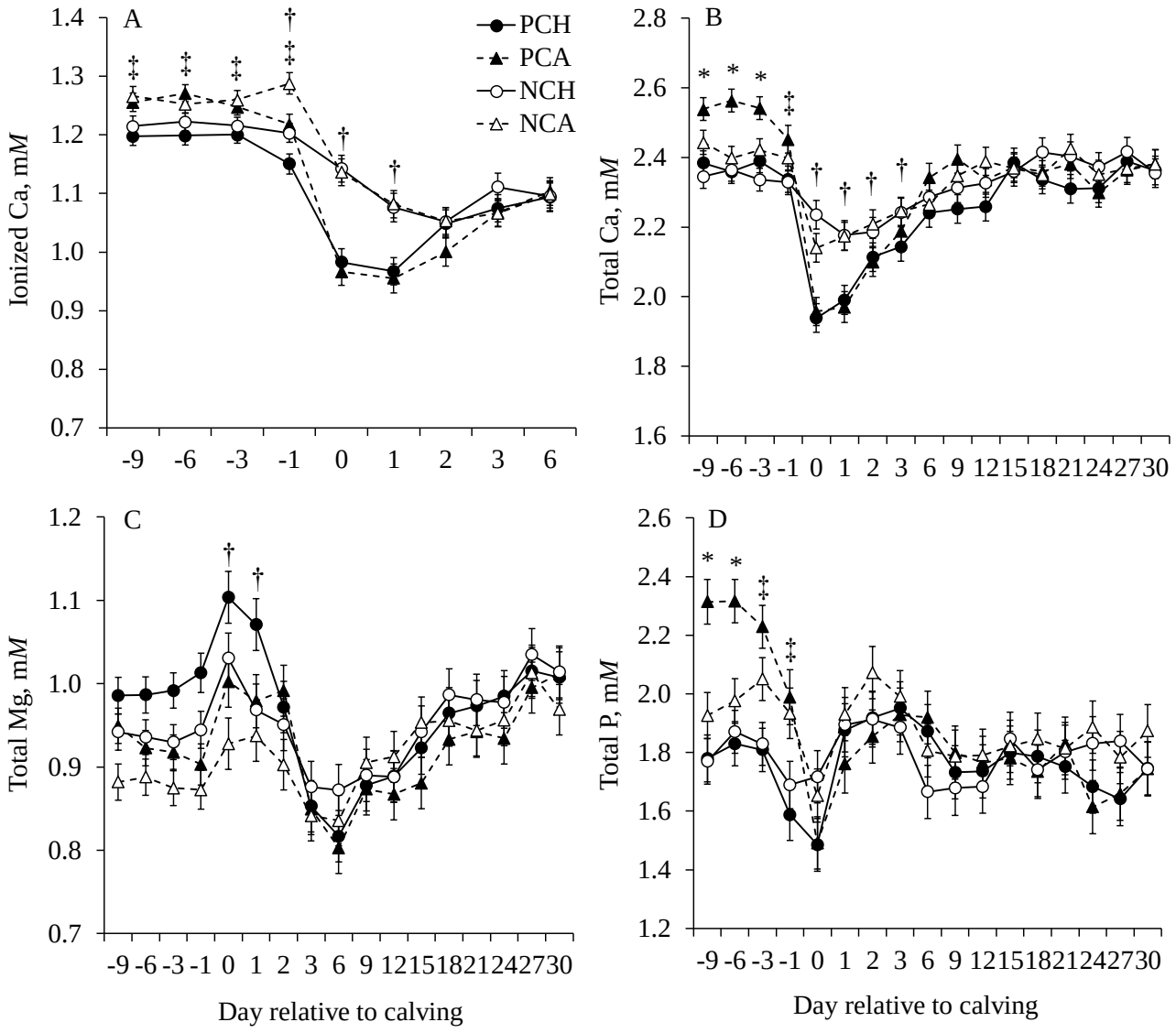

Figure 4. Concentrations of ionized Ca (A) in whole blood and total Ca (B), Mg (C), and P (D) in serum of cows fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Within a day, † denotes effect of DCAD (P < 0.05), ‡ effect of vitamin D (P < 0.05), and * interaction between DCAD and vitamin D (P < 0.05).

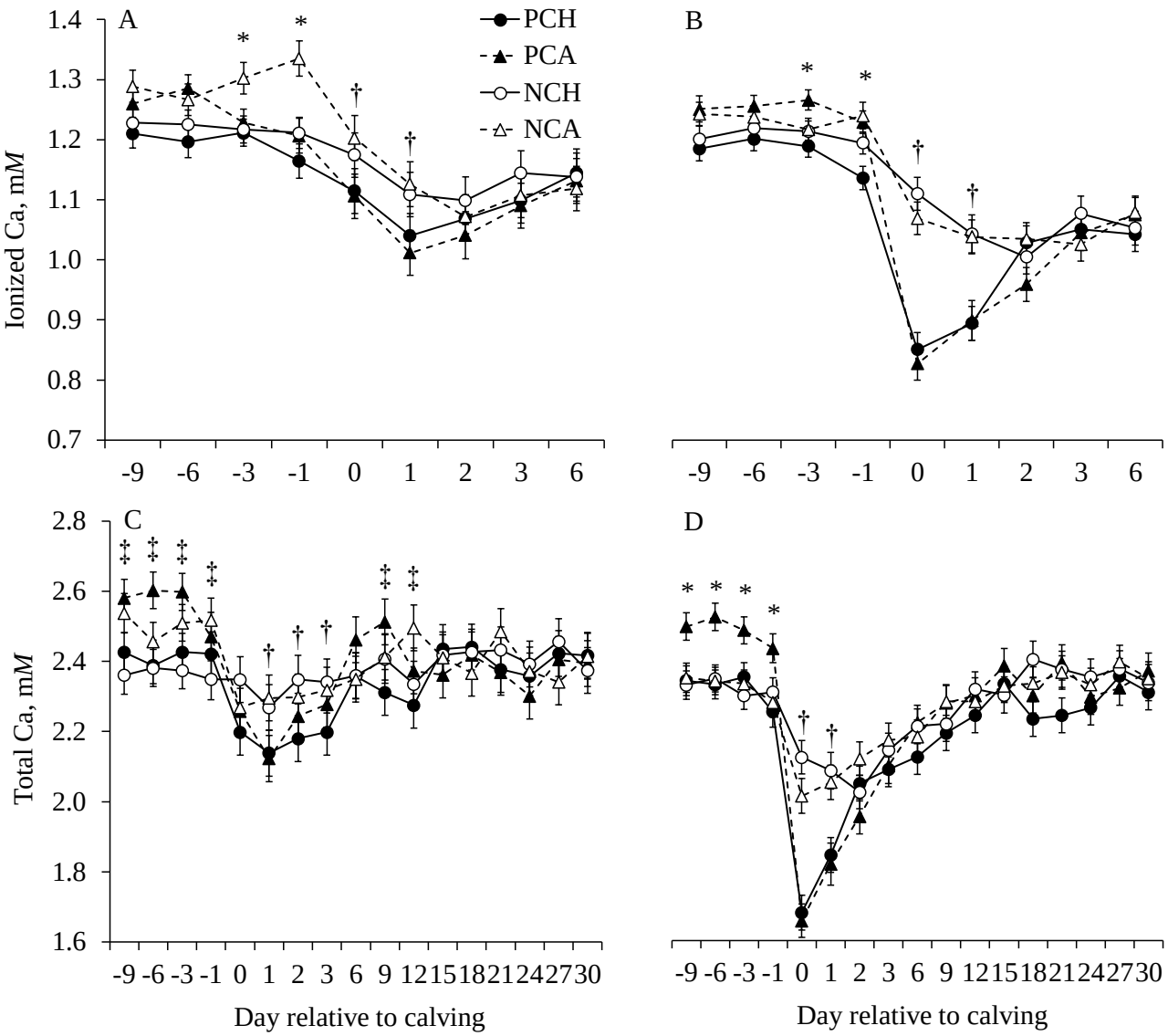

Figure 5. Concentrations of ionized Ca in whole blood of nulliparous (A) and parous cows (B) and total Ca in serum of nulliparous (C) and parous cows (D) fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Within a day, † denotes effect of DCAD ($P < 0.05$), ‡ effect of vitamin D ($P < 0.05$), and * interaction between DCAD and vitamin D ($P < 0.05$).

Prepartum concentrations of tMg in serum were greater ($P = 0.004$) for cows fed positive than negative DCAD (positive = 0.959 vs. negative = 0.909 m*M*) and greater ($P < 0.001$) for cows fed cholecalciferol than calcidiol (CH = 0.966 vs. CA = 0.901 m*M*; Table 4), and these differences were observed throughout the last 9 d of gestation (Figure 4C). Concentrations of tMg increased ($P < 0.001$) with day postpartum in all 4 treatments (Figure 4C). Only an

interaction (P = 0.05) between DCAD and parity was observed postpartum because within cows fed the diet with positive DCAD, nulliparous and parous had similar (P = 0.98) concentrations of tMg postpartum (nulliparous = 0.943 vs. parous = 0.942 m*M*); however, within cows fed negative DCAD, nulliparous had greater (P = 0.02) tMg than parous cows (nulliparous = 0.982 vs. parous = 0.900 m*M*).

Treatment affected concentrations of tP in serum prepartum and an interaction (P = 0.006) between DCAD and vitamin D was observed because feeding calcidiol increased concentrations of tP particularly in cows fed the diet with positive DCAD (Table 4). Concentrations of tP reached a nadir on the day of calving and became relatively stable after 2 DIM in all four treatments (Figure 4D). No effects of treatments were observed for postpartum tP concentrations (Table 5). Neither parity group nor interactions between parity and treatment influenced concentrations of tP in serum pre- and postpartum.

Concentrations of sodium (**Na**) in whole blood pre- and postpartum remained mostly unaffected by treatment (Tables 4 and 5); only a tendency (P = 0.06) for effect of parity was observed for blood Na postpartum because nulliparous had slightly smaller concentration than parous cows. Concentrations of potassium (**K**) in whole blood prepartum were greater (P = 0.003) in cows fed negative than positive DCAD (positive = 3.94 vs. negative = 4.06 m*M*; Table 4), whereas vitamin D only tended (P = 0.09) to affect prepartum blood K (CH = 4.04 vs. CA = 3.97 m*M*). As in the prepartum period, blood K concentration was greater (P = 0.05) for cows fed negative than positive DCAD (positive = 3.82 vs. negative = 3.92 m*M*; Table 5), but no other effects were observed of either treatment or parity.

Table 4. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on concentrations of minerals, hormones, and adipose metabolites in blood of Holstein cows prepartum[1]

	Positive		Negative			Parity			*P*-value[2]			
Item[3]	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Ionized Ca, m*M*[¥]	1.187	1.248	1.214	1.267	0.010	1.240	1.234	0.008	0.03	< 0.001	0.68	0.06
Total Ca, m*M*[¥]	2.367	2.524	2.344	2.415	0.028	2.463	2.362	0.028	0.005	< 0.001	0.07	0.02
Total Mg, m*M*	0.994	0.923	0.938	0.879	0.019	0.960	0.908	0.017	0.004	< 0.001	0.72	0.04
Total P, m*M*	1.751	2.212	1.790	1.971	0.059	1.933	1.930	0.059	0.04	< 0.001	0.006	0.97
Na, m*M*	144.3	144.0	144.0	144.3	0.3	144.0	144.3	0.3	0.64	0.68	0.61	0.40
K, m*M*	4.00	3.88	4.08	4.05	0.04	3.98	4.02	0.03	0.003	0.09	0.22	0.35
PTH, pg/mL	85.6	75.0	97.3	69.3	20.7	91.8	71.7	14.8	0.92	0.36	0.69	0.34
Serotonin, µg/mL	1.361	1.107	1.543	1.714	0.220	1.724	1.158	0.235	0.02	0.67	0.20	0.10
Adiponectin, ng/mL	17.7	14.0	14.9	16.1	2.4	17.3	14.1	2.5	0.90	0.49	0.17	0.38

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Blood samples collected on d -9, -6, -3, and -1 relative to calving. Whole blood analyzed for concentrations of ionized Ca, Na and Mg; serum analyzed for total Ca, total Mg, total P, and serotonin; plasma analyzed for adiponectin.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[3] PTH = parathyroid hormone; uOC = undercarboxylated osteocalcin; cOC = carboxylated osteocalcin; CTX-1 = C-terminal telopeptide of type 1 collagen.

[*] Interaction between DCAD and parity ($P < 0.05$).

[§] Interaction between source of vitamin D and parity ($P < 0.05$).

[¥] Interaction between DCAD, vitamin D and parity ($P < 0.10$).

Table 5. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on concentrations of minerals, hormones, and bone and adipose metabolites in blood of Holstein cows postpartum[1]

	Positive		Negative			Parity			*P*-value[2]			
Item[3]	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Ionized Ca, m*M*	1.033	1.019	1.095	1.087	0.015	1.107	1.010	0.009	< 0.001	0.45	0.82	< 0.001
Total Ca, m*M*	2.333	2.265	2.314	2.309	0.025	2.353	2.207	0.023	0.005	0.55	0.39	< 0.001
Total Mg, m*M* [*]	0.958	0.928	0.955	0.927	0.022	0.962	0.921	0.020	0.93	0.19	0.96	0.12
Total P, m*M*	1.768	1.760	1.788	1.850	0.052	1.812	1.770	0.062	0.17	0.49	0.38	0.60
Na, m*M*	141.4	141.5	141.8	142.0	0.3	141.3	142.0	0.3	0.17	0.63	0.87	0.06
K, m*M*	3.84	3.79	3.93	3.88	0.05	3.82	3.91	0.04	0.05	0.27	0.93	0.12
PTH, pg/mL	127.8	147.2	133.5	116.6	22.7	122.7	139.5	17.1	0.59	0.98	0.43	0.47
CTX-1, ng/mL[*§]	1.42	1.25	1.51	1.23	0.18	1.75	1.04	0.21	0.83	0.14	0.73	0.003
uOC, ng/mL	2.15	2.58	2.15	2.42	0.38	2.96	1.69	0.33	0.83	0.34	0.82	0.006
cOC, ng/mL[*]	29.7	30.1	32.6	32.4	2.5	44.9	17.5	2.7	0.21	0.96	0.88	< 0.001
Total OC, ng/mL[*]	31.8	32.6	34.8	34.9	2.6	47.8	19.2	2.8	0.24	0.83	0.86	< 0.001
cOC, % of total	91.7	89.6	92.7	91.2	2.0	93.0	89.6	1.6	0.51	0.38	0.88	0.09
Ratio cOC to CTX-1[*§]	26.2	39.4	25.7	32.9	5.0	32.8	29.3	5.1	0.43	0.02	0.51	0.60
Serotonin, µg/mL	1.351	1.305	1.495	1.621	0.242	1.607	1.286	0.276	0.20	0.85	0.64	0.31
Adiponectin, ng/mL	15.8	13.4	16.0	16.3	2.2	15.8	14.9	2.5	0.26	0.42	0.30	0.80

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Samples analyzed for ionized Ca in whole blood and serotonin in serum (d 0, 1, 2, 3, and 6), total Ca, Mg, and P in serum (d 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30), PTH in plasma (d 0, 1, 2, 3, 6, and 9), CTX-1, uOC and cOC in plasma (d -1, 0, 1, 2, and 3), and adiponectin in plasma (d 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30).

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[3] PTH = parathyroid hormone; uOC = undercarboxylated osteocalcin; cOC = carboxylated osteocalcin; CTX-1 = C-terminal telopeptide of type 1 collagen.

[*] Interaction between DCAD and parity ($P < 0.05$), [§] Interaction between source of vitamin D and parity ($P < 0.05$), [¥] Interaction between DCAD, vitamin D and parity ($P < 0.05$).

Concentrations of Hormones and Bone Markers

Concentrations of PTH peaked on the day of calving and then declined (P = 0.03) postpartum (Figure 6A); however, treatment did not affect concentrations either pre- or postpartum (Tables 4 and 5). Concentrations of CTX-1 increased (P < 0.001) with calving and remained elevated in the first 3 DIM (Figure 6B). Interactions (P < 0.05) between DCAD and parity and between vitamin D and parity were detected (Table 5). Within nulliparous cows, DCAD (positive = 1.99 vs. negative = 1.53 ng/mL; P = 0.15) or vitamin D (CH = 1.61 vs. CA = 1.90 ng/mL; P = 0.35) did not influence concentrations of CTX-1; however, within parous cows, those fed the diet with negative DCAD (positive = 0.90 vs. negative = 1.22 ng/mL; P = 0.02) or supplemented with cholecalciferol (CH = 1.34 vs. CA = 0.81 ng/mL; P < 0.001) had greater concentrations than cows fed the positive DCAD or those fed calcidiol.

Concentrations of uOC and cOC did not differ with treatment, but nulliparous cows had greater (P < 0.01) concentrations than parous cows for both bone metabolites (Table 5). Concentrations of cOC represented more than 90% of the total OC and were 13 to 15-fold greater than those of uOC, and both declined (P < 0.001) at calving and then slightly increased in the first 3 DIM (Figure 6C and 6D). Interactions (P = 0.05) between DCAD and parity were observed for cOC and total OC because within nulliparous cows, feeding the diet with negative DCAD increased cOC (positive = 41.5 vs. negative = 48.2 ng/mL) and total OC (positive = 44.3 vs. negative = 51.3 ng/mL), whereas no difference was observed within parous cows for cOC (positive = 18.2 vs. negative = 16.9 ng/mL) and total OC (positive = 20.1 vs. negative = 18.3 ng/mL). The ratios of cOC to CTX-1 were used as an index of bone turnover with interactions (P < 0.05) between DCAD and day and vitamin D (Figure 7). Cows fed the diet with positive DCAD had greater (P < 0.01) ratio on the day before calving than those fed the diet with negative DCAD. Similarly, cows fed calcidiol had greater (P < 0.01) ratio on the day before calving than those fed cholecalciferol. The ratio markedly decreased (P < 0.001) with calving and initiation of lactation. Parity did not affect the ratio (Table 5), but interactions (P < 0.05) between DCAD and parity and between vitamin D and parity were observed. Within nulliparous cows, DCAD did not affect the ratio that averaged 32.8, but feeding the diet with negative DCAD reduced (P = 0.003) the ratio in parous cows (positive = 37.7 vs. negative = 21.0). Also, within nulliparous, source of vitamin D did not influence the ratio, but feeding calcidiol increased (P < 0.001) the ratio in parous cows compared with cholecalciferol (CH = 19.7 vs. CA = 39.0).

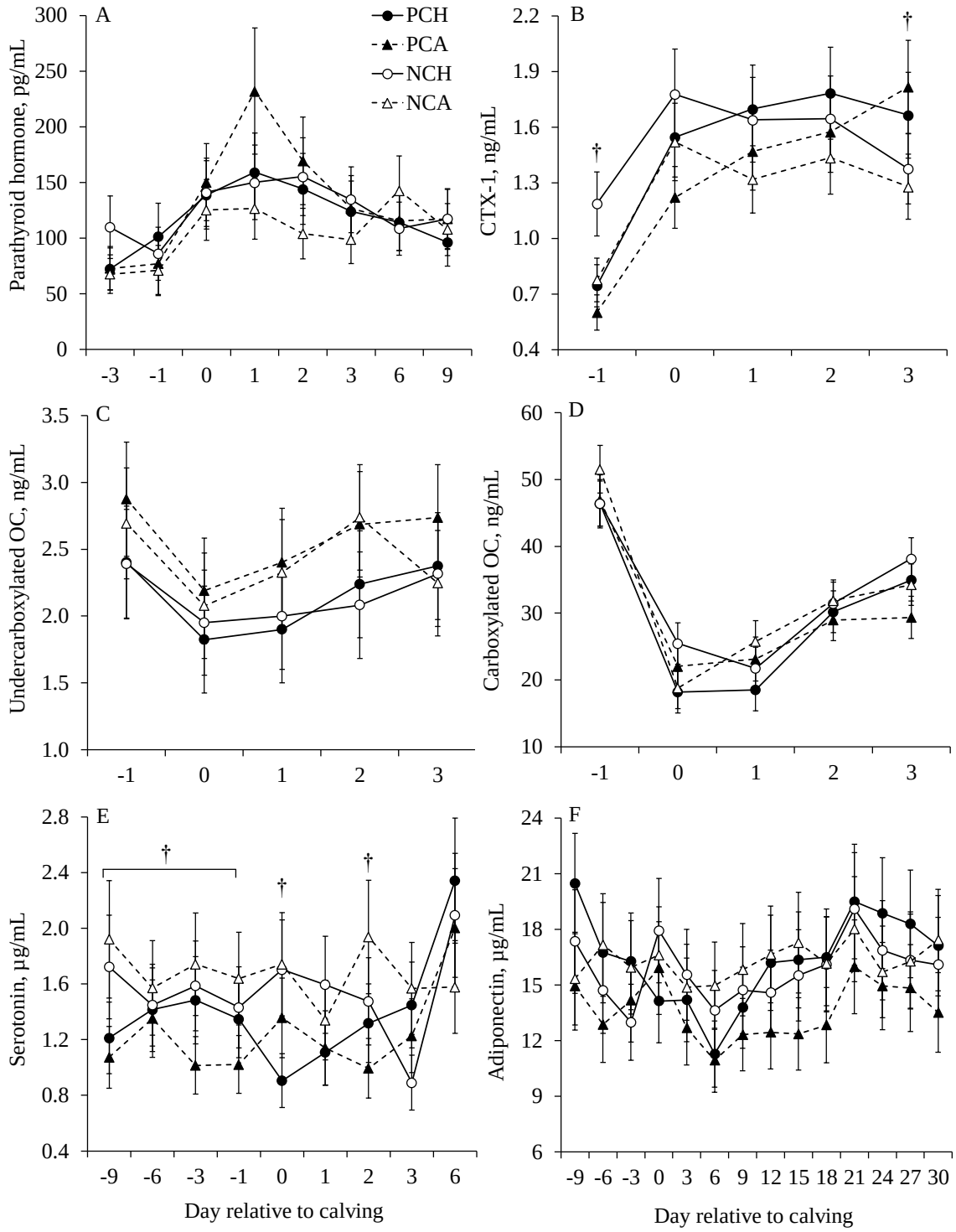

Figure 6. Concentrations of parathyroid hormone (A), C-terminal telopeptide of type 1 collagen (CTX-1; B), undercarboxylated osteocalcin (uOC; C), carboxylated osteocalcin (cOC), serotonin (E), and adiponectin (F) in plasma or serum of cows fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Within a day, † denotes effect of DCAD ($P < 0.05$), ‡ effect of vitamin D ($P < 0.05$), and * interaction between DCAD and vitamin D ($P < 0.05$).

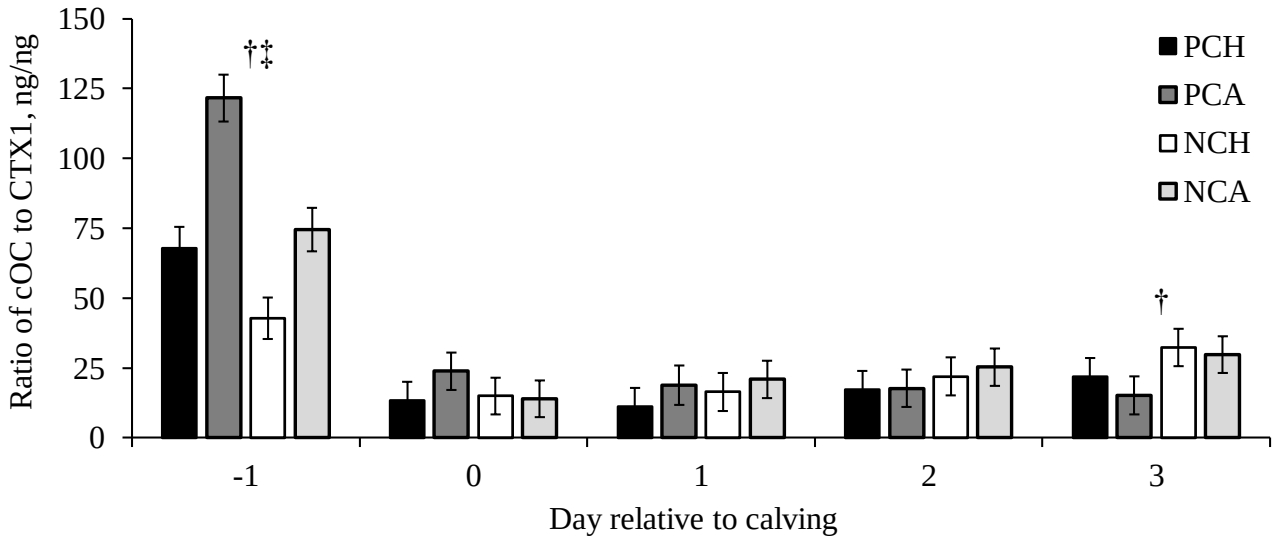

Figure 7. Ratios of carboxylated osteocalcin (cOC) to C-terminal telopeptide of type 1 collagen (CTX-1) in plasma of cows fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Within a day, † denotes effect of DCAD ($P < 0.05$), ‡ effect of vitamin D ($P < 0.05$).

Cows fed the diet with negative DCAD had greater ($P = 0.02$) concentrations of serotonin prepartum than those fed the positive DCAD (positive = 1.327 vs. negative = 1.557 µg/mL; Table 4), but neither vitamin D nor the interaction between DCAD and vitamin D affected prepartum serotonin. Postpartum, an interaction ($P = 0.02$) between DCAD and day was observed because cows fed the diet with negative DCAD had greater concentrations than those fed the positive DCAD on d 0 and 2 postpartum (Figure 6E). Nulliparous cows tended ($P = 0.10$) to have greater concentrations of serotonin prepartum than parous cows (Table 4), but this difference was no longer observed postpartum (Table 5). Treatment, parity, or the interaction between treatment and parity did not affect concentrations of adiponectin in the last 9 d of gestation or the first 30 DIM (Tables 4 and 5; Figure 6F).

Acid-Base Balance

Prepartum blood pH, HCO_3^-, and base excess were all reduced ($P < 0.001$) by feeding the acidogenic diet (Table 6). Interactions ($P < 0.03$) were observed for DCAD and vitamin D because the reductions in pH, HCO_3^-, and base excess were all more accentuated in cows fed calcidiol than cholecalciferol. Tendencies for interaction ($P < 0.10$) between DCAD and parity were observed for HCO_3^- and base excess because the reductions induced by feeding the diet with negative DCAD were less in nulliparous for HCO_3^- (positive = 30.5 vs. negative = 26.8 m*M*) and base excess (positive = 7.32 vs. negative = 2.90 m*M*) than for parous cows (positive =

30.1 vs. negative = 24.8 m*M*; HCO_3^-; positive = 6.66 vs. negative = 0.54 m*M* base excess). Prepartum pCO_2 was less ($P = 0.01$) for cows fed the negative than the positive DCAD diet, but treatments did not influence pO_2. Cows fed negative DCAD had smaller ($P < 0.001$) urinary pH prepartum than cows fed the positive DCAD, but no differences were observed for vitamin D.

Measures of acid-base balance postpartum were more influenced by parity than by treatments (Table 7). Blood pH did not differ among treatments, but it was greater ($P = 0.004$) for nulliparous than parous cows. Blood pH, base excess, pCO_2, and pO_2 did not differ among treatments. Cows fed the positive DCAD tended ($P = 0.08$) to have less blood HCO_3^- than cows fed the negative DCAD (positive = 32.2 vs. negative = 30.1 m*M*), and cows fed cholecalciferol tended ($P = 0.09$) to have smaller urinary pH postpartum than cows fed calcidiol (CH = 7.87 vs. CA = 8.05).

Table 6. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on measures of acid-base status in Holstein cows prepartum[1]

	Positive		Negative			Parity			*P*-value[2]			
Item	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Blood												
pH	7.489	7.495	7.458	7.429	0.009	7.478	7.458	0.008	< 0.001	0.16	0.03	0.07
HCO_3^-, m*M*[*]	29.5	31.1	26.2	25.4	0.5	28.7	27.5	0.5	< 0.001	0.42	0.02	0.05
BE, m*M*[*]	6.20	7.78	2.37	1.08	0.55	5.11	3.60	0.49	< 0.001	0.78	0.008	0.03
pCO_2, mm Hg	39.3	40.4	37.0	38.6	0.9	38.8	38.9	0.9	0.01	0.11	0.77	0.94
pO_2, mm Hg	57.4	56.9	58.4	52.6	4.5	57.8	54.9	3.6	0.73	0.49	0.56	0.53
Urine pH	8.03	7.93	5.69	5.73	0.15	6.82	6.87	0.11	< 0.001	0.84	0.67	0.72

[1] Prepartum cows starting at 255 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Whole blood samples collected and analyzed on d -9, -6, -3, and -1 relative to calving. Urine was collected twice weekly in the last 2 wk of gestation.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[*] Interaction between DCAD and parity ($P < 0.10$).

[§] Interaction between source of vitamin D and parity ($P < 0.05$).

[¥] Interaction between DCAD, vitamin D and parity ($P < 0.05$).

Table 7. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on measures of acid-base status in Holstein cows postpartum[1]

	Positive		Negative			Parity			*P*-value[2]			
Item[3]	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Blood												
Na, m*M*	141.4	141.5	141.8	142.0	0.3	141.3	142.0	0.3	0.17	0.63	0.87	0.06
K, m*M*	3.84	3.79	3.93	3.88	0.05	3.82	3.91	0.04	0.05	0.27	0.93	0.12
pH	7.500	7.502	7.500	7.498	0.009	7.513	7.487	0.006	0.83	0.99	0.83	0.004
HCO_3^-, m*M*	32.0	32.3	32.7	33.4	0.5	34.0	31.3	0.4	0.08	0.38	0.67	< 0.001
BE, m*M*	8.89	9.14	9.58	10.24	0.60	11.01	7.92	0.42	0.14	0.45	0.73	< 0.001
pCO_2, mm Hg	41.1	41.5	41.9	43.0	0.9	42.4	41.3	0.7	0.17	0.38	0.69	0.28
pO_2, mm Hg	49.4	51.2	50.9	49.2	3.4	53.5	47.1	2.5	0.95	0.99	0.62	0.07
Urine pH	7.86	8.07	7.88	8.04	0.11	7.97	7.96	0.07	0.93	0.09	0.82	0.92

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Whole blood samples collected and analyzed on d 0, 1, 2, 3, and 6 postpartum. Urine was sampled on d 2 and 6 postpartum.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[3] BE = base excess; pCO_2 = partial pressure of CO_2; pO_2 = partial pressure of O_2.

[*] Interaction between DCAD and parity ($P < 0.05$).

[§] Interaction between source of vitamin D and parity ($P < 0.05$).

[¥] Interaction between DCAD, vitamin D and parity ($P < 0.05$)

Table 8. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on concentrations and losses of minerals in urine and colostrum and estimated mineral balance in Holstein cows[1]

	Positive		Negative			Parity			*P*-value[2]			
Item	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Urine prepartum												
Creatinine, g/L	1.09	0.91	0.92	1.01	0.09	0.99	0.97	0.09	0.69	0.55	0.13	0.84
L/d	21.1	27.2	25.4	27.5	4.3	21.8	28.7	3.1	0.57	0.30	0.61	0.09
Ca, mg/L*	72	277	373	684	55	387	316	46	< 0.001	< 0.001	0.29	0.23
Ca, g/d	1.66	6.22	8.50	15.35	1.34	7.59	8.27	0.96	< 0.001	< 0.001	0.35	0.58
Mg, mg/L	726	644	407	598	73	658	529	68	0.004	0.37	0.03	0.15
Mg, g/d	13.52	13.50	9.43	12.10	1.10	12.11	12.16	0.99	0.004	0.15	0.15	0.97
Urine postpartum												
Creatinine, g/L	0.83	0.86	1.02	0.85	0.11	0.86	0.92	0.09	0.44	0.54	0.36	0.66
L/d	25.7	22.1	24.5	28.1	3.1	24.3	25.9	2.9	0.42	0.99	0.24	0.68
Ca, mg/L	21.7	13.7	15.2	28.6	7.4	19.1	20.5	6.0	0.57	0.71	0.16	0.86
Ca, g/d	0.53	0.33	0.39	0.70	0.20	0.46	0.51	0.16	0.55	0.76	0.21	0.82
Mg, mg/L	305	362	273	288	39	319	295	30	0.17	0.36	0.59	0.55
Mg, g/d	6.55	7.44	5.79	6.07	0.69	6.11	6.81	0.56	0.11	0.39	0.65	0.34
Colostrum												
Kg	5.86	7.68	6.21	7.96	1.06	5.29	8.56	0.86	0.77	0.10	0.97	0.003
Ca, g/L¥	2.76	3.30	3.23	3.12	0.12	3.28	2.93	0.12	0.17	0.05	0.004	0.04
Ca, g/d	15.6	24.5	19.8	25.1	3.6	16.9	25.5	2.9	0.51	0.05	0.62	0.02
Mg, g/L¥	0.48	0.55	0.52	0.52	0.02	0.51	0.54	0.02	0.57	0.10	0.09	0.64
Mg, g/d	2.69	4.09	3.25	4.19	0.61	2.60	4.51	0.49	0.59	0.06	0.71	0.002
Balance,[3] g/d												
Prepartum Ca*	31.8	24.1	14.7	10.3	1.8	18.8	21.6	1.3	< 0.001	< 0.001	0.33	0.09
Prepartum Mg	-6.2	-7.1	-3.3	-5.5	1.0	-5.8	-5.3	0.9	0.01	0.08	0.50	0.66
Postpartum Ca	15.3	-0.1	5.9	-4.4	7.1	8.7	-0.4	5.6	0.34	0.08	0.72	0.21
Postpartum Mg	-6.4	-9.6	-6.7	-8.9	1.3	-6.2	-9.6	1.1	0.89	0.04	0.72	0.03

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Urine was sampled on d -5 and 3 relative to calving.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[3] Calculated in the last week of gestation (prepartum) and first 3 DIM (postpartum). Estimated absorptions of Ca and Mg and accretion of fetal tissues accretion computed using the NRC (2001) according to diet composition and calf BW at birth. * Interaction between DCAD and parity ($P < 0.05$), § Interaction between source of vitamin D and parity ($P < 0.05$), ¥ Interaction between DCAD, vitamin D and parity ($P < 0.05$).

Urinary Excretion and Colostrum Secretion of Minerals and Estimated Mineral Balance

Treatment did not affect concentrations of creatinine in urine pre- and postpartum (Table 8). Cows excreted an estimated 25.3 and 25.1 L of urine per day in the last week prepartum and in the first week of lactation. Concentration of tCa and excretion of tCa in the urine prepartum were markedly increased ($P < 0.001$) by feeding a diet with negative DCAD or by supplementing calcidiol. For tMg, feeding a diet with negative DCAD reduced ($P = 0.004$) both concentration and excretion in the urine prepartum (Table 8). An interaction ($P = 0.03$) between DCAD and vitamin D was detected because the reduction in urinary loss of tMg induced by feeding the negative DCAD was only observed for cows fed NCH, but not in those fed NCA. Concentrations and losses of tCa and tMg in urine in the first week postpartum did not differ with treatment. On average, pre- and postpartum, cows excreted, respectively, 7.93 and 0.49 g of tCa/d and 12.14 and 6.46 g of tMg/d.

Colostrum yield did not differ between DCAD, but tended ($P = 0.10$) to be greater for cows fed calcidiol than cholecalciferol. Treatment affected concentrations and loss of tCa and tMg in colostrum (Table 8). Feeding calcidiol increased concentration of tCa in colostrum, but this effect was only observed in cows fed the diet with positive DCAD. Cows fed calcidiol lost 7.1 g additional tCa in colostrum compared with cows fed cholecalciferol (CH = 17.7 vs. CA = 24.8 g). An interaction ($P = 0.09$) between DCAD and vitamin D was observed for concentration of tMg in colostrum because within cows receiving the positive DCAD, those fed PCA had greater ($P = 0.02$) concentration of tMg than cows fed PCH, but no difference was observed between NCH and NCA. Secretion of tMg in colostrum tended ($P = 0.06$) to increase in cows fed calcidiol than those fed cholecalciferol (CH = 2.97 vs. CA = 4.14 g). Colostrum yield was greater ($P = 0.003$) for parous than nulliparous, thereby increasing ($P < 0.02$) the losses of tCa and tMg in colostrum.

The estimated balances of Ca and Mg in the last 7 d of gestation differed with treatments (Table 8). Cows were in positive Ca balance prepartum, but those fed diets with positive DCAD had greater ($P < 0.001$) Ca balance than cows fed the negative DCAD (positive = 28.0 vs. negative = 12.5 g/d), and cows fed cholecalciferol had greater had greater ($P < 0.001$) Ca balance than cows fed calcidiol (CH = 23.2 vs. CA = 17.2 g/d). Cows were in negative Mg balance prepartum, and it tended (P = 0.08) to be more negative for cows fed the positive than negative DCAD (positive = -6.7 vs. negative = -4.3 g/d), and it was less negative ($P = 0.01$) for cows fed cholecalciferol than calcidiol (CH = -4.8 vs. CA = -6.3 g/d). Calcium balance decreased ($P < 0.001$) with the

onset of lactation from 20.3 to 4.2 g/d and it tended (P = 0.08) to be greater for cholecalciferol than calcidiol (CH = 10.6 vs. CA = -2.3 g/d), but it was not affected by DCAD. Only 1 NCH cow was in negative Ca balance prepartum, whereas postpartum proportions of cows with negative Ca balance was 29.4% for PCH, 47.4% for PCA, 36.8 for NCH, and 55.0% for NCA. Magnesium balance became more (P = 0.002) negative postpartum, from -5.5 g/d prepartum, to -7.9 g/d with the onset of lactation. Altering the DCAD did not influence postpartum Mg balance, but feeding calcidiol further reduced Mg balance compared with cholecalciferol (CH = -6.5 vs. CA = -9.2 g/d).

DISCUSSION

Supplementing calcidiol in place of cholecalciferol during the last 3 wk of gestation was superior in increasing plasma concentrations of vitamin D metabolites pre- and postpartum and concentrations of minerals in whole blood and plasma prepartum in dairy cows. Concurrent with the changes observed with sources of vitamin D, feeding a diet with negative DCAD prepartum induced a compensated metabolic acidosis in dairy cows, which attenuated the decline in blood iCa and serum tCa after parturition with effects more pronounced in parous than nulliparous cows.

Vitamin D metabolism has critical roles in mineral homeostasis, particularly Ca metabolism. 25-hydroxyvitamin D_3 is the standard vitamin D metabolite quantified to determine adequacy of vitamin D status because it is the predominant metabolite found in plasma and has a long half-life (Jones, 2008). Concentrations considered adequate for cattle suggested by Horst et al. (1994) range from 20 to 50 ng/mL, although the minimum concentration of 25-hydroxyvitamin D_3 that optimizes mineral metabolism and health of dairy cattle have not been defined. Nelson et al. (2012) recently suggested a minimum of 30 ng/mL for proper immune function. A recent survey of 12 dairy herds in the US demonstrated that the mean (± SD) concentration of 25-hydroxyvitamin D_3 in lactating dairy cows sampled at all stages of lactation was 68 ± 22 ng/mL (Nelson et al., 2016). Unsurprisingly, feeding calcidiol increased plasma concentrations of 25-hydroxyvitamin D_3 to values much larger than those fed cholecalciferol, and concentrations remained elevated past the last day of supplementation. Even within cows fed cholecalciferol, concentrations of 25-hydroxyvitamin D_3 in plasma increased with day of supplementation, despite the decline in DM and cholecalciferol intakes as calving approached (Martinez et al., 2017a – Chapter 7), which likely reflect the natural conversion of vitamin D_3 to 25-

hydroxyvitamin D_3 (Ponchon et al., 1969). Concentrations of vitamin D_3 in plasma increased only in cows fed cholecalciferol, although those fed calcidiol had concentrations prepartum within what has been suggested as normal range in cattle, 1 to 3 ng/mL (Horst et al., 1981).

It is interesting that concentrations of 25-hydroxyvitamin D_3 in plasma of individual cows fed 3 mg of cholecalciferol did not go above 90 ng/mL, suggesting some limit in the conversion of dietary vitamin D_3 into plasma 25-hydroxyvitamin D_3 in dairy cows. Horst and Reinhardt (1982) injected 2 cows with 375 mg of cholecalciferol each and showed that plasma concentrations of vitamin D_3 increased to almost 50 ng/mL, approximately 3-fold greater than the values observed for PCH cows prepartum. Despite the massive increase in vitamin D_3, plasma concentrations of 25-hydroxyvitamin D_3 remained below 100 ng/mL in the subsequent 77 d (Horst and Reinhardt, 1982). Nelson et al. (2016) reported that herds supplementing lactating cows 0.75 to 1.25 mg of vitamin D_3 had cows with 25-hydroxyvitamin D_3 concentrations between 42 and 96 ng/mL (10^{th} and 90^{th} percentiles). No cow fed cholecalciferol, in which vitamin D_3 was supplemented at 3 mg/d had concentration of 25-hydroxyvitamin D_3 in plasma greater than 90 ng/mL either during supplementation in the prepartum period, or postpartum. Poindexter (2017) showed that increasing intake of vitamin D_3 from 1 to 3 mg/d for 28 d did not increase concentrations of 25-hydroxyvitamin D_3 in plasma of lactating dairy cows and concentrations in individual cows did not surpass 90 ng/mL; although, feeding 3 mg/d of cholecalciferol doubled vitamin D_3 concentrations in serum compared with feeding 1 mg/d. Conversion of vitamin D_3 into 25-hydroxyvitamin D_3 by 25-hydroxylase was first identified in the liver in rats almost 50 years ago (Ponchon et al., 1969); however, little is known about the regulation of this enzyme in bovine liver. Two forms of 25-hydroxylase have been identified, a mitochondrial and a microsomal enzyme, and serum concentrations of 25-hydroxyvitamin D_3 have been correlated with mitochondrial 25-hydroxylase activity in the liver of rats (Dahlback and Wikvall, 1987). In cattle, mutations in the gene for *CYP2J2* are associated with concentrations of 25-hydroxyvitamin D_3 in serum (Casas et al., 2013), but in general vitamin D 25-hydroxylase activity is considered an unregulated step in the vitamin D pathway. It is possible that bovine 25-hydroxylase activity is saturated by a large supply of vitamin D_3 or that other compounds of vitamin D metabolism control expression and activity of 25-hydroxylase such that conversion of vitamin D_3 into 25-hydroxyvitamin D_3 is inhibited. There is some evidence that 25-hydroxylase activity is influenced by 1,25-dihydroxyvitamin D_3 (Baran and Milne, 1986), and this effect might be mediated by cytosolic concentrations of iCa (Baran and Milne, 1986; Corlett et al., 1987).

Feeding the acidogenic diet reduced concentrations of both vitamin D_3 and 25-hydroxyvitamin D_3 in plasma of dairy cows. Because cows were supplemented with 3 mg of cholecalciferol or calcidiol for each 11 kg of DM, differences in DMI would influence the total intake of vitamin D supplements. Cows fed the negative DCAD consumed less DM prepartum than cows fed the positive DCAD diet (Martinez et al., 2017a – Chapter 7). This effect was only observed in parous cows (positive = 13.7 vs. negative = 11.5 kg/d), and not in nulliparous cows (positive = 11.0 vs. negative = 11.3 kg/d). The reduced intake of DM in parous cows fed NCH and NCA could reduce concentrations of vitamin D_3 and 25-hydroxyvitamin D_3 in plasma; however, the lack of interaction between DCAD and parity on concentrations of vitamin D_3 and 25-hydroxyvitamin D_3 indicates that the reductions in concentrations of those two vitamin D metabolites caused by feeding the diet with negative DCAD was not solely mediated by reduced intake of DM. Because nulliparous cows fed the diet with negative DCAD had the same DMI and, consequently same supplemental vitamin D intake, differences in plasma concentrations cannot be attributed to supply of the vitamin as in parous cows. Specifically, in nulliparous cows prepartum, the concentrations of vitamin D_3 in plasma decreased from 5.12 in positive to 3.87 ng/mL in negative DCAD, and those of 25-hydroxyvitamin D_3 decreased from 160.7 in positive to 131.9 ng/mL in negative DCAD, representing reductions in plasma concentrations of 18 to 24%, although intake did not differ. These data suggest that the metabolic acidosis induced by diets with negative DCAD might influence absorption or post-absorptive metabolism of vitamin D compounds provided in the diet.

Weiss et al. (2015) fed Holstein cows diets prepartum with either positive (+165 mEq/kg) or negative (-139 mEq/kg) DCAD and supplemented with 0.45 mg of vitamin D_3. Feeding the diet with negative DCAD only numerically reduced concentrations of 25-hydroxyvitamin D_3, but differences in 25-hydroxyvitamin D_3 concentrations between cows fed positive and negative DCAD increased with d in the experiment, suggesting a possible effect of DCAD on endogenous synthesis or catabolism of 25-hydroxyvitamin D_3. A metabolic acidosis induced by acidogenic diets increases blood iCa concentrations around calving in dairy cows (Charbonneau et al., 2006), which might affect subsequent release of PTH and thus the activity of 1-alpha-hydroxylase responsible for production of 1,25-dihydroxyvitamin D_3 (Fraser and Kodicek, 1970). Feeding acidogenic diets increased blood concentrations 1,25-dihydroxyvitamin D_3 and tCa in response to exogenous PTH (Goff et al., 2014). Nevertheless, we are not aware of data

demonstrating effects of acidogenic diets on absorption and concentrations of vitamin D_3 and on conversion of vitamin D_3 into 25-hydroxyvitamin D_3 in dairy cattle.

Concentrations of 25-hydroxyvitamin D_2 and 3-epi 25-hydroxyvitamin D_3 were affected by treatment. Vitamin D_2 is produced by UV light irradiation of ergosterol from fungi and is expected to be present in relatively low, but consistent concentrations in forages fed to dairy cows (Wallis et al., 1958), including those in the present experiment. Vitamin D_2 is hydroxylated in the liver by the microsomal 25-hydroxylase to 25-hydroxyvitamin D_2 (Bikle, 2014), and later catabolized by the 24-hydroxyase (Horst et al., 1986). It is unclear why cows fed NCA had a 27% increase in concentrations of 25-hydroxyvitamin D_2 throughout the experiment compared with cows fed the other three diets. The 3-epi 25-hydroxyvitamin D_3 is synthesized by a 3-epimerase that has identical molecular structure, but differ in stereochemical configuration (Bikle, 2014). The biological function of 3-epi 25-hydroxyvitamin D_3 is poorly described and we are unaware of dietary or metabolic factors that influence synthesis and metabolism of this vitamin D metabolite in cattle. Rodney et al. (2017 – Chapter 5) showed a linear increase in 3-epi 25-hydroxyvitamin D_3, but a quadratic decrease in 25-hydroxyvitamin D_2 concentrations in plasma of mid-lactation dairy cows supplemented with 0, 0.5, 1, 2 or 4 mg/d of calcidiol. Their data demonstrated that both metabolites are influenced by the supply of dietary calcidiol.

The conversion of 25-hydroxyvitamin D_3 into 1,25-dihydroxyvitamin D_3 is known to occur in response to metabolic demand independently of substrate availability; however, the results of this experiment show that concentrations of 1,25-dihydroxyvitamin D_3 can increase by feeding high doses of calcidiol to dairy cows. The increased 1,25-dihydroxyvitamin D_3 concentration prepartum in cows fed calcidiol might indicate that supplying large doses of 25-hdyroxyvitamin D_3 might over-ride the regulatory mechanisms for synthesis of 1,25-dihydroxyvitmain D_3. At 1 and 2 DIM, 1,25-dihydroxyvitamin D_3 was highest in cows fed PCH likely because those cows had reduced concentrations of iCa compared with cows fed NCH or NCA.

The increase in 1,25-dihydroxyvitamin D_3 with feeding calcidiol was minor compared with the massive increase in concentrations of the inactive metabolite 24,25-dihydoxyvitamin D_3. The increase in 24,25-dihydroxyvitamin D_3 above typically reported values of 2.6 ± 1.3 ng/mL (Horst et al., 1981), which was observed in cows fed calcidiol, is likely the result of increased supply of 25-hydroxyvitamin D_3 as substrate for the cytochrome P450 enzyme 24-hydroxylase. A linear relationship was observed between 25-hydroxyvitamin D_3 and 24,25-dihydroxyvitamin

D_3 concentrations in plasma in cows fed cholecalciferol and calcidiol, reinforcing the concept that supply of substrate increases the side-chain hydroxylation to convert 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 into inactive 24-hydroxylated vitamin D_3 metabolites. In fact, the ratio or 24,25-dihydroxyvitamin D_3 to 25-hydroxyvitamin D_3 increased 3-fold pre- and postpartum in cows fed calcidiol compared with cows fed cholecalciferol supporting the concept of induction of 24-hydroxylase abundance or activity as the supply of 25-hydroxyvitamin D_3 increased. Abundance of the 24-hydroxylase enzyme increases by 1,25-dihydroxyvitamin D_3 via vitamin D response elements in the *CYP24A1* gene promoter (Pike, 2011) and administration of 1,25-dihydroxyvitamin D_3 to cows immediately after calving increased concentrations of 24,25-dihydroxyvitamin D_3 (Viera-Neto et al., 2017); so, increased 24,25-dihydroxyvitamin D_3 concentrations in calcidiol-fed cows also may be caused by increased 24-hydroxylase abundance. Differences in concentrations 24,25-dihydroxyvitamin D_3 between parity might suggest that as cows age, activity of 24-hydroxylase increases perhaps because of uncoupling *CYP24A1* with age that might contribute to impairment of vitamin D metabolism that has been shown with aging of dairy cows (Horst et al., 1990). The side-chain hydroxylation of 25-dihydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 makes the two vitamin D metabolites more easily removed from circulation and is a key process in preventing excess accumulation of vitamin D metabolites (Horst et al., 1994). The finding that cows fed the diet with positive DCAD also had greater concentrations of 24,25-dihydroxyvitamin D_3 than those fed the negative DCAD diet likely reflects the increased concentrations of 25-hydoxyvitamin D_3 in cows fed the positive DCAD.

Feeding calcidiol increased concentration of tCa and tP prepartum but not postpartum indicating that increasing concentrations of 25-hydroxyvitamin D_3 by feeding calcidiol with either positive or negative is not effective in minimizing postpartum hypocalcemia. Furthermore, cows fed PCA had the smallest concentrations of Ca postpartum, indicating a potential negative consequence to high concentrations of 25-hydroxyvitamin D_3 if DCAD is not manipulated to minimize postpartum Ca loss. Wilkens et al. (2012) observed similar responses when cows were fed calcidiol at 3 mg/d, and Weiss et al. (2015) observed increased concentration of tCa prepartum, but no effect on postpartum tCa by feeding cows prepartum 6 mg/d of calcidiol. The inability of calcidiol to improve postpartum Ca likely stems from greater disruption of Ca and P homeostasis and vitamin D metabolism in cows fed calcidiol. Feeding calcidiol increased the loss of minerals in urine and colostrum, particularly tCa, by approximately 13 g/d, which probably contributed to low serum tCa and blood iCa when cows were fed PCA. On the other hand, when cows were fed

NCA, despite increased colostrum yield and losses of tCa in colostrum and urine, those cows maintained increased concentrations of tCa and iCa in blood in the first days of lactation. Because calcidiol tended to increase colostrum yield, the loss of both tCa and tMg was expected to increase. In addition, within cows fed the diet with positive DCAD, those supplemented with calcidiol had increased concentrations of both tCa and tMg in colostrum. Such a loss of tCa is anticipated to influence Ca homeostasis and the ability to maintain normocalcemia at the onset of lactation. This becomes important particularly when cows were fed the diet with positive DCAD because it is known that under alkalosis, 1,25-hydroxyvitamin D_3 synthesis in response to PTH and control of blood iCa and tCa is compromised (Charbonneau et al., 2006; Lean et al., 2006; Goff et al., 2014).

Wilkens et al. (2012) observed that supplementing calcidiol to cows fed a prepartum diet with positive DCAD resulted in the lowest blood iCa, particularly in cows of third or greater lactations, which tend to be the most susceptible to milk fever (Lean et al., 2006). Furthermore, feeding PCA resulted in the highest concentrations of serum tP prepartum, as observed by Wilkens et al. (2012), and increased concentrations of tP in plasma is known to stimulate production and secretion of fibroblast growth factor (**FGF**) 23 by osteoblasts and osteocytes, which regulates blood phosphate, but also inhibits 1α-hydroxylase, thereby suppressing the synthesis of 1,25-dihydroxyvitamin D_3 (Bikle, 2014). Also, FGF23 stimulates the catabolism of 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 by activating renal 24-hydroxylase (Bikle, 2014), thereby increasing the conversion into 24,25-dihydroxyvitamin D_3. Cows fed PCA also had the greatest pre- and postpartum concentrations of 24,25-dihydroxyvitamin D_3 and the largest ratio of 24,25-dihydroxyvitamin D_3 to 25-hydroxyvitamin D_3.

The onset of lactation and associated increase in demand for Ca triggered a marked, but expected, decrease in blood iCa and tCa concentrations across all treatments. Nevertheless, this decrease was less apparent in cows fed the acidogenic diet, which attenuated the decline in iCa and tCa particularly in parous cows. Furthermore, the acidogenic diet fed to cows supplemented with calcidiol minimized the decline in iCa and tCa compared with cows fed PCA despite the increased losses of tCa in colostrum and urine. The relatively more stable Ca concentrations in cows fed the diet with negative DCAD contributed to the reductions in the incidence of both clinical and subclinical hypocalcemia reported in the companion paper (Martinez et al., 2017b – Chapter 8).

Feeding the diet with negative DCAD induced a typical compensated metabolic acidosis in cows in the prepartum period with reductions in blood and urinary pH and in concentrations of HCO_3^-, base excess, and pCO_2 in whole blood. Most of those responses were no longer detected in the first 6 DIM, which reflects the similar and alkalogenic diet fed upon calving to all cows. The acid-base responses to feeding acidogenic diets prepartum have been well documented in the literature (Charbonneau et al., 2006), and metabolic acidosis has been shown to promote response to PTH in dairy cows (Goff et al., 2014), which explains the benefits to Ca homeostasis and the resulting increments in urinary mineral losses.

One of the goals of this experiment was to identify integration of vitamin D, mineral and bone metabolism as the cow transitions into lactation that would be reflected into subsequent benefits to health and lactation observed with feeding acidogenic diets and/or calcidiol prepartum (Lean et al., 2014; Martinez et al., 2017a – Chapter 7). Feeding the diet with negative DCAD increased concentrations of serotonin prepartum. Serotonin plays a role in bone remodeling as it is required for osteoclastogenesis (Chabbi-Achengli et al., 2012), and it regulates Ca transport into mammary epithelial cells during lactation (Laporta et al., 2014a). In response to lactation, serotonin produced by the mammary gland triggers production of PTH related-protein which stimulates osteoclast activity and increased bone mobilization (Laporta et al., 2014b). Because DCAD was shown to influence serotonin as cows approached calving, and treatment with 1,25-dihydroxyvitamin D_3 immediately after calving has been shown to also increase serotonin in dairy cows (Vieira-Neto et al., 2017), it is clear that these dietary interventions can influence endocrine signals that affect nutrient flux to the mammary gland.

Integration of bone and energy metabolism has been proposed in murine and human studies (Lee et al., 2007, Wolf, 2008), and more recently in cattle (Lean et al., 2014). Lee et al. (2007) showed that trangenic mice lacking osteocalcin had reduced pancreatic beta-cell proliferation, glucose intolerance, and insulin resistance, suggesting a crosstalk between osteoblast-secreted molecules and control of energy metabolism. Numerous other experiments have shown that molecules derived from skeleton to influence energy metabolism, and molecules produced by adipocytes can affect bone formation (Wolf, 2008). Although the treatments imposed in the current experiment had remarkable influences on vitamin D and mineral metabolism, affected aspects of energy metabolism with enhanced yields of milk and milk components (Martinez et al., 2017a – Chapter 7), and reduced the incidence of health disorders (Martinez et al., 2017b –

Chapter 8), the direct interplay between bone-secreted molecules and energy metabolism were not clearly evident.

Concentrations of uOC and cOC did not differ with treatments, except for an increase in nulliparous cows when fed the diet with negative DCAD. Osteocalcin is an osteoblast-derived protein present at high concentration in the bone extracellular matrix that undergoes a posttranslational vitamin K-dependent γ-carboxylation that converts glutamic acid residues to γ-carboxyglutamic acid in the molecule which increases affinity for Ca and hydroxyapatite in bones (Wolf, 2008). It has been identified in bovine (Price et al., 1976), and later shown to be released into the bloodstream by osteoblasts as new bone is formed; however, uOC plays major roles on energy metabolism influencing insulin release and glucose homeostasis (Lee et al., 2007). In the present experiment, most OC identified in blood plasma was cOC, representing more than 90%. Because only cOC was affected by negative DCAD in nulliparous, it might suggest that the manipulations imposed affect bone metabolism, but the changes in energy metabolism might not be mediated by bone in transition cows. Also, treatments did not affect the concentrations of adiponectin reported herein, as well as those of leptin and insulin reported by Martinez et al. (2017a – Chapter 7). Although differences in bone markers with treatment were minor, CTX-1 increased prepartum in cows fed the diet with negative DCAD, but marked differences were observed with parity, and nulliparous had greater concentrations of uOC, cOC, and CTX-1, and tended to have a larger portion of the total OC as cOC than parous cows. It is not surprising the differences between parity groups for bone-related markers as nulliparous are growing, accreting, and remodeling bone to a greater extent than older cows (Sato et al., 2011), and are better able to cope with the demands for Ca with the onset of lactation (Lean et al., 2006).

The ratio of cOC to CTX-1 was used as an index of bone turnover (Wilkens et al., 2014), and an increase in the ratio suggests increments in bone acretion relative to resorption, whereas a reduction suggests a decrease in bone accretion relative to resorption. As expected, dynamic changes were observed as cows calved and lactation initiated with a marked reduction in the ratio starting on the day of calving likely to support the irreversible losses of Ca in colostrum and milk. In fact, secretion of colostrum and milk caused 42.7% of the cows to be in negative Ca balance, which agrees with balance studies showing positive Ca balance preparutm, but negative in the first week of lactation (Ender et al., 1971). Similar to our findings, Wilkens et al. (2014) documented a marked decline in the ratio of OC to CTX-1 in dairy goats as they transition from

the dry period into lactation. Effects of treatment were observed primarily before calving, and calcidiol increased the ratio, suggeting increased bone accretion relative to resorption, whereas feeding a diet with negative DCAD reduced the ratio suggesting improved bone resportion with acidogenic compared with alkalogenic diets. Parous cows cows were responsive to the effects of DCAD or vitamin D affecting the ratio of cOC to CTX-1 perhaps because of the demands for Ca are greater or because the effect of vitamin D on interstinal cells stimulating Ca absorption diminishes with age (Horst et al., 1990), thereby making the animal more dependent on bone turnover to meet the sudden needs for Ca with the onset of lactation.

Supplementing cholecalciferol or calcidiol as a large single bolus dose of 15 mg did not affect concentrations of total OC in cows (Taylor et al., 2008). On the other hand, a subcutaneous or intramuscular injection of 0.5 μg of calcitriol/kg BW in nonlactating cows elevated plasma concentrations of OC over several days after treatment (Kim et al., 2011). Treatment with calcitriol induced a rapid increase of blood iCa and tCa, within approximately 12 to 24 h (Kim et al., 2011; Vieira-Neto et al,. 2017), but it did not seem to alter markers of bone resorption (Kim et al., 2011; Vieira-Neto et al,. 2017). Nevertheless, injectable calcitriol increased concentrations of cOC in nonpregnant, nonlactating cows (Kim et al., 2011), which differ considerably in metabolism to peri-parturient cows. Positive associations between plasma 25-hydroxyvitamin D_3 and OC concentrations were identified in mid-lactation dairy cows using time series statistical methods (Rodney et al., 2017 – Chapter 5), suggesting potential associations between the two molecules, but these were lagged by 3 d, rather than being simultaneous. At this point, it remains unclear if the treatments implemented to alter vitamin D and mineral metabolism are capable of influencing bone markers that cross-talk with other endocrine signals such as adiponectin, leptin, and insulin in transition dairy cows.

CONCLUSIONS

Supplementing diets of prepartum dairy cows with 3 mg of calcidiol increased concentrations of vitamin D metabolites in plasma throughout the transition period compared with the same amount of cholecalciferol. Concurrently, feeding prepartum cows an acidogenic diet induced a compensated metabolic acidosis that attenuated the decline in iCa and tCa with the onset of lactation. Calcidiol increased prepartum concentrations of iCa, tCa, and tP, but decreased those of tMg, which resulted in increased urinary excretion of tCa, but not that of tMg. On the other hand, feeding a diet with negative DCAD increased excretion of tCa and tMg in urine

particularly when fed concurrent with calcidiol. Because calcidiol tended to increase colostrum yield, the losses of Ca and Mg in colostrum were greater than those observed for cows fed cholecalciferol. Feeding the diet with negative DCAD reduced plasma concentrations of 25-hydroxyvitamin D_3 in cows supplemented with cholecalciferol suggesting that conversion of vitamin D_3 into 25-hydroxyvitamin D_3 might be influenced by acid-base status of dairy cows or the consequences of metabolic acidosis on PTH action and vitamin D metabolism. Treatments had effects on concentrations of serotonin and metabolites secreted by bone, suggesting some interplay between the dietary interventions imposed and regulatory hormones that influence mineral and energy metabolism.

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CHAPTER SEVEN: EFFECTS OF PRE-PARTUM DIETARY CATION-ANION DIFFERENCE AND SOURCE OF VITAMIN D ON DAIRY COWS: LACTATION PERFORMANCE AND ENERGY METABOLISM

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OVERVIEW OF CHAPTER SEVEN

As a companion Chapter to Chapter 6, this paper presents the results of manipulation of vitamin D supplementation and dietary cation-anion difference in the pre-partum period on production outcomes and variables related to energy metabolism.

The collaborative nature of research meant that the writing of the papers presented in Chapters 7 and 8 was the primary responsibility of Dr Natalia Martinez, at the time, a PhD candidate within the University of Florida, and Prof. Jose Santos. The inclusion of these papers as Chapters 7 and 8 of this thesis is necessary to present the full context of the work presented in Chapter 6 and to indicate contributions to the total work that the candidate undertook during the completion of this PhD.

ABSTRACT

The objectives of the experiment were to evaluate the effects of feeding diets with two dietary cation-anion difference (DCAD) supplemented with either cholecalciferol (CH) or calcidiol (CA) during late gestation on lactation performance and energetic metabolism in dairy cows. The hypothesis was that combining prepartum acidogenic diet with calcidiol supplementation would benefit peripartum Ca metabolism and, thus, improve energy metabolism and lactation performance compared with cows fed an alkalogenic diet or CH. Holstein cows at 252 d gestation were blocked by parity (28 nulliparous and 51 parous cows) and milk yield within parous cows, and randomly assigned to one of 4 treatments arranged as a 2 x 2 factorial, with two levels of DCAD (positive, +130 vs. negative, -130 mEq/kg) and two sources of vitamin D, cholecalciferol or calcidiol fed at 3 mg per 11 kg of diet DM. The resulting treatment combinations were positive DCAD with cholecalciferol (PCH), positive DCAD with calcidiol (PCA), negative DCAD with cholecalciferol (NCH), or negative DCAD with calcidiol (NCA), which were fed for the last 21 d of gestation. After calving, cows were fed the same lactation diet. Body weight, and body condition were evaluated prepartum and for the first 49 d postpartum. Blood was sampled thrice weekly prepartum, and on d 0, 1, 2, 3, and every 3 d thereafter until 30 d postpartum for quantification of hormones and metabolites. Lactation performance was evaluated for the first 49 d postpartum. Feeding a diet with negative DCAD reduced DM intake in parous cows by 2.1 kg/d, but no effect was observed in nulliparous cows. The negative DCAD reduced concentrations of glucose (positive = 4.05 vs. negative = 3.95 m*M*), insulin (positive = 0.57 vs. negative = 0.45 ng/mL) and IGF-1 (positive = 110 vs. negative = 95 ng/mL) prepartum. Treatments did not affect DMI postpartum, but CA-supplemented cows tended to produce more colostrum (PCH = 5.86, PCA = 7.68 NCH = 6.21, NCA = 7.96 ± 1.06 kg), and produced more fat-corrected milk (PCH = 37.0, PCA = 40.1 NCH = 37.5, NCA = 41.9 ± 1.8 kg) and milk components compared with cholecalciferol cows. Feeding the negative DCAD only numerically increased yield of FCM by 1.0 kg/d in both nulliparous and 1.4 kg/d in parous cows. Minor differences were observed in postpartum concentrations of hormones and metabolites linked to energy metabolism among treatments. Results from this experiment indicate that replacing cholecalciferol with calcidiol supplemented at 3 mg/d during the prepartum period improved postpartum lactation performance in dairy cows.

Keywords: dairy cow, DCAD, vitamin D, lactation

INTRODUCTION

Adoption of feeding acidogenic salts and products to manipulate the DCAD prepartum minimizes the decline in blood total calcium (**tCa**) immediately after calving and reduces the incidence of milk fever in dairy cows (Enders et al., 1971; Block et al, 1984), although subclinical hypocalcemia remains prevalent in dairy herds (Reinhardt et al., 2011; Chapinal et al., 2012; Martinez et al., 2016). Subclinical hypocalcemia reduces DM intake (Martinez et al., 2014), impairs energy metabolism (Chamberlin et al., 2013; Martinez et al., 2014), and suppresses immune function (Kehrli and Goff, 1989; Martinez et al., 2014). Cows induced to have subclinical hypocalcemia had signs of insulin resistance with reduced insulin concentrations and increased lipid mobilization despite increased blood glucose concentrations (Martinez et al., 2014). Furthermore, neutrophils from cows induced to have subclinical hypocalcemia had less cytosolic ionized Ca (**iCa**) and impaired phagocytic and killing activities (Martinez et al., 2014). Such changes in metabolism and immune function might explain the increased risk of diseases observed in cows that suffer from clinical and subclinical hypocalcemia (Seifi et al., 2011; Martinez et al., 2012). Therefore, the inability to maintain proper Ca homeostasis affects energy metabolism and immune function, which likely predisposes cows to diseases beyond milk fever as observed by Curtis et al. (1983). Altering the DCAD of prepartum diet to negative values improves peripartum Ca metabolism and increases milk yield in the first months of the subsequent lactation (Lean et al., 2014).

Cholecalciferol or vitamin D_3 is one of the inactive forms of the vitamin, and it is the product of ultraviolet light reacting with 7-dehydrocholesterol (Horst et al., 1994). Activation of vitamin D_3 into 1,25-dihydoxyvitamin D_3 or calcitriol occurs after two hydroxylation steps mediated by cytochrome P450 enzymes. The first hydroxylation occurs in the hepatic mitochondria and microsomes and effected by vitamin D-25-hydroxylases (CYP2R1, CYP2J2, CYP27A1) forming 25-hydroxyvitamin D_3, or calcidiol. The second hydroxylation takes place in the kidney, and it is carried out by the cytochrome P450 enzyme CYP27B1, also known as 1α-hydroxylase. The 1α-hydroxylase is tightly regulated by the coordinated actions of parathyroid hormone, calcitonin, and 1,25-dihydroxyvitamin D_3 (Yoshida et al., 2001; Liu et al., 2006).

Most prepartum diets for dairy cows are supplemented with cholecalciferol, with recommended dose of approximately 0.5 mg for a 650-kg cow (NRC, 2001). However, despite cholecalciferol supplementation and other prepartum dietary manipulations, the prevalence of subclinical

hypocalcemia during the first days of lactation remains high (Reinhardt et al., 2011; Chapinal et al., 2012; Martinez et al., 2016). Recent findings demonstrated improvements in peripartum Ca metabolism in cows supplemented daily with 3 mg of calcidiol per day, or 120,000 IU combined with a diet containing a low DCAD (Wilkens et al., 2012). Cows fed a combination of 3 mg of calcidiol concurrent with a diet with negative DCAD had greater mean plasma concentrations of iCa during the last days of gestation and first days of lactation compared with cows fed a diet with positive DCAD or not supplemented with calcidiol (Wilkens et al., 2012). Nevertheless, feeding 5.4 mg/d of calcidiol for the last 13 d prepartum seemed to cause more detrimental, than beneficial effects on cows (Weiss et al., 2015). Thus, available data indicate that feeding more than 3 mg/d of calcidiol in the last 2 wk of gestation might be excessive and not benefit transition cows.

It was hypothesized that supplementation with calcidiol is superior to cholecalciferol in maintaining blood Ca concentrations during the periparturient period, which would benefit metabolism and lactation performance. It also was hypothesized that the benefits of calcidiol are potentiated when fed with an acidogenic diet. The objectives of this experiment were to evaluate the effects of feeding diets with distinct DCAD and supplemented with two sources of vitamin D during late gestation on productive performance and energy metabolism in dairy cows.

MATERIALS AND METHODS

This manuscript is one of a series of three companion papers (Martinez et al., 2017; Rodney et al., 2017- Chapters 6 and 8). The University of Florida Institutional Animal Care and Use Committee approved all procedures involving cows in the experiment under the protocol number 201408331. Throughout the manuscript, the vitamins fed will be referred to as cholecalciferol (**CH**) and calcidiol (**CA**), whereas measurements in blood plasma will be referred as vitamin D_3, 25-dihydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3.

Cows and Housing

The experiment was conducted in the University of Florida Dairy Unit from February to July 2014. Eighty pregnant dry Holstein cows, 28 nulliparous and 52 parous cows were enrolled in the experiment. Selection criteria included only apparently healthy cows, with no history of disease within 30 days before enrollment. Throughout the manuscript, cows of lactation 0 at enrollment will be designated as nulliparous cows, whereas those enrolled of lactation > 0 will

be designated as parous cows. Nulliparous cows were enrolled in the experiment because of the scarce data of the effects of manipulating DCAD prepartum on postpartum lactation performance (Moore et al., 2000).

The mean ± SD BW and BCS were, respectively, 600.9 ± 34.5 kg and 3.58 ± 0.25 for nulliparous, and 738.6 ± 88.6 kg and 3.55 ± 0.41 for parous cows on the day of enrollment, and the mean ± SD lactation number for parous cows prepartum was 1.96 ± 0.98. Cows at 252 d of gestation were moved to the experimental free-stall barn to acclimate to the facilities and to the individual feeding gates (Calan Broadbent feeding system, American Calan Inc., Northwood, NH). The first 3 d of feed intake were not considered for statistical analysis because cows were learning how to use the feeding gates. Therefore, measurements started at 255 d of gestation.

All prepartum cows were housed together in a free-stall barn with sand bedded stalls and each cow was randomly assigned to an individual feeding gate. Immediately after calving, cows were moved to a second pen within the same barn and cows were assigned to an individual feeding gate based on the sequence of calving. The experimental pens were equipped with two rows of fans (1 fan/6 linear meters), one facing the feed lane immediately above the feed bunk and the other immediately above the beds. Fans were equipped with low pressure nozzles and both fans and nozzles were activated once ambient temperature reached 18^{o}C.

Feeding Management and Treatments

Cows were fed once daily, at 0730 h during the prepartum period and twice daily postpartum at 0730 h and again at 1230 h. The amounts of feed offered to individual cows were adjusted daily to result in at least 5% refusals, which were weighed once daily, before the morning feeding. Description of diets is presented in Table 1.

Table 1. Dietary ingredients and nutrient composition of diets fed pre- and postpartum

	Prepartum diets[1]				
	Positive DCAD		Negative DCAD		
Item	Cholecalciferol	Calcidiol	Cholecalciferol	Calcidiol	Postpartum diet
Ingredients, % of DM					
Corn silage	61.80	61.80	61.80	61.80	25.8
Bermuda hay	9.10	9.10	9.10	9.10	7.5
Brewer's grains, wet	---	---	---	---	8.6
Corn grain, finely ground	---	---	---	---	25.9
Citrus pulp	9.10	9.10	9.10	9.10	5.2
Soybean hulls	---	---	---	---	8.6
Whole cottonseed	6.40	6.40	6.40	6.40	3.4
Soybean meal, solvent extract	---	---	4.50	4.40	8.2
Soybean meal, cooker-processing[2]	11.18	11.08	---	---	3.3
Acidogenic supplement[3]	---	---	7.25	7.25	--
Cholecalciferol mixture[4]	0.08	---	0.08	---	--
Calcidiol mixture[5]	---	0.18	---	0.18	--
MgO + NaCl	0.54	0.54	---	---	---
Prepartum mineral[6]	1.80	1.80	1.80	1.80	--
Postpartum protein and mineral[7]	---	---	---	---	3.5
DM, %	55.4 ± 1.0	55.6 ± 1.0	55.4 ± 1.0	55.4 ± 1.0	69.5 ± 0.6
Nutrients, DM basis (± SD)[8]					
Net energy,[9] Mcal/kg	1.65	1.65	1.65	1.65	1.67
OM, %	94.0 ± 0.4	93.9 ± 0.4	94.2 ± 0.4	94.1 ± 0.4	94.0 ± 0.1
CP, %	13.5 ± 0.3	12.9 ± 0.3	13.5 ± 0.3	13.4 ± 0.3	15.7 ± 0.6
Starch, %	20.2 ± 0.2	20.1 ± 0.2	20.8 ± 0.2	20.9 ± 0.2	27.6 ± 1.0
Non-fibrous carbohydrates,[10] %	38.7 ± 1.1	38.1 ± 1.1	38.3 ± 1.1	38.5 ± 1.1	40.8 ± 1.2
NDF, %	37.8 ± 0.6	39.0 ± 0.6	38.3 ± 0.6	38.2 ± 0.6	33.3 ± 0.5
NDF from forage, %	30.8 ± 0.7	30.8 ± 0.7	30.8 ± 0.7	30.8 ± 0.7	15.8 ± 0.4
Fatty acids, %	3.28 ± 0.03	3.33 ± 0.03	3.45 ± 0.03	3.37 ± 0.03	3.93 ± 0.22
Ca, %	0.61 ± 0.08	0.62 ± 0.08	0.54 ± 0.08	0.55 ± 0.08	0.59 ± 0.03
P, %	0.32 ± 0.01	0.31 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	0.36 ± 0.01
Mg, %	0.39 ± 0.02	0.37 ± 0.02	0.38 ± 0.02	0.39 ± 0.02	0.27 ± 0.01
K, %	1.22 ± 0.08	1.19 ± 0.08	1.15 ± 0.08	1.15 ± 0.08	1.15 ± 0.06
Na, %	0.20 ± 0.01	0.20 ±0.01	0.16 ± 0.01	0.16 ± 0.01	0.46 ± 0.04
Cl, %	0.54 ± 0.04	0.55 ± 0.04	0.94 ± 0.04	0.90 ± 0.04	0.30 ± 0.01
S, %	0.17 ± 0.004	0.16 ± 0.004	0.37 ± 0.004	0.36 ± 0.004	0.18 ± 0.01
DCAD,[11] mEq/kg	145 ± 11	130 ± 119	-129 ± 11	-124 ± 11	293 ± 28

[1] Prepartum cows starting at 252 d of gestation were fed diets with either a positive (+130 mEq/kg) or a negative (-130 mEq/kg) dietary cation-anion difference (DCAD). Within each DCAD diet, cows were fed either 3 mg of cholecalciferol or 3 mg of calcidiol.
[2] Amino Plus (cooker-processing soybean meal; Ag Processing Inc., Emmetsburg, IA).
[3] Bio-Chlor (a fermentation product containing dried condensed extracted glutamic acid fermentation product, dried condensed corn fermentation solubles, processed grain by-products, and magnesium chloride; Arm & Hammer Animal Nutrition, Princeton, NJ).
[4] Rovimix D3 (a product containing 300 mg of cholecalciferol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ).
[5] Hy-D (a product containing 153 mg of calcidiol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ).
[6] Each kg contains (DM basis) 10.3% Ca, 0.7% P, 4.0% Mg, 0.9% K, 0.25% S, 1.8% Na, 2.7% Cl, 1,750 mg Zn, 600 mg Cu, 1,090 mg Mn, 21 mg Se, 75 mg Co, 21 mg I, 260,000 IU of vitamin A, and 7,500 IU of vitamin E.
[7] A supplement containing 30% blood meal enriched with rumen-protected lysine and methionine (LysAAMet, Perdue Ag Solutions, LLC, Salisbury, MD). Each kg contains (DM basis) 26.4% CP, 5.1% Ca, 1.6% P, 4.1% Mg, 6.8% K, 0.3% S, 10.7% Na, 2.5% Cl, 665 mg Zn, 230 mg Cu, 416 mg Mn, 7.2 mg Se, 24 mg Co, 13.6 mg I, 110,000 IU of vitamin A, 33,000 IU of cholecalciferol (0.825 mg), 1,100 IU of vitamin E, and 460 mg of monensin (Rumensin 90, Elanco Animal Health, Eli Lilly and Co, Indianapolis, IN).
[8] Samples collected weekly and composited monthly for chemical analyses.
[9] Calculated based on the chemical analysis of dietary ingredients and using the NRC (2001) for a DM intake of 12.0 kg/d prepartum and 18 kg/d postpartum.
[10] Calculated using the equation DM – [(CP + NDF + fat + ash – (NDF insoluble protein)].
[11] Calculated using the equation [(mEq of Na + mEq of K) - (mEq of Cl + mEq of S)].

The experiment followed a randomized complete block design with cow as the experimental unit. Weekly cohorts of prepartum cows at 252 d of gestation were blocked by parity (0 vs. > 0) and previous lactation 305-d milk yield (parous cows) and, within each block, assigned randomly to one of the four treatments. Treatments were arranged as a factorial with two levels of DCAD,positive (+130 mEq/kg) or negative (-130 mEq/kg), and two sources of vitamin D, cholecalciferol or calcidiol that were fed at 3 mg for each 11 kg of diet DM. It was anticipated that the prepartum cows would consume on average 11 kg of DM/d for the last 21 d of gestation, which would result in an intake of vitamin D of 3 mg/d. Therefore, the four treatments were positive DCAD with cholecalciferol (**PCH**; 7 nulliparous, 5 lactation 1, 6 lactation 2, and 2 lactation 3 or greater), positive DCAD with calcidiol (**PCA;** 7 nulliparous, 6 lactation 1, 4 lactation 2, and 3 lactation 3), negative DCAD with cholecalciferol (**NCH**; 7 nulliparous, 4 lactation 1, 6 lactation 2, and 3 lactation 3 or greater), and negative DCAD with calcidiol (**NCA**; 7 nulliparous, 4 lactation 1, 5 lactation 2, and 4 lactation 3 or greater). Treatment diets were fed from 252 d of gestation to calving. Upon calving, cows were fed the same lactation ration for the first 49 DIM. All diets were fed as TMR.

Ingredient Sampling, Chemical Analyses, and Calculation of DM Intake

Forages and concentrate mixtures were collected weekly, dried at 55^{o}C and moisture loss recorded and stored for later analyses as monthly composites. Dried samples were ground to pass a 1 mm screen of a Wiley mill (Thomas Scientific, Swedesboro, NJ), and analyzed for DM (105 °C for 12 h). Dried samples were composited monthly and then analyzed for OM (512 °C for 8 h), sequential analysis of NDF using a heat stable α-amylase (Van Soest et al., 1991), N using an automated quantitative combustion digestion method (LECO FP628, LECO Corp. St. Joseph, MI), starch after acid hydrolysis (Vidal et al., 2009), total fatty acids (Sukhija and Palmquist, 1988), and minerals by inductively-coupled plasma mass spectrometry. The energy density of the diets was estimated using chemical analysis of dietary ingredients and calculated for 12.0 and 18.0 kg of DM intake for the pre- and postpartum periods, respectively, using the NRC (2001) model (Table 1). Intake of DM for each cow was calculated daily for the first 42 DIM based on the DM content measured weekly at 105 ^{o}C of the ingredients and the respective composition of diets.

Body Weight and Body Condition Score

Cows were weighed on the day of experiment enrollment and then once weekly prepartum, in the morning, during the last 3 wk of gestation. Body condition was scored on the day of

enrollment and then once weekly by the same trained evaluator using a 1 to 5 scale (Ferguson et al., 1994) with increments of 0.25 units as depicted in the Elanco BCS chart (Elanco, 2009). During the postpartum period, immediately after each milking, cows were weighed on a walk-though scale (AfiWeigh, S.A.E. Afikim, Israel) located on the exit lane of the milking parlor. Body condition was scored once weekly as described previously.

Blood Samples

Blood was collected 3 times per week from 265 d of gestation until calving, and postpartum at 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 d postpartum by puncture of the coccygeal blood vessels into evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing no anticoagulant agents for serum separation, or in tubes containing K_2 EDTA for plasma separation. Samples were collected after the morning feeding between 0800 and 1000 h. For the prepartum period, the samples collected on d -9, -6, -3 and -1 relative to calving were used for assaying metabolites and hormones. Postpartum, all samples collected from 0 to 30 DIM were assayed for nonesterified fatty acids (**NEFA**), BHB, total protein, and glucose; samples collected every 3 d from 0 to 30 DIM were assayed for insulin IGF-1 and leptin; and samples collected every 6 d from 0 to 30 DIM were assayed for total cholesterol. Serum samples were allowed to clot and then placed on ice until processing. Samples with anticoagulant were placed in ice immediately upon collection and were processed within 4 h of collection. Tubes were centrifuged and serum and plasma were harvested and split into multiple aliquots and frozen at -20°C until analyses.

Blood Assays

Plasma concentrations of NEFA (NEFA-C kit; Wako Diagnostics Inc., Richmond, VA; according to Johnson and Peters, 1993) and BHB (Wako Autokit 3-HB; Wako Diagnostics, Inc., Richmond, VA) were analyzed using colorimetric enzymatic assays. The intra- and inter-assays CV were, respectively, 9.2 and 4.8% for NEFA, and 9.8 and 5.9% for BHB. Concentrations of glucose in plasma were determined by colorimetric continuous flow analysis (Autoanalyzer II, SEAL Analytical, UK) using a modification of the method described by Gochman and Schmitz (1972). Intra- and inter-assays CV were 2.6 and 3.8 % for glucose. Concentrations of insulin (Mercodia Bovine Insulin ELISA, Mercodia Inc., Uppsala, Sweden) and IGF-1 in plasma (R&D Systems, Inc., Minneapolis) were analyzed by enzyme immune assays. The intra- and inter-assays CV were, respectively, 3.1 and 4.7 % for insulin, and 6.0 and 10.3% for IGF-1. Concentrations of leptin in plasma were analyzed by an ELISA at Commonwealth Scientific and

Industrial Research Organisation (CSIRO) laboratory in Australia. Concentrations of total protein in serum were quantified using a digital clinical refractometer (TS Meter-D Automatic Digital Clinical Refractometer, Reichert Technologies, Buffalo, NY). Concentrations of cholesterol in serum were analyzed by an enzymatic colorimetric assay (Wako Cholesterol E; Wako Diagnostics, Inc., Richmond, VA). The intra and inter-assays CV were 4.5 and 4.7%, respectively. Details of assays of vitamin D metabolites in plasma and iCa in whole blood are presented in a companion paper (Rodney et al., 2017 – Chapter 6).

Measurements of Colostrum, Milk, and Milk Components

Cows were milked within the first 6 h after calving and colostrum yield was measured and duplicate samples were collected and analyzed for concentrations of fat, true protein, lactose, SNF, total solids, urea N, and SCC at the USDA Milk Market Administrator Laboratory (Lawrenceville, GA). In addition, colostrum samples were analyzed for IgG using a bovine IgG ELISA kit (ZMC Catalog #: 0801198; ZeptoMetrix, Franklin, MA) according to manufacturer instructions. Duplicate values were averaged for each cow.

Cows were milked twice daily at 0700 h and 1900 h, and yields of milk were recorded automatically (AfiFlo milk meters, S.A.E. Afikim, Israel) for the first 49 DIM. Samples of milk were collected once weekly in two sequential milkings, morning and afternoon, for measurements of concentrations of fat, true protein, lactose, and SCC at the Southeast Milk laboratory (Belleview, FL). Milk yield from each sampling was taken into account to calculate the final concentrations of milk components. Yields of milk corrected for 3.5% fat content and for energy, and the NE content of milk were calculated according to NRC (2001) as: 3.5% FCM = 0.4324 x milk kg + (16.218 x milk fat kg); ECM = [(0.3246 x Milk yield) + (12.86 x fat yield) + (7.04 x protein yield)]; NE = (0.0929 x fat %) + (0.0563 x protein %) + (0.0395 x lactose %).

Measurement of Net Energy Balance

Energy balance was calculated using daily caloric intake from DM intake and the energy content of the diets according to NRC (2001) using the NE_L system. The needs for maintenance were calculated based on the formula of NRC (2001) and according to metabolic BW ($0.08 \times BW^{0.75}$). Calories required for gestation for prepartum cows were estimated at 3.7 Mcal of NE_L/d for a calf that would eventually be born with 43 kg (NRC, 2001). Calories secreted as milk were calculated according to yields of fat, protein, and lactose (milk yield x [(0.0929 x fat %) +

(0.0563 x protein %) + (0.0395 x lactose %)]) based on NRC (2001). Daily values were averaged into weekly means for statistical analyses.

Statistical Analysis

The experiment followed a randomized complete block design with cow as the experimental unit. Prepartum cows at 252 d of gestation were blocked by parity (0 vs. > 0) and previous lactation 305 d milk (parous cows) and, within each block, assigned randomly to one of the four treatments. Sample size was calculated with the POWER procedure of SAS (SAS ver. 9.4, SAS/STAT, SAS Institute Inc., Cary, NC) to detect differences in blood concentrations of tCa and iCa and vitamin D metabolites described in Rodney et al. (2017 – Chapter 6). For those responses, only 5 cows per treatment were needed at $\alpha = 0.05$ and $\beta = 0.20$ if the SD followed values observed previously (Martinez et al., 2012; Wilkens et al., 2012). Nevertheless, because our goal also was to determine if dietary treatments affect production, we calculated the number of experimental units to be able to detect a minimum difference in milk yield of 2.6 kg/d assuming a SD of 4 kg. The sample size calculated assumed equal number of cows per main effects of DCAD or source of vitamin D at 39. Total of 40 cows per main effect was chosen to allow 20 cows per individual treatment.

Data were analyzed by ANOVA for the pre- and postpartum periods separately. Normality of residuals and homogeneity of variance were examined for each continuous dependent variable analyzed after fitting the final model. Responses that violated the assumptions of normality were subjected to power transformation according to the Box-Cox procedure (Box and Cox, 1964) using the PROC TRANSREG in SAS (SAS/STAT). The LSM and SEM were back transformed for presentation according to Jørgensen and Pedersen (1998).

The composition of colostrum was analyzed with the MIXED procedure of (SAS/STAT) with mixed models that included the fixed effects of level of DCAD (positive vs. negative), source of vitamin D (CH vs. CA), parity (nulliparous vs. parous), the interactions between DCAD and vitamin D, DCAD and parity, vitamin D and parity, and DCAD and vitamin D and parity, and the random effect of block. The Kenward-Roger method was used to compute the approximate denominator degrees of freedom for the F tests in the statistical models. When an interaction was significant, pairwise comparisons among treatments were performed after adjusting by the method of Tukey.

Data with repeated measures within experimental units were analyzed with mixed models using the MIXED procedure of SAS (SAS/STAT). Models included the fixed effects of level of DCAD, source of vitamin D, interaction between level of DCAD and source of vitamin D, parity, day of measurement, and interactions between DCAD and parity, vitamin D and parity, DCAD and day, vitamin D and day, parity and day, DCAD and vitamin D and parity, DCAD and vitamin D and day, DCAD and parity and day, vitamin D and parity and day, and DCAD and vitamin D and parity and day. Random effects included block and cow nested within level of DCAD and source of vitamin D. The covariance structure selected for each model was based on spacing of measurements and the smallest corrected Akaike's information criterion. The Kenward-Roger method was used to compute the approximate denominator degrees of freedom for the F tests in the statistical models. When an interaction was significant, pairwise comparisons among treatments were performed after adjusting by the method of Tukey. The models for BW and BCS included the values measured on the day of experiment enrolment as covariates.

Additional statistical analyses of postpartum DMI and yield of ECM were performed with the same models described above, but also including morbidity in the first 30 DIM (yes vs. no) and the interaction between morbidity and parity to determine if changes in lactation performance caused by treatments were in part mediated by differences in morbidity in early lactation reported by Martinez et al. (2017 – Chapter 8).

Statistical significance was considered at $P \leq 0.05$, and tendency was considered at $0.05 < P \leq 0.10$.

RESULTS

Twenty-eight nulliparous and 52 parous cows were enrolled in the experiment, but one parous cow fed PCH was removed from the data analyses because of diagnosis of lymphosarcoma during late gestation. Therefore, 79 cows were included in all statistical analyses. One PCA cow developed pneumonia because of aspiration of an oral Ca drench used to treat clinical hypocalcemia and she had to be euthanized and was removed prematurely from the experiment and contributed with data from enrollment to 2 DIM. The length of gestation (± SD) was 275 ± 4.4 d and days receiving the prepartum diets did not differ with treatments and averaged 22.7 ±

5.3. All cows in the experiment stayed a minimum of 2 wk in the prepartum diets and one cow remained a maximum of 34 d.

Details of concentrations of vitamin D metabolites and minerals in blood are reported elsewhere (Rodney et al., 2017 – Chapter 6). Briefly, feeding cholecalciferol increased ($P < 0.001$) the concentrations of vitamin D_3 in plasma pre- (CH = 14.7 vs. CA = 1.1 ± 0.6 ng/mL) and postpartum (CH = 5.6 vs. CA = 1.4 ± 0.3 ng/mL), whereas feeding calcidiol increased ($P < 0.001$) the concentrations of 25-hydroxyvitamin D_3 in plasma pre- (CH = 59.7 vs. CA = 237.0 ± 6.8 ng/mL) and postpartum (CH = 58.5 vs. CA = 218.3 ± 5.3 ng/mL). Feeding the diet with negative DCAD reduced ($P < 0.05$) the concentrations of vitamin D_3 and 25-hydroxyvitamin D_3 pre- and postpartum compared with feeding the diet with positive DCAD (Rodney et al., 2017 – Chapter 6). Both negative DCAD and calcidiol increased the concentrations of iCa prepartum (PCH = 1.19, PCA = 1.25, NCH = 1.22, NCA = 1.27 m*M*); however, at calving and on day 1 postpartum, concentrations of iCa increased ($P < 0.001$) with feeding the diet with negative compared with positive DCAD (positive = 0.968 vs. negative = 1.110 ± 0.008 m*M*), but no difference was observed with source of vitamin D.

Prepartum Intake and Measures of Energy Status

The DM intake prepartum was less ($P = 0.04$) in cows fed the negative compared with those fed the positive DCAD (Table 2); however, an interaction ($P < 0.01$) between DCAD and parity was detected because the depression in intake caused by the negative DCAD diet was observed only in parous cows (positive = 13.7 vs. negative = 11.5 ± 0.4 kg/d), but not in nulliparous cows (positive = 11.0 vs. negative = 11.3 ± 0.5 kg/d). Similarly, the caloric intake of cows consuming the negative DCAD treatment decreased ($P = 0.03$) because of an interaction ($P = 0.01$) between DCAD and parity (Figure 1, A and B). Dietary cation-anion difference did not affect caloric intake prepartum in nulliparous cows (positive = 18.2 vs. negative = 18.6 ± 0.8 Mcal NE_L/d), but decreased ($P < 0.001$) that in parous cows (positive = 22.6 vs. negative = 18.9 ± 0.6 Mcal NE_L/d). The changes in caloric intake resulted in differences in energy balance, which was less ($P < 0.01$) for parous cows fed the diet with negative DCAD compared with those fed the diet with positive DCAD (positive = 6.2 vs. negative = 2.5 ± 0.6 Mcal NE_L/d), but no difference was observed for nulliparous cows (positive = 3.9 vs. negative = 2.5 ± 0.7 Mcal NE_L/d). Despite the effects on intake of prepartum parous cows, the mean NE_L balance remained positive until 2 d before calving in nulliparous or parous cows fed the diet with positive or the negative DCAD, and only on the day before calving did the mean NE_L balance became negative for all cows fed

either DCAD level. Source of vitamin D had no effect on prepartum DM, caloric intake, or NE_L balance (Table 2).

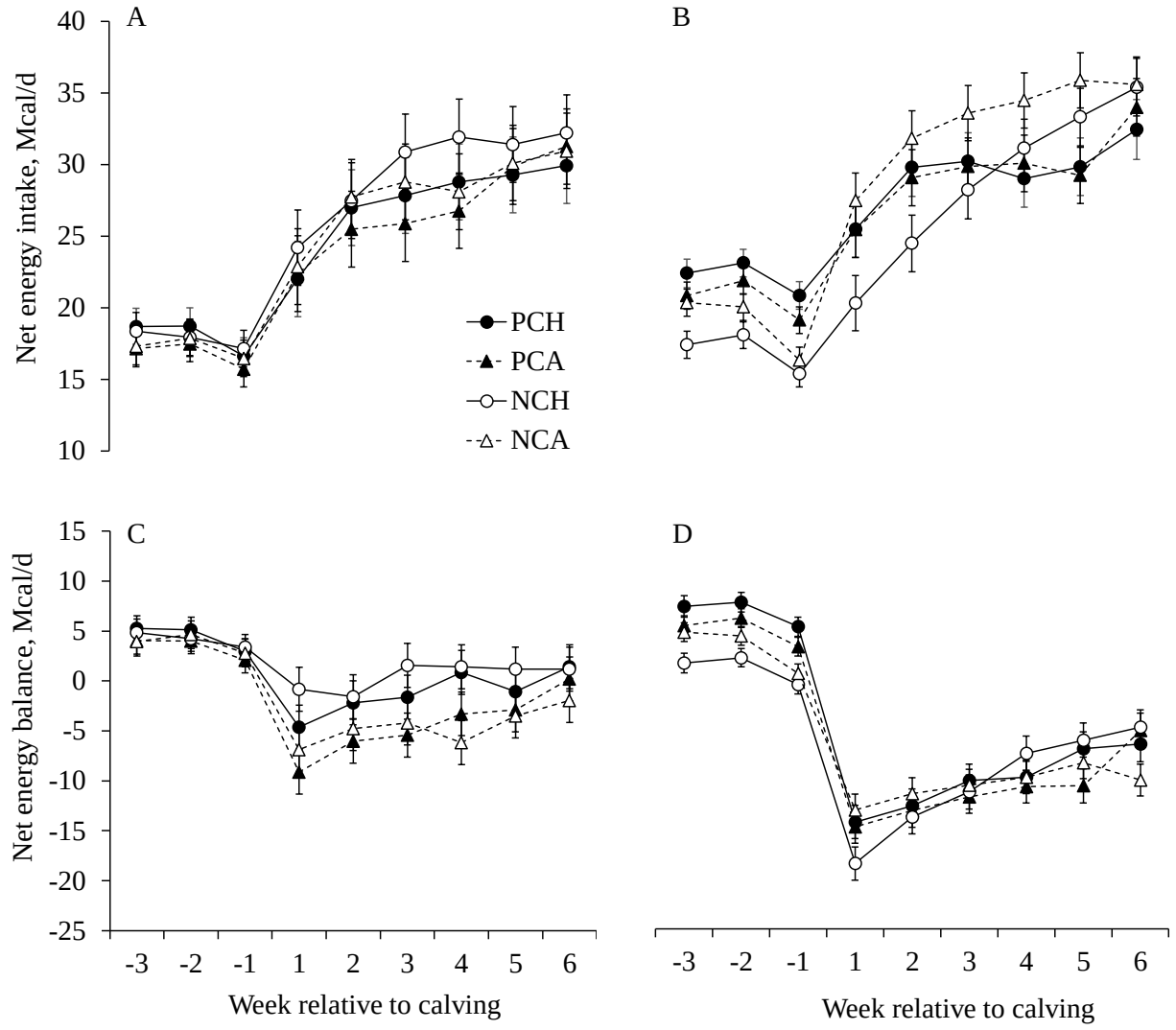

Figure 1. Net energy intake in nulliparous (A) and parous cows (B) and NE balance in nulliparous (C) and parous cows (D) fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Panels A and B: Prepartum, effects of parity (P = 0.003), DCAD and parity (P = 0.01), vitamin D and parity (P = 0.43), DCAD and vitamin D and parity (P = 0.33), DCAD and parity and week (P = 0.27), vitamin D and parity and week (P = 0.52), and DCAD and vitamin D and parity and week (P = 0.66). Postpartum, effects of parity (P = 0.11), and interactions between DCAD and parity (P = 0.93), vitamin D and parity (P = 0.24), DCAD and vitamin D and parity (P = 0.35), DCAD and parity and week (P = 0.06), vitamin D and parity and week (P = 0.49), and DCAD and vitamin D and parity and week (P = 0.81). Panels C and D: Prepartum, effects of parity (P = 0.54), DCAD and parity (P = 0.005), vitamin D and parity (P = 0.52), DCAD and vitamin D and parity (P = 0.22), DCAD and parity and week (P = 0.61), vitamin D and parity and week (P = 0.77), and DCAD and vitamin D and parity and week (P = 0.72). Postpartum, effects of parity (P < 0.001), DCAD and parity (P = 0.76), vitamin D and parity (P = 0.11), DCAD and vitamin D and parity (P = 0.55), DCAD and parity and week (P = 0.13), vitamin D and parity and week (P = 0.09), and DCAD and vitamin D and parity and week (P = 0.26).

Table 2. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on measures of energy status in the last 21 d of gestation in Holstein cows[1]

	Positive DCAD		Negative DCAD			*P*-value[2]		
Prepartum	CH	CA	CH	CA	SEM	DCAD	Vitamin D	DCAD x Vitamin D
DM intake,[*] kg/d	12.7	12.0	11.2	11.6	0.5	0.04	0.70	0.20
Caloric intake,[*] Mcal/d	21.0	19.7	18.4	19.1	0.8	0.03	0.71	0.19
NE balance,[*] Mcal/d	5.8	4.4	2.8	3.7	0.7	0.01	0.68	0.08
Body weight								
Kg	695.6	696.3	686.3	694.8	5.1	0.27	0.34	0.42
Change,[*] kg/d	1.57	1.70	0.99	1.68	0.27	0.26	0.12	0.30
Body condition, 1 to 5	3.59	3.55	3.58	3.64	0.05	0.36	0.79	0.29

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).
[2] DCAD = effect of DCAD (positive vs. negative); Vitamin D = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x vitamin D = interaction between DCAD and vitamin D.
[*] Interaction between DCAD and parity ($P < 0.01$).

Nulliparous and parous cows gained similar amounts of BW in the last 3 wk of gestation, an average 33.4 ± 4.6 kg (Figure 2 panels A and B). Nevertheless, an interaction ($P < 0.01$) between DCAD and parity was observed for daily BW change. Within parous cows, those fed the diet with positive DCAD gained more ($P < 0.01$) BW than those fed the diet with negative DCAD (positive = 1.96 vs. negative = 0.93 ± 0.24 kg/d), whereas DCAD did not affect daily BW gain in nulliparous cows (positive = 1.31 vs. negative = 1.74 ± 0.32 kg/d). The changes in BW with DCAD were not replicated in changes in BCS. Treatments did not affect the mean BCS or the patterns of change in BCS prepartum in both nulliparous and parous cows (Table 2, Figure 1 panels C and D).

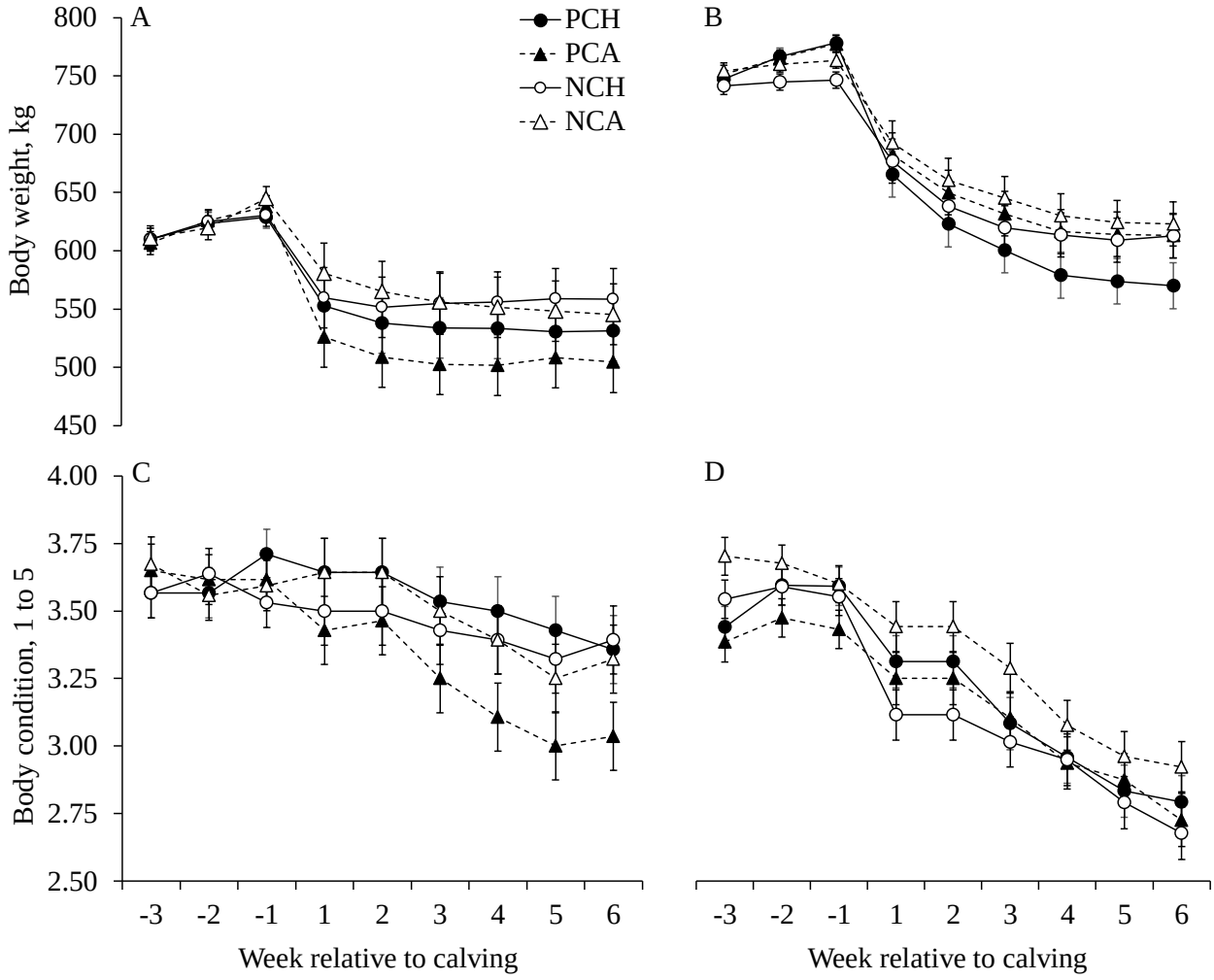

Figure 2. Body weight in nulliparous (A) and parous cows (B) and body condition score in nulliparous (C) and parous cows (D) fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Panels A and B: Prepartum, effects of parity ($P < 0.001$), and interactions between DCAD and parity ($P = 0.19$), vitamin D and parity ($P = 0.64$), DCAD and vitamin D and parity ($P = 0.41$), DCAD and parity and week ($P = 0.07$), vitamin D and parity and week ($P = 0.35$), and DCAD and vitamin D and parity and week ($P = 0.63$). Postpartum, effects of parity ($P < 0.001$), and interactions between DCAD and parity ($P = 0.63$), vitamin D and parity ($P = 0.22$), DCAD and vitamin D and parity ($P = 0.48$), DCAD and parity and week ($P = 0.84$), vitamin D and parity and week ($P = 0.29$), and DCAD and vitamin D and parity and week ($P = 0.81$). Panels C and D: Prepartum, effects of parity ($P = 0.31$), and interactions between DCAD and parity ($P = 0.12$), vitamin D and parity ($P = 0.82$), DCAD and vitamin D and parity ($P = 0.35$), DCAD and parity and week ($P = 0.70$), vitamin D and parity and week ($P = 0.94$), and DCAD and vitamin D and parity and week ($P = 0.47$). Postpartum, effects of parity ($P < 0.001$), and interactions between DCAD and parity ($P = 0.71$), vitamin D and parity ($P = 0.05$), DCAD and vitamin D and parity ($P = 0.69$), DCAD and parity and week ($P = 0.98$), vitamin D and parity and week ($P = 0.38$), and DCAD and vitamin D and parity and week ($P = 0.23$).

Blood Concentrations of Metabolites Prepartum

Concentrations of NEFA in plasma increased ($P < 0.01$) as cows approached calving (Figure 3 panel A). A tendency for interaction ($P = 0.06$) between DCAD and source of vitamin D was detected for concentrations of NEFA prepartum because in cows fed the positive DCAD, those receiving cholecalciferol tended ($P = 0.09$) to have increased NEFA concentrations than cows receiving CH, whereas no difference in NEFA concentrations was observed between sources of vitamin D in cows fed the diet with negative DCAD (Table 3). Treatment did not affect the concentrations of BHB, cholesterol, and total protein prepartum and they remained relatively constant until the day before calving (Figure 3, panels B-D).

Concentrations of glucose increased ($P < 0.01$), whereas those of insulin, IGF-1, and leptin decreased ($P < 0.05$) as cows approached calving (Figure 4, panels A-D). Feeding the diet with negative DCAD reduced the concentrations of glucose, insulin, and IGF-1 (Table 3). Nevertheless, interactions between DCAD and vitamin D were detected for insulin and IGF-1. For cows fed CH, the diet with negative DCAD reduced ($P < 0.01$) the concentrations of insulin and IGF-1 in plasma compared with the diet with positive DCAD; however, no differences were observed for insulin and IGF-1 between cows fed PCA and NCA. Treatment did not influence concentrations of leptin prepartum (Table 3).

Table 3. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on concentrations of energy metabolites in the last 9 d of gestation in Holstein cows[1]

	Positive DCAD		Negative DCAD			*P*-value[2]		
							Vitamin	DCAD x Vitamin
Prepartum	CH	CA	CH	CA	SEM	DCAD	D	D
NEFA,[§] m*M*	0.19	0.24	0.24	0.22	0.02	0.35	0.54	0.06
BHB, m*M*	0.49	0.48	0.46	0.45	0.02	0.17	0.65	0.73
Cholesterol, mg/dL	72.0	76.2	80.0	82.4	4.5	0.05	0.36	0.80
Total protein, mg/dL	6.22	6.21	6.11	6.09	0.09	0.19	0.89	0.92
Glucose, m*M*	4.10	4.05	3.90	3.99	0.06	0.03	0.72	0.26
Insulin, ng/mL	0.61	0.54	0.40	0.50	0.05	0.02	0.68	0.08
IGF-1, ng/mL	117.4	103.2	89.6	101.4	6.1	0.003	0.80	0.009
Leptin, ng/mL	3.68	3.64	3.07	3.98	0.42	0.61	0.17	0.14

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Blood sampled on d -9, -6, -3 and -1 relative to calving.
[2] DCAD = effect of DCAD (positive vs. negative); Vitamin D = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x vitamin D = interaction between DCAD and vitamin D. [§] Interaction between source of vitamin D and parity ($P = 0.08$).

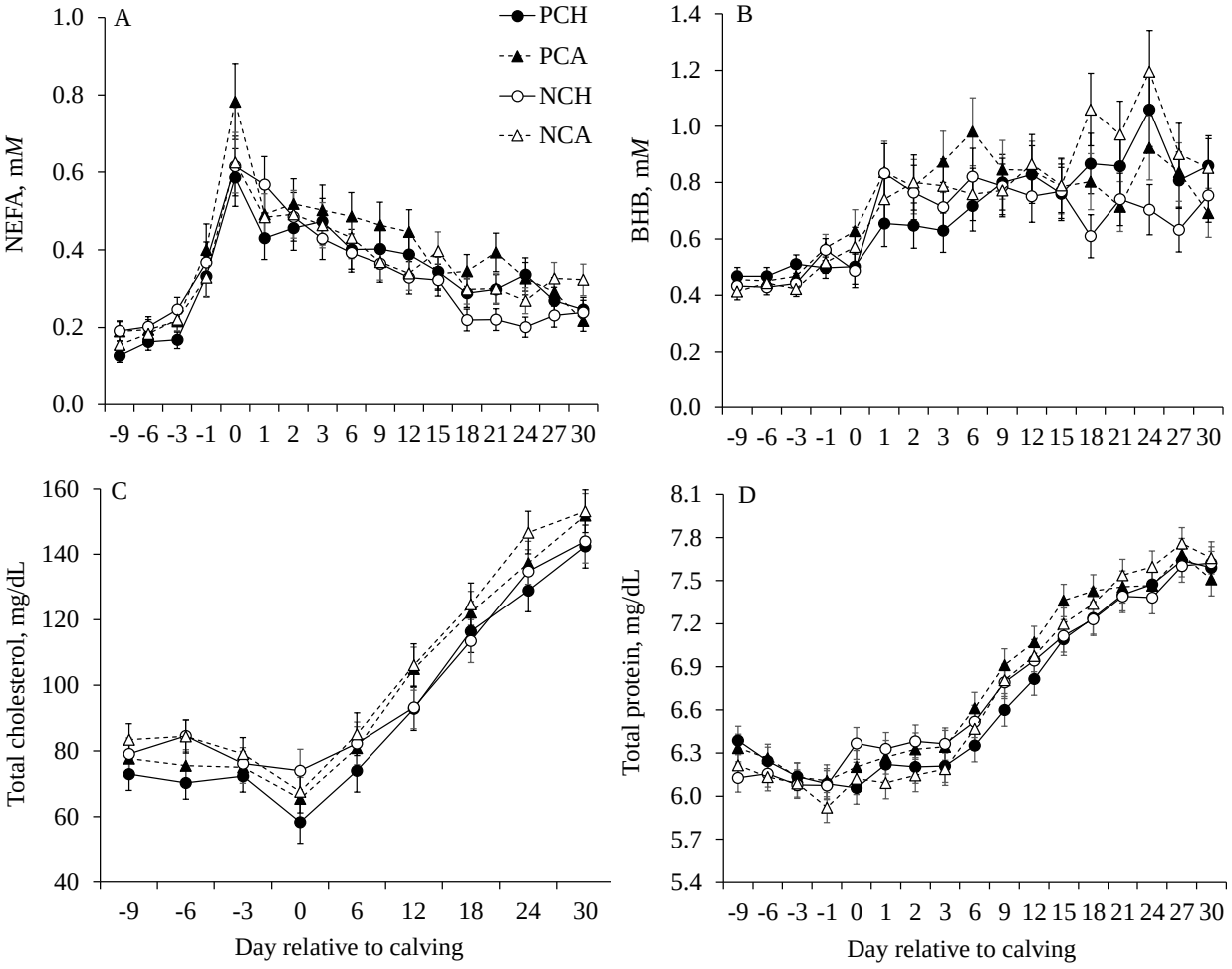

Figure 3. Concentrations of NEFA (A) and BHB (B) in plasma, and total cholesterol (C) and protein (D) in serum of cows fed prepartum diets containing either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and supplemented with either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

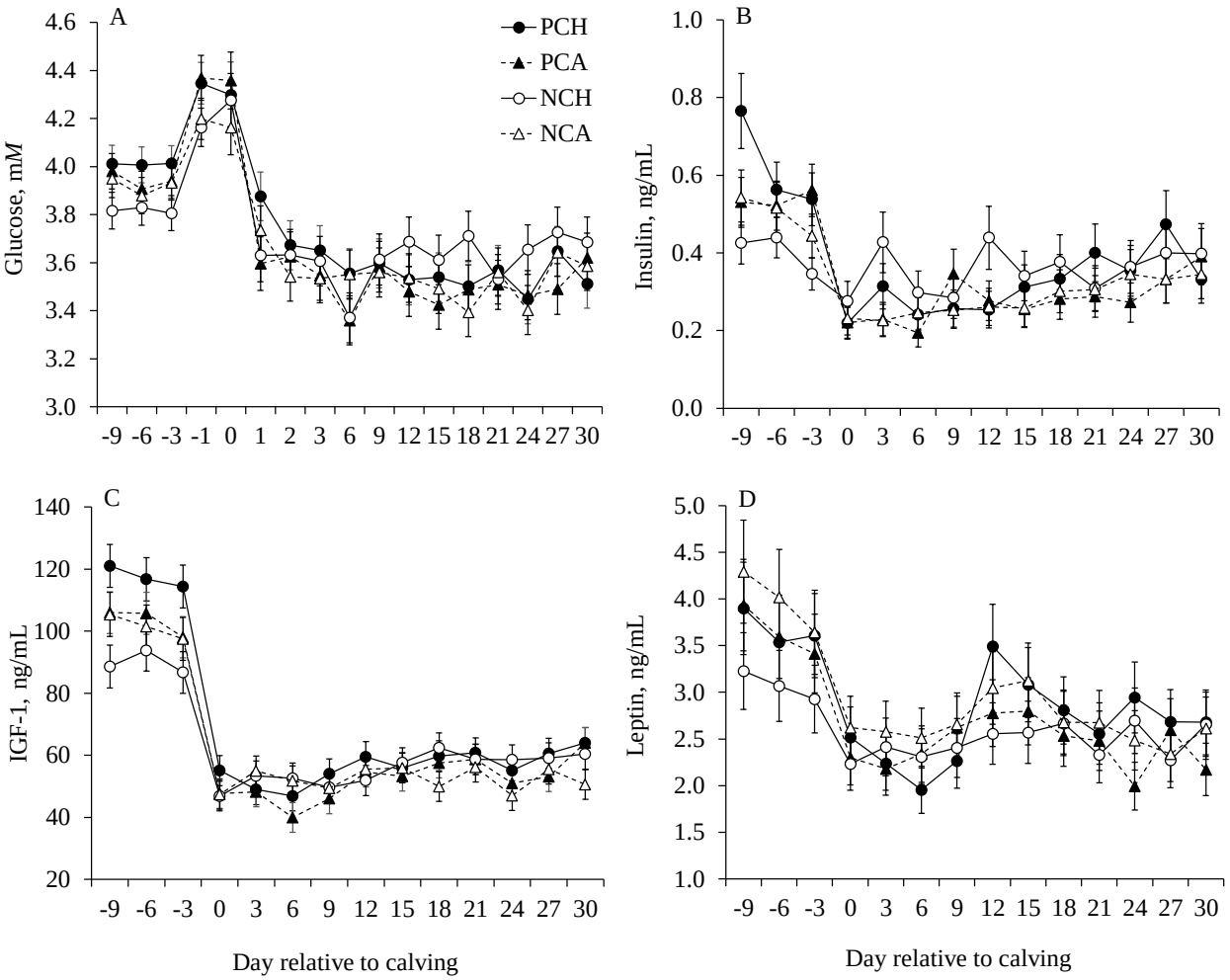

Figure 4. Concentrations of glucose (m*M*, A), insulin (ng/mL, B), and IGF-1 (ng/mL, C) in plasma of cows fed prepartum diets containing either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and supplemented with either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

Table 4. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on colostrum yield and composition in Holstein cows[1]

	Positive DCAD		Negative DCAD			*P*-value[2]		
Item	CH	CA	CH	CA	SEM	DCAD	Vitamin D	DCAD x Vitamin D
Colostrum yield, kg	5.86	7.68	6.21	7.96	1.06	0.77	0.10	0.97
Fat								
%	4.02	5.37	5.40	4.24	0.54	0.83	0.87	0.02
Yield, kg	0.25	0.43	0.30	0.39	0.08	0.93	0.12	0.58
True protein								
%	11.9	15.8	14.9	14.9	0.89	0.23	0.03	0.04
Yield, kg	0.66	1.20	0.88	1.17	0.17	0.57	0.02	0.47
Lactose								
%[¥]	2.94	2.47	2.49	2.41	0.14	0.07	0.05	0.16
Yield,[¥] kg	0.18	0.19	0.17	0.19	0.03	0.87	0.50	0.82
Solids-not-fat								
%[¥]	16.8	20.6	19.6	19.6	0.86	0.27	0.03	0.03
Yield, kg	0.95	1.57	1.19	1.55	0.22	0.62	0.03	0.56
Total solids								
%[¥]	20.9	26.0	25.1	23.9	1.00	0.31	0.05	0.002
Yield, kg	1.21	2.00	1.49	1.94	0.29	0.70	0.04	0.55
Net energy								
Content,[¥] Mcal/kg	1.16	1.48	1.44	1.33	0.07	0.35	0.10	0.001
Yield, Mcal	6.7	11.5	8.4	11.0	1.7	0.73	0.04	0.53
Urea N, mg/dL	35.2	39.5	35.4	38.7	2.3	0.90	0.10	0.82
IgG, g/L	45.3	57.7	50.6	60.1	3.8	0.31	0.005	0.70
Somatic cell score[*]	6.45	6.96	6.38	7.18	0.44	0.87	0.14	0.74

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

[2] DCAD = effect of DCAD (positive vs. negative); Vitamin D = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x vitamin D = interaction between DCAD and vitamin D.

[*] Interaction between DCAD and parity ($P = 0.09$).

[¥] Interaction between DCAD, vitamin D and parity ($P < 0.05$).

Colostrum Yield and Composition

Cows fed calcidiol tended (P = 0.10) to produce 1.8 kg more colostrum than those fed cholecalciferol (CH = 6.0 vs. CA = 7.8 ± 0.8 kg), but DCAD had no effect on yield of colostrum (Table 4). As expected, parous cows produced more (P < 0.01) colostrum than nulliparous cows, which averaged 8.6 and 5.3 ± 0.8 kg, respectively. An interaction (P = 0.02) between DCAD and vitamin D was observed for fat content in colostrum. In cows fed the positive DCAD, calcidiol tended (P = 0.07) to increase milk fat content compared with CH, but within the diet with negative DCAD, those fed calcidiol had numerically less (P = 0.11) fat content than cows fed CH. Treatment did not affect yield of fat in colostrum, which averaged 0.34 kg. An interaction (P = 0.04) between DCAD and vitamin D was observed for true protein content in colostrum because supplementing cows with calcidiol increased (P < 0.01) true protein when fed the diet with positive but not the diet with negative DCAD. Protein yield increased (P = 0.02) in cows fed calcidiol compared with CH, but it did not differ with level of DCAD. Lactose content in colostrum tended (P = 0.07) to be greater for cows fed the positive compared with the negative DCAD, and it was greater (P = 0.05) for cows fed cholecalciferol than CA, but yield of lactose was not affected by treatment. The concentrations of SNF and of total solids in colostrum increased (P < 0.05) with feeding calcidiol only in cows fed the positive, but not the negative DCAD; however, yields of SNF and total solids in colostrum increased (P < 0.04) with calcidiol compared with cholecalciferol in both, the diets with positive and negative DCAD.

Interactions (P < 0.05) between DCAD and vitamin D and parity were detected for concentrations of lactose, SNF and total solids in colostrum. In nulliparous, within those fed the positive DCAD, supplementing calcidiol reduced (P < 0.05) lactose content (PCH = 3.03 vs. PCA = 2.30 ± 0.22%), whereas within those fed the negative DCAD, supplementing calcidiol increased lactose content (NCH = 2.33 vs. NCA = 2.71 ± 0.22%). On the other hand, no differences were observed for parous cows. For SNF and total solids, the same responses were observed. In nulliparous cows, feeding calcidiol within the positive DCAD increased (P < 0.05) SNF (PCH = 15.6 vs. PCA = 21.8 ± 1.4%), but the opposite response was observed for the diet with negative DCAD (NCH = 19.8 vs. NCA = 17.9 ± 1.4%). On the other hand, no differences were observed within parous cows. Similarly, supplementing calcidiol within the positive DCAD increased (P < 0.05) total solids in colostrum of nulliparous cows (PCH = 20.6 vs. PCA = 28.5 ± 1.6%), but the opposite response was observed for the diet with negative DCAD (NCH = 26.9 vs. NCA = 22.4 ± 1.6%). No differences were observed for total solids within parous cows.

An interaction (P = 0.001) between DCAD and vitamin D was observed for NE_L content of colostrum. Supplementing calcidiol increased the NE_L content in colostrum of cows fed the positive DCAD, but the opposite response was observed when cows were fed the negative DCAD (Table 4). Nevertheless, the calories secreted as colostrum increased (P = 0.04) with supplementing calcidiol and this response was observed in both levels of DCAD fed. The concentration of urea N in colostrum tended (P = 0.10) to increase with feeding calcidiol compared with CH, but no effect of DCAD was observed. Cows fed calcidiol produced colostrum with greater (P = 0.005) IgG content than those fed CH, but DCAD had no effect on colostrum IgG concentration. Treatments did not affect the somatic cell score (**SCS**) in colostrum.

Lactation Performance

Supplementing diets with calcidiol improved (P < 0.05) yields of milk, 3.5% FCM and ECM in nulliparous and parous cows (Table 5), and the differences in production were observed throughout the 49-d experiment (Figure 5, panels A and B). Cows supplemented with calcidiol produced approximately 3.70 kg/d more (P = 0.008) milk than those fed cholecalciferol. In addition, cows supplemented with calcidiol tended (P < 0.07) to have increased yields of fat and true protein, and increased (P = 0.03) that of lactose because of changes in milk yield, but not because of changes in content of those compounds in milk. Feeding a diet with negative compared with positive DCAD increased (P = 0.05) the content of fat in milk, but DCAD only numerically increased yields of 3.5% FCM (P = 38.5 vs. N = 39.7 ± 1.2 kg/d) and ECM (P = 37.1 vs. N = 38.3 ± 1.2 kg/d) by 1.2 kg/d. Treatments did not affect the SCS in milk.

Additional analyses of DMI and production included morbidity described by Martinez et al. (2017 – Chapter 8) in the statistical models. Cows with morbidity produced 6.3 kg/d less (P < 0.001) milk than healthy cows (34.2 vs. 40.5 ± 1.1 kg/d) and the differences were observed in both nulliparous (28.5 vs. 33.7 ± 1.8 kg/d) and parous cows (40.0 vs. 47.2 ± 1.6 kg/d).

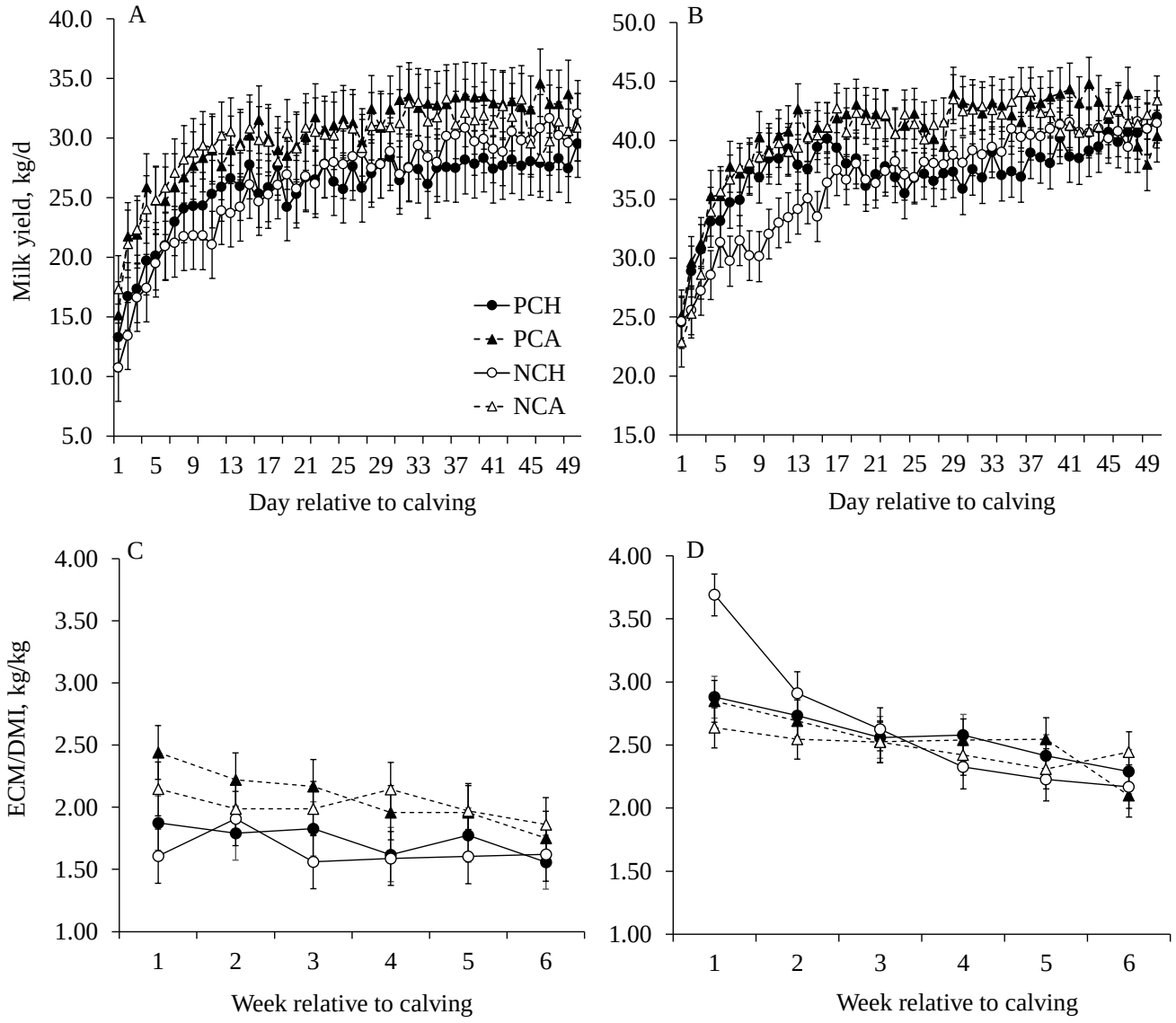

Figure 5. Milk yield in nulliparous (A) and parous cows (B) and efficiency of feed conversion into energy-corrected milk in nulliparous (C) and parous cows (D) fed prepartum diets with either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Panel A, nulliparous cow averaged (kg/d): PCH = 25.7 ± 2.2; PCA = 30.2 ± 2.2; NCH = 26.1 ± 2.2; 29.6 ± 2.2. Panel B, parous cows averaged (kg/d): PCH = 37.3 ± 1.7; PCA = 40.6 ± 1.7; NCH = 36.6 ± 1.6; NCA = 40.1 ± 1.6. Effects of parity ($P < 0.001$), interactions between DCAD and parity ($P = 0.85$), vitamin D and parity ($P = 0.83$), and DCAD and vitamin D and parity ($P = 0.85$). Panel C, nulliparous cows averaged: PCH = 1.74 ± 0.17; PCA = 2.08 ± 0.17; NCH = 1.65 ± 0.17; NCA = 2.02 ± 0.17. Panel D, parous cows averaged: PCH = 2.58 ± 0.13; PCA = 2.54 ± 0.13; NCH = 2.66 ± 0.13; NCA = 2.48 ± 0.12. Effects of parity ($P < 0.001$), interactions between DCAD and parity ($P = 0.68$), vitamin D and parity ($P = 0.04$), and DCAD and vitamin D and parity ($P = 0.70$).

Table 5. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on lactation performance in the first 49 d postpartum in Holstein cows[1]

	Positive DCAD		Negative DCAD			*P*-value[2]		
Item	CH	CA	CH	CA	SEM	DCAD	Vitamin D	DCAD x Vitamin D
Yield, kg/d								
Milk	31.5	35.4	31.4	34.9	1.4	0.79	0.008	0.90
3.5% FCM	37.0	40.1	37.5	41.9	1.8	0.50	0.04	0.72
ECM	35.6	38.6	36.0	40.4	1.7	0.53	0.03	0.68
Fat								
%	4.56	4.37	4.62	4.77	0.12	0.05	0.89	0.15
Yield, kg	1.43	1.53	1.46	1.66	0.81	0.33	0.07	0.54
True protein								
%	3.16	3.10	3.14	3.25	0.09	0.48	0.73	0.36
Yield, kg	0.98	1.07	0.97	1.11	0.06	0.82	0.06	0.70
Lactose								
%	4.70	4.74	4.77	4.76	0.04	0.24	0.73	0.59
Yield, kg	1.49	1.67	1.54	1.67	0.07	0.78	0.03	0.73
Somatic cell score	2.30	2.51	2.25	2.77	0.36	0.77	0.31	0.67

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

[2] DCAD = effect of DCAD (positive vs. negative); Vitamin D = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x vitamin D = interaction between DCAD and vitamin D.

Table 6. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on measures of energy status in the first 42 d postpartum in Holstein cows[1]

	Positive DCAD		Negative DCAD			*P*-value[2]		
Item	CH	CA	CH	CA	SEM	DCAD	Vitamin D	DCAD x Vitamin D
DM intake, kg/d	17.2	17.2	17.6	18.4	0.8	0.33	0.64	0.63
ECM/DMI,[§] kg/kg	2.16	2.31	2.15	2.25	0.11	0.75	0.26	0.79
Milk NE								
Mcal/kg	0.787	0.767	0.794	0.814	0.011	0.02	0.96	0.09
Mcal/d	24.7	26.8	25.1	28.2	1.2	0.48	0.03	0.67
NE intake, Mcal/d	28.5	28.3	29.3	30.7	1.4	0.27	0.67	0.58
NE balance, Mcal/d	-5.5	-7.6	-4.8	-7.5	1.1	0.69	0.03	0.80
Body weight								
Kg	570.0	572.3	593.4	602.9	16.0	0.09	0.70	0.86
Change, kg/d	-1.7	-1.3	-1.0	-1.5	0.3	0.37	0.76	0.11
Body condition,[§] 1 to 5	3.25	3.08	3.16	3.29	0.07	0.42	0.79	0.03

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

[2] DCAD = effect of DCAD (positive vs. negative); Vitamin D = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x vitamin D = interaction between DCAD and vitamin D.

[§] Interaction between source of vitamin D and parity ($P < 0.05$).

Postpartum DM Intake and Measures of Energy Status

Treatments did not affect the intakes of DM and calories during the first 42 DIM (Table 6). As expected, cows with morbidity consumed 2.7 kg/d less (P = 0.003) DM than healthy cows (16.0 vs. 18.7 ± 0.6 kg/d), and the detrimental impact of morbidity was observed in both nulliparous (15.8 vs. 17.9 ± 0.9 kg/d) and parous cows (15.9 vs. 19.7 ± 0.7 kg/d).

An interaction (P < 0.05) between source of vitamin D and parity was detected for efficiency of feed conversion into ECM (Figure 5, panels C and D). Within nulliparous cows, feeding calcidiol increased (P = 0.04) feed efficiency (CH = 1.69 vs. CA = 2.05 ± 0.12), but no effect was observed for parous cows (CH = 2.62 vs. CA = 2.51 ± 0.12). Feeding the diet with negative DCAD increased (P = 0.02) the NE concentration in milk and the increment with the negative DCAD tended (P = 0.09) to be greater in cows receiving calcidiol than cholecalciferol (Table 6). Nevertheless, DCAD did not influence the amount of NE secreted as milk. The increased milk yield in cows fed calcidiol resulted in greater (P = 0.03) daily NE secretion as milk compared with cows fed cholecalciferol (Table 6). As expected, the onset of lactation induced a period of negative NE balance and the nadir was observed in the first week of lactation and averaged -5.4 ± 1.1 and -15.0 ± 0.8 Mcal/d in nulliparous and parous cows, respectively (Figure 1, panels C and D). Altering the prepartum DCAD did not affect NE_L balance, but the increased caloric secretion in milk with feeding calcidiol resulted in smaller (P = 0.03) NE_L balances in nulliparous and parous cows (Table 6, Figure 1, panels C and D). Nulliparous returned to positive NE_L balance by week 6 postpartum, whereas parous cows remained in negative energy status by then. No interactions between parity and DCAD or vitamin D were observed for NE_L balance postpartum.

As anticipated, cows lost BW postpartum (Figure 2, panels A and B). Despite the differences in energy balance, treatments did not affect the changes in BW in the first 6 wk of lactation (Table 6). Parous cows lost more BW (P < 0.001) than nulliparous cows (nulliparous = -0.56 ± 0.19 vs. parous = -2.15 ± 0.25 kg/d) in the first 6 wk postpartum. Analogous to BW, cows lost body condition in the first 42 DIM, but interactions between DCAD and vitamin D (P = 0.03) and between vitamin D and parity (P = 0.05) were detected, although differences among treatments were small. For cows fed the diet with positive DCAD, those fed PCH had greater BCS than cows fed PCA; however, for cows fed the diet with negative DCAD, PCA had greater BCS than PCH (Table 6). Within nulliparous, those fed cholecalciferol had greater BCS than cows

supplemented with calcidiol (CH = 3.46 vs. CA = 3.30 ± 0.08); however, the opposite response was observed in parous cows (CH = 2.95 vs. CA = 3.07 ± 0.06).

Blood Concentrations of Energy Metabolites Postpartum

Plasma NEFA reached the maximum mean concentrations on the day of calving, 0.65 ± 0.04 m*M*, and then constantly decreased (effect of day, P < 0.001) until the end of the first month postpartum to approximately 0.26 ± 0.03 m*M* (Figure 3A). An interaction (*P* < 0.05) between source of vitamin D and parity was detected because in nulliparous cows, those supplemented with calcidiol had greater (*P* = 0.04) NEFA concentrations than those supplemented with cholecalciferol (CH = 0.25 vs. CA = 0.33 ± 0.03 m*M*); however, no difference was observed between source of vitamin for parous cows (CH = 0.49 vs. CA = 0.48 ± 0.03 m*M*). Dietary cation-anion difference did not affect concentrations of NEFA in plasma postpartum (Table 7).

The concentrations of BHB increased (*P* < 0.01) with the onset of lactation and the maximum values were not observed until 24 to 27 DIM (Figure 3, panel B). An interaction (*P* < 0.001) between vitamin D and parity was detected because BHB concentrations were greater (*P* < 0.01) in nulliparous cows supplemented with calcidiol compared with those supplemented with cholecalciferol (CH = 0.69 vs. CA = 0.50 ± 0.05 m*M*, respectively), but no difference was observed in parous cows (CH = 0.98 vs. CA = 1.07 ± 0.05 m*M*). The level of DCAD did not affect postpartum BHB concentrations (Table 7).

The concentrations of cholesterol sharply increased (*P* < 0.001) with DIM from 66.5 to 147.9 ± 4.1 mg/dL (Figure 3, panel C). Dietary cation-anion difference did not affect concentrations of cholesterol, but cows fed calcidiol tended (*P* = 0.09) to have greater concentrations than those fed cholecalciferol (Table 7). The concentrations of total protein in serum increased (*P* < 0.001) with DIM, but there were no effects of treatment (Figure 3D). The concentrations of glucose decreased (*P* < 0.01), whereas those of insulin, IGF-1, and leptin increased (*P* < 0.01) from the day of calving to 30 DIM (Figure 4, panels A-D); however, none of them were affected by treatment (Table 7).

Table 7. Effect of dietary cation-anion difference (DCAD) and source of vitamin D fed prepartum on concentrations of energy metabolites in the first 30 d postpartum in Holstein cows[1]

	Positive DCAD		Negative DCAD			*P*-value[2]		
Postpartum	CH	CA	CH	CA	SEM	DCAD	Vitamin D	DCAD x Vitamin D
NEFA,[§] m*M*	0.37	0.41	0.33	0.38	0.03	0.30	0.13	0.86
BHB,[§] m*M*	0.76	0.81	0.71	0.84	0.05	0.88	0.09	0.47
Cholesterol, m*M*	102.2	110.5	107.0	113.9	5.3	0.36	0.09	0.88
Total protein, mg/dL	6.84	6.97	6.93	6.92	0.09	0.86	0.48	0.40
Glucose, m*M*	3.68	3.61	3.73	3.60	0.08	0.80	0.20	0.71
Insulin, ng/mL	0.31	0.28	0.35	0.28	0.03	0.54	0.13	0.62
IGF-1, ng/mL	56.4	52.2	55.6	52.2	3.7	0.89	0.26	0.90
Leptin, ng/mL	2.62	2.42	2.46	2.66	0.26	0.54	0.13	0.62

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Blood sampled on d 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 postpartum.
[2] DCAD = effect of DCAD (positive vs. negative); Vitamin D = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x vitamin D = interaction between DCAD and vitamin D.
[§] Interaction between source of vitamin D and parity ($P < 0.05$).

DISCUSSION

The present experiment revealed that supplementing prepartum diets with calcidiol in place of cholecalciferol increased yields of colostrum and colostrum nutrients and yields of milk, 3.5% FCM and ECM in early lactation. Although the highest DMI and production of ECM was observed in cows fed NCA, the increments in productive performance observed with feeding calcidiol were detected in both diets, the positive and negative DCAD. Also, feeding a diet with negative compared with positive DCAD in the last 3 weeks of gestation reduced DMI prepartum, but only numerically increased DMI postpartum and yields of 3.5% FCM and ECM.

Reducing the DCAD of the diet fed prepartum usually depresses DMI (Charbonneau et al., 2006), and cows fed the diet with -130 mEq/kg consumed 1.0 kg/d less DM than cows fed the diet containing +130 mEq/kg, but the depression in DMI was attributable to the decrease of 2.2 kg/d in parous cows. Consequently, caloric intake decreased, which resulted in a less positive NE_L balance prepartum. Charbonneau et al. (2006) showed that as the DCAD decreased, so did DMI prepartum. Applying the same DCAD formula of Charbonneau et al. (2006) to the current diets would result in DCAD values of +181 and -32 mEq/kg for the positive and negative DCAD diets, a shift of 212 mEq per kg of DM. Each unit of decrease in DCAD (mEq/kg) was anticipated to reduce DMI by 0.004288 kg/d (Charbonneau et al., 2006), therefore, approximately 0.91 kg/d. This is similar to the observed 1.0 kg depression by feeding the diet with negative DCAD, from 12.4 to 11.4 kg/d. However, it remains unclear why the depression was observed only in parous cows, a 2.2 kg difference, but not in nulliparous cows. Joyce et al. (1997) showed that addition of acidogenic salts to reduce the DCAD from +350 to -70 mEq/kg reduced DMI of prepartum parous cows by approximately 2.0 kg/d prepartum. On the other hand, Moore et al. (2000) found that reducing the DCAD from +150 to -150 mEq/kg reduced DMI in nulliparous cows by 2.5 kg/d (10.5 vs. 8.0 kg/d), whereas in parous cows the reduction was only numerical and of 1.5 kg/d (14.5 to 13.0 kg/d). Likely, the reduction in DMI induced by acidogenic diets was caused by metabolic acidosis (Vagnoni and Oetzel, 1998), which was observed in the current experiment as presented by Rodney et al. (2017 – Chapter 6). Nevertheless, parous cows fed the diet with negative DCAD were still consuming on average 16.2 Mcal of NE/d at 2 d before calving, and only on the day before calving caloric intake decreased enough that resulted in an average negative NE balance. The effects of treatment on DMI were not observed during the postpartum period, although cows that received NCA had the highest intake of DM, although no statistical effect was detected.

Parous cows fed the negative DCAD had greater concentrations of NEFA prepartum than those fed the diet with positive DCAD. Also, cows fed the diet with positive DCAD had greater prepartum concentrations of glucose, insulin, and IGF-1 compared with those fed the diet with negative DCAD and the increases in insulin and IGF-1 with the positive compared with the negative DCAD diet were more exacerbated in cows supplemented with cholecalciferol. One possible explanation for the observed differences is that cows in the positive DCAD had greater DMI prepartum and, therefore, had increased absorption of short-chain fatty acids including propionate, which would stimulate hepatic uptake and gluconeogenesis, thereby increasing liver glucose output (Aschenbach et al., 2010). Increased circulating glucose stimulates insulin release by the pancreatic β-cells, and insulin has been shown to recouple the growth hormone-IGF axis, thereby stimulating IGF-1 secretion (Butler et al., 2003). Exogenous insulin suppresses lipolysis in dairy cows (Léonard and Block, 1997; Sechen et al., 1989), so endogenous increases in insulin are expected to reduce NEFA concentrations, which was observed in cows fed the diet with positive DCAD.

Another possible explanation is that diet-induced metabolic acidosis reduces blood concentrations of IGF-1 (Challa et al., 1993; Brüngger et al., 1997), although results were confounded with either depressions in DMI or loss of BW caused by metabolic acidosis. The impact of metabolic acidosis on insulin release is equivocal. In mice, metabolic alkalosis (blood pH = 7.8) reduced pancreatic insulin secretion by the perfused pancreas compared with controls (blood pH = 7.4). On the other hand, metabolic acidosis (blood pH = 7.0) either increased insulin secretion under hyperglycemia (6.6 m*M*) or attenuated insulin secretion under normoglycemia (3.3 m*M*; Reboledo et al., 1978). In dairy cows, reducing the DCAD from +143 to -405 reduced basal insulin concentrations and attenuated insulin release in response to a glucose tolerance test, although authors did not report DMI (Bigner et al., 1996). However, a reduction in DCAD from +113 to -87 mEq/kg did not influence insulin concentrations after a glucose challenge prepartum (Grünberg et al., 2011). Collectively, it seems that with the changes in DCAD imposed, the differences in insulin and IGF-1 are likely related to the changes in DMI and not acid-base status of cows. Only under very low DCAD and exacerbated metabolic acidosis, do insulin concentrations seem to change in dairy cows (Bigner et al., 1996).

The differences in glucose, insulin and IGF-1 observed prepartum were not maintained during the postpartum period likely because all cows were fed the same diet with a positive DCAD and

caloric intake did not differ between those fed the positive or the negative DCAD prepartum. On the other hand, feeding the diet with negative DCAD improved blood iCa and total Ca concentrations at the onset of lactation (Rodney et al., 2017 – Chapter 6) and reduced the prevalence of subclinical hypocalcemia in dairy cows (Martinez et al., 2017 – Chapter 8). In sheep, hypocalcemia reduces endogenous glucose production, particularly during a period of intense ketogenesis (Schlumbohm et al., 1997; Schlumbohm and Harmeyer, 2003). Also, subclinical hypocalcemia blunts insulin release in dairy cows (Martinez et al., 2014). Therefore, despite the reduced concentrations of glucose, insulin and IGF-1 prepartum in cows fed the diet with negative DCAD, it is possible that by preventing hypocalcemia, the differences observed prepartum were attenuated at the initiation of lactation, and energy metabolism improved by prevention of hypocalcemia. In fact, postpartum concentrations of energy metabolites did not differ with prepartum DCAD, which reflects the lack of differences in NE_L balance, daily BW change, and BCS in those cows. Even the smaller NE_L balance observed for cows fed calcidiol compared with cholecalciferol was not sufficient to influence concentrations of glucose, insulin and IGF-1 postpartum.

Lactation performance was only numerically influenced by prepartum DCAD. Cows fed the diet with negative DCAD produced 1.1 kg more ECM than cows fed the positive DCAD diet. Lean et al. (2014) reviewed the literature on altering the DCAD in diets fed to prepartum cows and observed an increase in production of 1.15 kg/d for the first 65 DIM with reducing the DCAD. Nevertheless, the authors identified only a limited number of experiments that used nulliparous cows and responses were heterogeneous, but feeding diets with negative DCAD to nulliparous cows resulted in a mean depression in production of 1.48 kg/d. In the present experiment, no interaction between DCAD and parity was detected, and the same numerical increases in ECM were observed with the negative DCAD diet in nulliparous (1.0 kg/d) and parous cows (1.2 kg/d). Using the mean milk response to feeding acidogenic diets prepartum of 1.15 kg/d (Lean et al., 2014), then a much larger experiment would be needed with transition cows to accommodate the intrinsic variability in production in early lactation to detect statistical effects caused by the negative DCAD. Nevertheless, the responses observed are within the expected changes to manipulating the prepartum DCAD (Lean et al., 2014).

Feeding calcidiol tended to improve yield of colostrum and improved concentrations and yields of N fractions in colostrum such as true protein, IgG, and urea N. These changes resulted in increased yields of total solids in colostrum compared with cows fed cholecalciferol. Similar to

colostrum yield, feeding calcidiol improved yields of milk, 3.5% FCM and ECM, and tended to improve yields of fat and true protein in the first 49 DIM. One of the benefits of feeding calcidiol in the present experiment was that it reduced morbidity in dairy cows (Martinez et al., 2017 – Chapter 8), and peripartum diseases are known to have devastating impacts on lactation performance, with the greater depression in yields of milk and ECM observed in the first weeks relative to the diagnosis of disease (Østergaard and Gröhn, 1999). When additional statistical analyses were performed in the current experiment including morbidity reported by Martinez et al. (2017 – Chapter 8) in the models for postpartum DMI and production of ECM, then it was clear that cows that developed clinical diseases in early lactation consumed 2.7 kg/d less DM and produced 6.3 kg/d less ECM during the experiment. Therefore, the reduction in morbidity observed in cows fed calcidiol (Martinez et al., 2017 – Chapter 8) likely explains some of the improvements in production performance.

Nevertheless, because most diseases occur in the days following calving, it is less logical that they would influence colostrum yield and composition. Therefore, in addition to the reduction in diseases, one could hypothesize that calcidiol might have had direct effects on mammary cells. Activation of vitamin D requires two sequential hydroxylations, with the second hydroxylation primarily taking place in the kidney, and carried out by CYP27B1, also known as 1α-hydroxylase (Horst et al., 1994). Recent research shows that the conversion of 25-hydroxyvitamin D_3 into 1,25-dihydroxyvitamin D_3 also takes place in cell tissues other than kidney. For instance, human mammary epithelial cells locally express *CYP27B1*, therefore they are capable of synthesizing its own 1,25-dihydroxyvitamin D_3 from 25-hydroxyvitamin D_3 (Kemmis et al., 2006). Mammary epithelial cells also express the vitamin D receptor (**VDR**). Expression of *CYP27B1* and the *VDR* increased in the murine mammary gland during pregnancy, and maximum *VDR* expression in the mammary gland was observed during lactation (Zinser and Welsh, 2004), suggesting the likelihood that local synthesis of 1,25-dihydroxyvitamin D_3 contributes to mammary development. Furthermore, VDR knockout mice exhibited impaired mammary development during pregnancy, although milk yield was not affected (Zinser and Welsh, 2004). Activation of VDR by 1,25-hydroxyvitamin D_3 has been shown to trigger extensive genomic changes in epithelial cells (Beaudina et al., 2015) and, in general, is known to contribute to control of proliferation and differentiation of mammary epithelial cells (Welsh, 2007) Although most data on the role of vitamin D on mammary cell biology has been related to tumorigenesis, one could speculate that physiological roles during preparation for a new lactation when there is a decrease in the rate of epithelial cell death relative

to epithelial cell proliferation (Sorensen et al., 2006). Bovine mammary epithelial cells are very sensitive to 1,25-dihydroxyvitamin D_3 in culture (Merriman et al., 2015), and one could speculate that increased synthesis in mammary tissue because of increased substrate availability, combine with increased Ca prepartum, in cows fed calcidiol contributed to increased colostrum and milk yield.

The 25-hydroxyvitamin D_3 has a long half-life in circulation, approximately 15 d, compared with only 2 to 3 d for vitamin D_3 (Jones, 2008), which may prolong the effects of calcidiol into the postpartum period, after cessation of supplementation. Indeed, elevated concentrations of 25-hydroxyvitamin D_3 in plasma of dairy cows supplemented with calcidiol prepartum extended at least up to 30 DIM compared with cows supplemented with cholecalciferol (Rodney et al., 2017 – Chapter 6). Perhaps, continuous autocrine and paracrine effects of mammary produced 1,25-dihydroxyvitamin D_3 might have influenced mammary cell proliferation and activity in early lactation in cows supplemented with calcidiol prepartum. Also, 1,25-dihydroxyvitamin D_3 stimulates synthesis and secretion of prolactin from pituitary, decidua and even from immune cells in rats, and from human endometrium (Delvin et al., 1990; Díaz et al., 2011). Although prolactin is not critical for milk production in dairy cattle, it has permissive effects for steroids and a surge in prolactin occurs hours before parturition (Ingalls et al., 1973), which has been demonstrated to be critical for subsequent milk yield (Akers et al., 1981). Perhaps, cows fed calcidiol had changes in prolactin secretion induced by 1,25-dihydroxyvitamin D_3 benefited milk production. Also, 1,25-dihydroxyvitamin D_3 might increase the expression of *RANKL*, which is considered an important paracrine-mediator of progesterone induced-proliferation during alveologenesis (Macias and Hinck, 2012). Collectively, the improved production of milk observed in cows supplemented with calcidiol might have been the result of reduced morbidity combined with potential paracrine and autocrine effects on mammary cells during the periparturient period.

Feeding calcidiol increased prepartum concentrations of 1,25-dihydroxyvitamin D_3 prepartum in plasma of dairy cows (Rodney et al., 2017 – Chapter 6), and calcidiol improved colostrum yield and yields of components in colostrum, particularly the protein fractions. Indeed, an interesting finding of the present experiment was that supplementation with calcidiol increased concentrations of IgG in colostrum. Among the many functions of 1,25-dihydroxyvitamin D_3 is the modulation of innate and adaptive immune responses. Calcitriol binds to VDR and recognizes cognate DNA motifs called vitamin D response elements in the nucleus of the cell,

with high affinity inducing different responses (White, 2011). For instance, in activated human B cells, 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 increased IL-10 production by 3-fold (Heine et al., 2008), and IL-10 can promote B cell differentiation into plasmablasts to secrete Ig (Defrance et al., 1992). Reinhardt et al. (1999) reported that administration of 1,25-dihydroxyvitamin D_3 to cows at the time of vaccination with *Escherichia coli* increased IgG1 antibody titers suggesting improved humoral immune response. Perhaps, cows fed calcidiol had increased serum concentrations of IgG during late gestation, concurrent with vaccinations that took place prepartum, which increased the availability of these proteins to be taken up by mammary cells and transferred into colostrum. Also, it is known that 1,25-dihydroxyvitamin D_3 improves Ca uptake by mammary cells (Mezzetti et al., 1988; Sun et al., 2016), and Rodney et al. (2017 – Chapter 6) showed that cows fed calcidiol had increased concentration and secretion of Ca in colostrum. Perhaps, the increased flux of Ca into the mammary gland to be secreted in colostrum involves an increased amount casein, which eventually results in larger amounts of proteins secreted in milk. In goats mammary cells, 1,25-dihydroxyvitamin D_3 stimulated Ca and glucose uptakes because of stimulation of cell proliferation and expression of *VDR* and genes involved in Ca uptake and transport and genes for glucose transporters (Sun et al., 2016). Therefore, it is possible that the positive effects of increased concentrations of 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 observed in cows fed calcidiol might have stimulated mammary epithelial cell function as discussed previously. Nevertheless, we are unaware of a specific role of vitamin D on transport and incorporation of proteins in bovine milk. In mice, vitamin D deficiency attenuated milk protein synthesis (Bhattacharjee et al., 1987). The authors suggested that, although vitamin D was not critical for morphological development of the mammary gland in mice, it was required for normal function and protein secretion (Bhattacharjee et al., 1987).

CONCLUSIONS

Feeding a diet with a DCAD of -130 mEq/kg of DM during the last 21 d of gestation reduced DM intake prepartum only in parous cows. The reduced DMI prepartum in cows fed the diet with negative DCAD resulted in reduced concentrations of glucose, insulin and IGF-1 in plasma prepartum. Nevertheless, cows fed the negative DCAD remained in positive energy balance until 2 d before calving. Cows fed the negative DCAD produced numerically more ECM, 1.1 kg/d, which is in line with responses reported in the literature. Feeding calcidiol in place of cholecalciferol prepartum at 3 mg/d improved yields of colostrum, milk, 3.5% FCM, and ECM

with a numerical increase DMI. Colostrum protein output, including IgG was enhanced by calcidiol. Because of the increased production, cows fed calcidiol were under more negative NE balance, although changes in BW and BCS did not differ among treatments. The greatest yields of 3.5% FCM and ECM were observed in cows fed the negative DCAD combined with calcidiol. Improvements in production in cows fed calcidiol were attributed to differences in morbidity, although the changes in colostrum yield and composition leads us to speculate that increases in 25-hydroxyvitamin D_3 in plasma pre- and postpartum in cows fed calcidiol might influence mammary biology. Further studies are warranted to investigate the underlying mechanism by which calcidiol supplementation influences production in dairy cows.

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CHAPTER EIGHT: EFFECTS OF PRE-PARTUM DIETARY CATION-ANION DIFFERENCE AND SOURCE OF VITAMIN D ON DAIRY COWS: HEALTH AND REPRODUCTIVE RESPONSES

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OVERVIEW OF CHAPTER EIGHT

This Chapter completes the series of companion papers presented in Chapters 6, 7 and 8. It focuses on the effects of vitamin D supplementation and dietary cation-anion difference in the pre-partum period on health and reproductive outcomes of peri-parturient dairy cows.

ABSTRACT

The objectives of the experiment were to evaluate the effects of feeding diets with distinct dietary cation-anion difference (**DCAD**) supplemented with two sources of vitamin D during the prepartum transition period on postpartum health and reproduction in dairy cows. The hypotheses were that feeding acidogenic diets prepartum would reduce the risk of hypocalcemia and other diseases and the benefits of a negative DCAD treatment on health are potentiated by supplementing calcidiol compared with cholecalciferol. Cows at 252 d gestation were blocked by parity (28 nulliparous and 52 parous cows) and milk yield within parous cows, and randomly assigned to one of 4 treatments arranged as a 2 x 2 factorial, with two levels of DCAD, positive (+130 mEq/kg) or negative (-130 mEq/kg) and two sources of vitamin D, cholecalciferol or calcidiol fed at 3 mg for each 11 kg of diet DM. The resulting treatment combinations were positive DCAD with cholecalciferol (**PCH**), positive DCAD with calcidiol (**PCA**), negative DCAD with cholecalciferol (**NCH**), and negative DCAD with calcidiol (**NCA**), which were fed from 252 d of gestation to calving. After calving, cows were fed the same lactation diet supplemented with cholecalciferol at 0.70 mg for every 20 kg of DM. Blood was sampled 7 d before parturition, and 2 and 7 d postpartum to evaluate cell counts and measures of neutrophil function. Postpartum clinical and subclinical diseases and reproductive responses were evaluated. Feeding a diet with negative DCAD eliminated clinical hypocalcemia (23.1 vs. 0%) and drastically reduced the incidence and daily risk of subclinical hypocalcemia, and these effects were observed in the first 48 to 72 h after calving. The diet with negative DCAD tended to improve the intensity of oxidative burst activity of neutrophils in all cows prepartum and increased the intensity of phagocytosis in parous cows prepartum and the proportion of neutrophils with killing activity in parous cows postpartum (58.5 vs. 67.6%). Feeding calcidiol improved the proportion of neutrophils with oxidative burst activity (60.0 vs. 68.7%) and reduced the incidence of retained placenta (30.8 vs. 2.5%) and metritis (46.2 vs. 23.1%), and reduced the proportion of cows with multiple diseases in early lactation. Combining the negative DCAD with calcidiol reduced morbidity by at least 60% compared with any of the other treatments. Cows with morbidity had less blood ionized and serum total Ca concentrations than healthy cows. Treatments did not affect the daily risk of hyperketonemia in the first 30 d in lactation. Despite the changes in cow health, manipulating the prepartum DCAD did not influence reproduction, but feeding calcidiol tended to increase the rate of pregnancy by 55% which reduced the median days open by 19. In conclusion, feeding prepartum cows with a diet containing a negative DCAD combined with 3 mg of calcidiol benefited health in early lactation.

Keywords: dairy cow, DCAD, hypocalcemia, vitamin D

INTRODUCTION

The onset of lactation increases irreversible losses of Ca in colostrum and milk and many cows in early lactation are unable to adapt to this sudden loss of Ca in colostrum and succumb to hypocalcemia. Although the incidence of clinical hypocalcemia has declined with the adoption of acidogenic diets to manipulate the DCAD prepartum (Block, 1984; Charbonneau et al., 2006; Lean et al., 2006), the prevalence of subclinical hypocalcemia remains elevated in dairy herds (Reinhardt et al., 2011; Chapinal et al., 2012). Hypocalcemia reduces DM intake and impairs energy metabolism and immune function (Kimura et al., 2006; Martinez et al., 2014). The changes in metabolism and immune function observed in cows that suffer from hypocalcemia likely explain the increased risk of diseases such metritis, hyperketonemia, displaced abomasum, and culling (Chapinal et al., 2011; Seifi et al., 2011; Martinez et al., 2012). Therefore, the inability to maintain proper Ca homeostasis predisposes cows to diseases beyond milk fever. On the other hand, early postpartum diseases, especially metritis, negatively affect fertility (Ribeiro et al., 2016). Low total Ca (**tCa**) concentrations during the last week prepartum and early postpartum decreases the probability of pregnancy at first AI and increases the time to pregnancy (Chapinal et al., 2012; Martinez et al., 2012). Hence, dietary strategies that mitigate the incidence of subclinical hypocalcemia might benefit health and reproduction in dairy cows.

Some of the current strategies to reduce hypocalcemia include feeding acidogenic diets prepartum and supplementation with vitamin D metabolites (Block, 1984; Thilsing-Hansen et al., 2002). It is well established that acidogenic diets markedly reduces the risk of milk fever in multiparous cows (Ender et al., 1971; Charbonneau et al, 2006; Lean et al., 2006), although the impacts on other diseases and the potential benefits to health of nulliparous cows are not well documented. The intermediate metabolite of vitamin D, 25-hydroxyvitamin D_3, also known as calcidiol, has a long half-life of approximately 15 d (Jones, 2008), and its final hydroxylation to 1,25-dihydroxyvitamin D_3 or calcitriol is catalyzed by the enzyme cytochrome p450 27B1 (**CYP27B1**) also known as 1-α hydroxylase. Because conversion of vitamin D to its active form 1,25-dihydroxyvitamin D_3 is tightly regulated by CYP27B1 via endocrine control, feeding of calcidiol provides a greater margin of safety than that of calcitriol. Early work with injectable calcidiol at 4 to 8 mg/cow as a single dose prepartum reduced the incidence of clinical hypocalcemia, particularly in those that calved within 3 and 10 d after treatment (Olson et al., 1973). Recent data demonstrated improvements in peripartum Ca metabolism in cows supplemented daily with 3 mg of calcidiol, or approximately 120,000 IU, when combined with a

diet containing a low DCAD (Wilkens et al., 2012). On the other hand, feeding twice this amount, 6 mg/d of calcidiol with a negative DCAD increased vitamin D status of cows, but did not improve Ca status or reduce hypocalcemia (Weiss et al., 2015).

We hypothesized that feeding acidogenic diets would reduce the incidence of clinical and subclinical hypocalcemia and the peripartum health benefits of a negative DCAD ration would be enhanced when supplemented with calcidiol compared with cholecalciferol. Calcidiol can more effectively increase concentrations of 25-hydroxyvitamin D_3 in plasma of cattle (Wilkens et al., 2012), which has been shown to influence innate immune response (Nelson et al., 2012). Therefore, the objectives of the experiment were to evaluate the effects of feeding diets with distinct DCAD and supplemented with two sources of vitamin D during the last 3 wk of gestation on health and reproduction in dairy cows.

MATERIALS AND METHODS

This manuscript is one of a series of three companion papers (Martinez et al., 2017; Rodney et al., 2017 – Chapters 6 and 7). The University of Florida Institutional Animal Care and Use Committee approved all procedures involving cows in the experiment under the protocol number 201408331. Throughout the manuscript, the vitamins fed will be referred as cholecalciferol and calcidiol, whereas measurements in blood plasma will be referred as vitamin D_3, 25-dihydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3.

Cows, Housing, Feeding Management, and Treatments

The experiment was conducted from February to July 2014 at the University of Florida Dairy Unit. Details of cow housing and general management are presented in Martinez et al. (2017 – Chapter 7) and Rodney et al. (2017- Chapter 6). Eighty pregnant dry Holstein cows, 28 nulliparous and 52 parous cows were enrolled in the experiment. For consistency of terminology throughout the manuscript, prepartum nulliparous, that became primiparous cows postpartum, and prepartum parous, that became postpartum multiparous cows, were designated as nulliparous and parous, respectively. Cows were fed once daily prepartum and twice daily postpartum and amounts offered and refused were measured daily. Description of diets is presented in Table 1 and details of feed ingredients and chemical analyses are presented elsewhere (Martinez et al., 2017- Chapter 7).

Table 1. Dietary ingredients and nutrient composition of diets fed pre- and postpartum

	Prepartum diets[1]				
	Positive DCAD		Negative DCAD		
Item	Cholecalciferol	Calcidiol	Cholecalciferol	Calcidiol	Postpartum diet
Ingredients, % of DM					
Corn silage	61.80	61.80	61.80	61.80	25.8
Bermuda hay	9.10	9.10	9.10	9.10	7.5
Brewer's grains, wet	---	---	---	---	8.6
Corn grain, finely ground	---	---	---	---	25.9
Citrus pulp	9.10	9.10	9.10	9.10	5.2
Soybean hulls	---	---	---	---	8.6
Whole cottonseed	6.40	6.40	6.40	6.40	3.4
Soybean meal, solvent extract	---	---	4.50	4.40	8.2
Soybean meal, cooker-processing[2]	11.18	11.08	---	---	3.3
Acidogenic supplement[3]	---	---	7.25	7.25	--
Cholecalciferol mixture[4]	0.08	---	0.08	---	--
Calcidiol mixture[5]	---	0.18	---	0.18	--
MgO + NaCl	0.54	0.54	---	---	---
Prepartum mineral[6]	1.80	1.80	1.80	1.80	--
Postpartum protein and mineral[7]	---	---	---	---	3.5
DM, %	55.4 ± 1.0	55.6 ± 1.0	55.4 ± 1.0	55.4 ± 1.0	69.5 ± 0.6
Nutrients, DM basis (± SD)[8]					
Net energy,[9] Mcal/kg	1.65	1.65	1.65	1.65	1.67
OM, %	94.0 ± 0.4	93.9 ± 0.4	94.2 ± 0.4	94.1 ± 0.4	94.0 ± 0.1
CP, %	13.5 ± 0.3	12.9 ± 0.3	13.5 ± 0.3	13.4 ± 0.3	15.7 ± 0.6
Starch, %	20.2 ± 0.2	20.1 ± 0.2	20.8 ± 0.2	20.9 ± 0.2	27.6 ± 1.0
Non-fibrous carbohydrates,[10] %	38.7 ± 1.1	38.1 ± 1.1	38.3 ± 1.1	38.5 ± 1.1	40.8 ± 1.2
NDF, %	37.8 ± 0.6	39.0 ± 0.6	38.3 ± 0.6	38.2 ± 0.6	33.3 ± 0.5
NDF from forage, %	30.8 ± 0.7	30.8 ± 0.7	30.8 ± 0.7	30.8 ± 0.7	15.8 ± 0.4
Fatty acids, %	3.28 ± 0.03	3.33 ± 0.03	3.45 ± 0.03	3.37 ± 0.03	3.93 ± 0.22
Ca, %	0.61 ± 0.08	0.62 ± 0.08	0.54 ± 0.08	0.55 ± 0.08	0.59 ± 0.03
P, %	0.32 ± 0.01	0.31 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	0.36 ± 0.01
Mg, %	0.39 ± 0.02	0.37 ± 0.02	0.38 ± 0.02	0.39 ± 0.02	0.27 ± 0.01
K, %	1.22 ± 0.08	1.19 ± 0.08	1.15 ± 0.08	1.15 ± 0.08	1.15 ± 0.06
Na, %	0.20 ± 0.01	0.20 ±0.01	0.16 ± 0.01	0.16 ± 0.01	0.46 ± 0.04
Cl, %	0.54 ± 0.04	0.55 ± 0.04	0.94 ± 0.04	0.90 ± 0.04	0.30 ± 0.01
S, %	0.17 ± 0.004	0.16 ± 0.004	0.37 ± 0.004	0.36 ± 0.004	0.18 ± 0.01
DCAD,[11] mEq/kg	145 ± 11	130 ± 119	-129 ± 11	-124 ± 11	293 ± 28

[1] Prepartum cows starting at 252 d of gestation were fed diets with either a positive (+130 mEq/kg) or a negative (-130 mEq/kg) dietary cation-anion difference (DCAD). Within each DCAD diet, cows were fed either 3 mg of cholecalciferol or 3 mg of calcidiol.
[2] Amino Plus (cooker-processing soybean meal; Ag Processing Inc., Emmetsburg, IA).
[3] Bio-Chlor (a fermentation product containing dried condensed extracted glutamic acid fermentation product, dried condensed corn fermentation solubles, processed grain by-products, and magnesium chloride; Arm & Hammer Animal Nutrition, Princeton, NJ).
[4] Rovimix D3 (a product containing 300 mg of cholecalciferol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ).
[5] Hy-D (a product containing 153 mg of calcidiol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ).
[6] Each kg contains (DM basis) 10.3% Ca, 0.7% P, 4.0% Mg, 0.9% K, 0.25% S, 1.8% Na, 2.7% Cl, 1,750 mg Zn, 600 mg Cu, 1,090 mg Mn, 21 mg Se, 75 mg Co, 21 mg I, 260,000 IU of vitamin A, and 7,500 IU of vitamin E.
[7] A supplement containing 30% blood meal enriched with rumen-protected lysine and methionine (LysAAMet, Perdue Ag Solutions, LLC, Salisbury, MD). Each kg contains (DM basis) 26.4% CP, 5.1% Ca, 1.6% P, 4.1% Mg, 6.8% K, 0.3% S, 10.7% Na, 2.5% Cl, 665 mg Zn, 230 mg Cu, 416 mg Mn, 7.2 mg Se, 24 mg Co, 13.6 mg I, 110,000 IU of vitamin A, 33,000 IU of cholecalciferol (0.825 mg), 1,100 IU of vitamin E, and 460 mg of monensin (Rumensin 90, Elanco Animal Health, Eli Lilly and Co, Indianapolis, IN).
[8] Samples collected weekly and composited monthly for chemical analyses.
[9] Calculated based on the chemical analysis of dietary ingredients and using the NRC (2001) for a DM intake of 12.0 kg/d prepartum and 18 kg/d postpartum.
[10] Calculated using the equation DM – [(CP + NDF + fat + ash – (NDF insoluble protein)].
[11] Calculated using the equation [(mEq of Na + mEq of K) - (mEq of Cl + mEq of S)].

The experiment followed a randomized complete block design with cow as the experimental unit. Weekly cohorts of cows at 252 d of gestation were blocked by lactation number (0 vs. > 0) and previous lactation 305-d milk for parous cows and, within each block, assigned randomly to one of the four treatments. Treatments were arranged as a factorial with two levels of DCAD, positive (+130 mEq/kg) or negative (-130 mEq/kg), and two sources of vitamin D, cholecalciferol or calcidiol that were fed at 3 mg for each 11 kg of diet DM. The amount of vitamin D selected to be fed was based on the work of Wilkens et al. (2012) who fed quantities above current guidelines established by the NRC (2001). Therefore, the four treatments were positive DCAD with cholecalciferol (**PCH**), positive DCAD with calcidiol (**PCA**), negative DCAD with cholecalciferol (**NCH**), and negative DCAD with calcidiol (**NCA**). Treatment diets were fed from 252 d of gestation to calving. Upon calving, cows were fed the same lactation ration for the first 49 DIM. All diets were fed as TMR.

Blood Samples

Blood was sampled 3 times per week from 265 d of gestation until calving, and postpartum, on d 0, 1, 2, and 3, and then every 3-d postpartum until 30 DIM, by puncture of the coccygeal vein or artery into evacuated tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing no anticoagulant agent for serum separation or in tubes containing K_2 EDTA for plasma separation. Tubes with no anticoagulant were left at room temperature to clot and then placed on ice until processing. Tubes were centrifuged and aliquots of serum and plasma were frozen at -20°C until analysis of tCa and BHB, respectively. Concentration of BHB in plasma was measured using a commercial kit (Wako Autokit 3-HB; Wako Diagnostics, Inc., Richmond, VA) per manufacturer's guideline. The intra- and inter-assays CV were, respectively, 5.9 and 9.8%. Concentration of tCa in serum was analyzed using an atomic absorption spectrophotometer (AAnalyst 200, Perkin-Elmer Inc., Waltham, MA) as described previously (Martinez et al., 2012). The and intra- and inter-assays CV were, respectively, 1.8 and 2.0%. For the prepartum period, samples collected on d -9, -6, -3, and -1 relative to calving were analyzed. Additional whole blood was sampled on d -9, -6, -3, -1, 0, 1, 2, 3, and 6 relative to calving and analyzed for concentrations of ionized Ca (**iCa**) using a handheld analyzer (VetScan i-STAT, Abaxis, Union City, CA).

Differential Leukocyte Count and Assay for Neutrophil Function

Whole blood was collected at 269 d of gestation, which averaged 7.0 ± 3.4 d prepartum, and again at 2 and 7 DIM and analyzed for total and differential leukocyte counts using an automated

hematology analyzer (ProCyte Dx Hematology Analyzer, IDDEX Laboratories, Westbrook, ME). The percentage of neutrophils exhibiting phagocytosis and oxidative burst activities of labeled *Escherichia coli* was measured *in vitro* on d -7, 2 and 7 relative to calving according to procedures described in detail by Martinez et al. (2012, 2014). The responses quantified were the percentage of neutrophils containing phagocytized propidium iodide-labeled *E. coli*, the percentage of neutrophils with oxidative burst activity, the mean fluorescence intensity for phagocytosis as a proxy for the number of bacteria phagocytized per neutrophil, and the mean fluorescence intensity for oxidative burst, as an indicator of the amount of oxygen reactive species generated per neutrophil.

Definition and Diagnosis of Clinical and Subclinical Diseases and Survival

A complete physical examination of all cows was performed at 4, 7, and 12 DIM. In addition, cows were observed daily for the first 30 DIM and any abnormal symptom or a substantial decrease in DM intake or milk yield resulted in cows undergoing further physical examinations for the diagnosis of clinical diseases. Dystocia was recorded when calving assistance lasted longer than 15 min. Retained placenta was diagnosed in cows that failed to expel the fetal membranes within 12 h after delivery of the calf. Clinical hypocalcemia was diagnosed when a cow was unable to rise and confirmed by blood iCa < 0.80 m*M*. Metritis was diagnosed based on transrectal palpation of a flaccid enlarged uterus with the presence of watery, fetid, reddish/brownish discharge. Mastitis was diagnosed based on visible abnormalities in the milk. Displacement of the abomasum was diagnosed based on auscultation and percussion of the flank and confirmed by laparotomy that was used for surgical correction of the disease. Morbidity was considered when a cow had one of more clinical diseases that included retained placenta, clinical hypocalcemia, metritis, mastitis, or displaced abomasum in the first 30 DIM. Cow survival was evaluated up to 305 DIM.

Subclinical metabolic diseases evaluated were hypocalcemia and ketosis. Three distinct thresholds were selected to define subclinical hypocalcemia using either whole blood iCa ≤ 1.0 m*M* (Oetzel et al., 1988), or serum tCa ≤ 2.0 m*M* (Reinhardt et al., 2011) or tCa < 2.15 m*M* (Martinez et al., 2012). Hyperketonemia was defined as serum BHB concentrations greater than 1.20 m*M* in at least one day on days 0, 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 DIM based on the threshold used by others (McArt et al., 2011; Martinez et al., 2016).

Reproductive Management and Reproductive Responses

All cows had their estrous cycles presynchronized with 2 i.m. injections of 25 mg of $PGF_{2\alpha}$ (Lutalyse Sterile Solution, 5 mg/mL dinoprost as tromethamine salt; Zoetis, Florham Park, NJ) administered 14 d apart, at 41 ± 3 and 55 ± 3 DIM. Cows were then enrolled in the Ovsynch protocol at 67 ± 3 DIM. The protocol consisted of an i.m. injection of 100 μg of GnRH (Factrel, 50 μg/mL gonadorelin hydrochloride, Zoetis) at 67 ± 3 DIM, followed by an injection of $PGF_{2\alpha}$ at 74 ± 3 DIM, and a final injection of GnRH 56 h after the $PGF_{2\alpha}$. Cows were inseminated approximately 16 h after the GnRH, at 77 ± 3 DIM. Pregnancy was diagnosed on d 32 after the first AI based on the presence of an amniotic vesicle with an embryo with heartbeat by transrectal ultrasonography. All cows received GnRH on d 25 after each AI and those nonpregnant on d 32 completed the Ovsynch protocol for reinsemination. Pregnant cows were reexamined for pregnancy by transrectal palpation on d 70 of gestation. Pregnancy loss between d 32 and 70 of gestation was recorded. Interval to pregnancy up to 305 DIM was also recorded. Cows that became "do not inseminate", were sold or died, or remained nonpregnant by 305 DIM were censored. Responses measured included pregnancy at first AI and interval to pregnancy.

Statistical Analysis

The experiment followed a randomized complete block design with cow as the experimental unit. Prepartum cows at 252 d of gestation were blocked by parity as nulliparous or parous and previous lactation 305-d milk yield for parous cows and, within each block, they were assigned randomly to one of the four treatments. Therefore, 7 blocks of with 4 nulliparous cows each and 13 blocks with 4 parous cows each were enrolled in the experiment.

Normality of residuals and homogeneity of variance were examined for each continuous dependent variable analyzed after model fitting. Responses without normal distribution had data transformed according to the power transformation suggested by the Box-Cox procedure (Box and Cox, 1964) using the PROC TRANSREG in SAS (SAS/STAT, SAS Institute Inc.) before final analyses. For transformed data, the LSM were back transformed and the respective SEM were calculated (Jørgensen and Pedersen, 1998).

Continuous data were analyzed with the MIXED procedure of SAS and the statistical models included the fixed effects of DCAD (positive vs. negative), vitamin D (cholecalciferol vs. calcidiol), parity (nulliparous vs. parous), and the two- and three-way interactions between DCAD, vitamin D, and parity, and the random effect of block. For responses with repeated

measures within the same experimental unit, then the models also included the fixed effects of day, and the interactions of DCAD and day, vitamin D and day, parity and day, DCAD and parity and day, vitamin D and parity and day, and DCAD and vitamin D and parity and day, and the random effect cow nested within level of DCAD and source of vitamin D. The Kenward-Roger method was used to approximate the denominator degrees of freedom for the F tests in the statistical models. Model fit was assessed based on the smallest corrected Akaike's information criterion. For repeated measures, the covariance structure was selected for each model based on spacing of measurements and the smallest corrected Akaike's information criterion. When an interaction was significant, pairwise comparisons were performed with the adjustment by Tukey. Additional statistical analyses were performed for concentrations of whole blood iCa and serum total tCa for the pre- and postpartum periods separately with the MIXED procedure of SAS according to the same model described previously, but also including morbidity (yes vs. no) and the interaction between morbidity and day of blood sampling.

Categorical data were analyzed by logistic regression using the GLIMMIX procedure of SAS with either binary or binomial distributions. The models included the fixed effects of level of DCAD, source of vitamin D, interaction between level of DCAD and source of vitamin D, and parity, and the random effect of block.

Time to an event such as pregnancy or leaving the herd was analyzed with the Cox's proportional hazard regression using the PHREG procedure of SAS. The model included the fixed effects of level of DCAD, source of vitamin D, interaction between DCAD and vitamin D, and parity. When the interaction between DCAD and vitamin D was nonsignificant ($P > 0.10$), it was dropped from the final model. The adjusted hazard ratio (**HR**) and the 95% CI were calcualted.

Statistical significance was considered at $P \leq 0.05$, and tendency was considered at $0.05 < P \leq 0.10$.

RESULTS

Twenty-eight nulliparous and 52 parous cows were enrolled in the experiment, but one parous cow fed PCH was removed from the data analyses because of diagnosis of lymphosarcoma during the prepartum period. Therefore, 79 cows were included in all statistical analyses. One

PCA cow that developed clinical hypocalcemia received i.v. Ca borogluconate solution and an oral Ca drench, but developed aspiration pneumonia and had to be euthanized and was removed prematurely from the experiment and contributed with data from enrollment to 2 DIM. The length of gestation (± SD) was 275 ± 4.4 d and days in the prepartum diets did not differ with treatments and averaged 22.7 ± 5.3. All cows in the experiment stayed a minimum of 14 d in the prepartum diets and one cow stayed a maximum of 34 d.

Details of concentrations of vitamin D metabolites and minerals in blood according to treatments are reported elsewhere (Rodney et al., 2017 – Chapter 6). Briefly, feeding cholecalciferol increased ($P < 0.001$) the concentrations of vitamin D_3 in plasma pre- (cholecalciferol = 14.7 vs. calcidiol = 1.1 ± 0.6 ng/mL) and postpartum (cholecalciferol = 5.6 vs. calcidiol = 1.4 ± 0.3 ng/mL), whereas feeding calcidiol increased ($P < 0.001$) the concentrations of 25-hydroxyvitamin D_3 in plasma pre- (cholecalciferol = 59.7 vs. calcidiol = 237.0 ± 6.8 ng/mL) and postpartum (cholecalciferol = 58.5 vs. calcidiol = 218.3 ± 5.3 ng/mL). Feeding the diet with negative DCAD reduced ($P < 0.05$) the concentrations of vitamin D_3 and 25-hydroxyvitamin D_3 pre- and postpartum compared with feeding the diet with positive DCAD (Rodney et al., 2017 – Chapter 6). At calving and on day 1 postpartum, concentrations of whole blood iCa increased ($P < 0.001$) with feeding the diet with negative compared with positive DCAD (positive = 0.968 vs. negative = 1.110 ± 0.008 m*M*), but source of vitamin D did not influence iCa concentrations in those days (cholecalciferol =1.042 vs. calcidiol = 1.035 ± 0.008 m*M*). Similarly, at calving and on day 1 postpartum, concentrations of tCa in serum increased ($P < 0.001$) with feeding the diet with negative compared with positive DCAD (positive = 1.964 vs. negative = 2.181 ± 0.02 m*M*), but source of vitamin D did not influence tCa concentrations in those days (cholecalciferol =2.085 vs. calcidiol = 2.060 ± 0.02 m*M*).

Total and Differential Leukocyte Counts and Neutrophil Function

The concentration of leukocytes in blood decreased ($P < 0.01$) after parturition, mainly associated with a reduction ($P < 0.01$) in circulating neutrophils and a slight reduction ($P = 0.09$) in the circulating lymphocytes. Pre- and postpartum concentrations of total leukocytes, neutrophils, lymphocytes, and monocytes in blood were not affected by prepartum DCAD or source of vitamin D (Tables 2 and 3).

Table 2. Effect of dietary cation-anion difference (DCAD) and source of vitamin D (VitD) fed prepartum on leukocyte counts and neutrophil function prepartum

	Positive DCAD		Negative DCAD			*P*-value[2]		
	CH	CA	CH	CA	SEM	DCAD	VitD	DCAD x VitD
Leukocytes, x 10^3/µL								
Total	13.1	12.7	14.2	13.8	1.8	0.50	0.80	0.97
Neutrophils	4.73	4.86	4.46	4.95	0.40	0.82	0.41	0.63
Lymphocytes	6.00	5.74	7.03	6.15	1.19	0.53	0.62	0.80
Monocytes	1.62	1.62	1.81	1.71	0.23	0.51	0.81	0.81
Neutrophil function								
Phagocytosis, % PMN	72.2	68.1	73.8	71.9	3.9	0.19	0.12	0.93
Oxidative burst, % PMN	57.4	57.8	63.3	60.9	4.6	0.31	0.60	0.75
Phagocytosis MFI[3], Log_{10}	4.86	4.86	4.88	4.89	0.04	0.41	0.81	0.72
Oxidative burst MFI, Log_{10}	4.56	4.55	4.65	4.62	0.05	0.07	0.72	0.88

[1] Prepartum cows at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

[2] DCAD = effect of level of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between level of DCAD and source of vitamin D

[3] MFI = mean fluorescence intensity of the red (indicator of number of bacteria phagocytized per neutrophil) and green dyes (indicator of intensity of oxidative burst produced per neutrophil).

Table 3. Effect of dietary cation-anion difference (DCAD) and source of vitamin D (VitD) fed prepartum on leukocyte count and neutrophil function postpartum

	Positive DCAD		Negative DCAD			*P*-value[2]		
	CH	CA	CH	CA	SEM	DCAD	VitD	DCAD x VitD
Leukocytes, x 10^3/µL								
Total	11.2	12.0	13.3	12.7	1.5	0.31	0.89	0.62
Neutrophils	3.39	4.01	4.03	4.26	0.38	0.24	0.27	0.61
Lymphocytes	5.46	5.77	6.89	5.87	1.09	0.51	0.82	0.57
Monocytes	1.89	1.57	1.76	1.66	0.21	0.96	0.28	0.59
Neutrophil function								
Phagocytosis, % PMN	73.7	75.4	72.9	76.7	2.9	0.99	0.49	0.82
Oxidative burst, % PMN	58.5	67.4	61.7	70.1	3.5	0.36	< 0.01	0.94
Phagocytosis MFI[3], Log_{10}	4.94	4.96	4.96	4.96	0.03	0.74	0.82	0.73
Oxidative burst MFI, Log_{10}	4.77	4.75	4.79	4.69	0.05	0.67	0.17	0.34

[1] Prepartum cows at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

[2] DCAD = effect of level of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between level of DCAD and source of vitamin D.

[3] MFI = mean fluorescence intensity of the red (indicator of number of bacteria phagocytized per neutrophil) and green dyes (indicator of intensity of oxidative burst produced per neutrophil).

Treatments did not affect the percentages of neutrophils prepartum with phagocytosis or displaying oxidative burst (Table 2). The intensity of phagocytosis measured prepartum did not differ with level of DCAD or source of vitamin D fed; however, a tendency for interaction (P = 0.07) between level of DCAD and parity was observed for intensity of phagocytosis prepartum because feeding the diet with negative DCAD improved (P = 0.04) phagocytic MFI in parous cows, but no difference was observed in nulliparous cows (Table 4). Feeding the negative DCAD diet also tended (P = 0.07) to increase prepartum intensity of oxidative burst of neutrophils compared with the positive DCAD diet. No interactions between level of DCAD or source of vitamin D were observed for the measures of neutrophil function prepartum (Table 2).

Treatment or interaction between treatment and parity did not affect the proportions of neutrophils displaying phagocytosis postpartum (Tables 3 and 4). Phagocytosis tended (P = 0.10) to increase with day in the experiment (Figure 1A). The percentage of neutrophils with oxidative burst activity increased (P < 0.01) in cows fed calcidiol compared with cholecalciferol (Table 3). Also, a tendency for interaction (P = 0.06) between level of DCAD and parity was observed for oxidative burst because feeding the negative DCAD increased (P = 0.02) the percentage of neutrophils with oxidative burst postpartum compared with feeding the positive DCAD in parous cows, but not in nulliparous (Table 4). The proportion of neutrophils with oxidative burst activity increased (P = 0.02) with day in the experiment (Figure 1B). The intensity of neutrophil phagocytosis or oxidative burst measured postpartum did not differ with level of DCAD or source of vitamin D (Table 3).

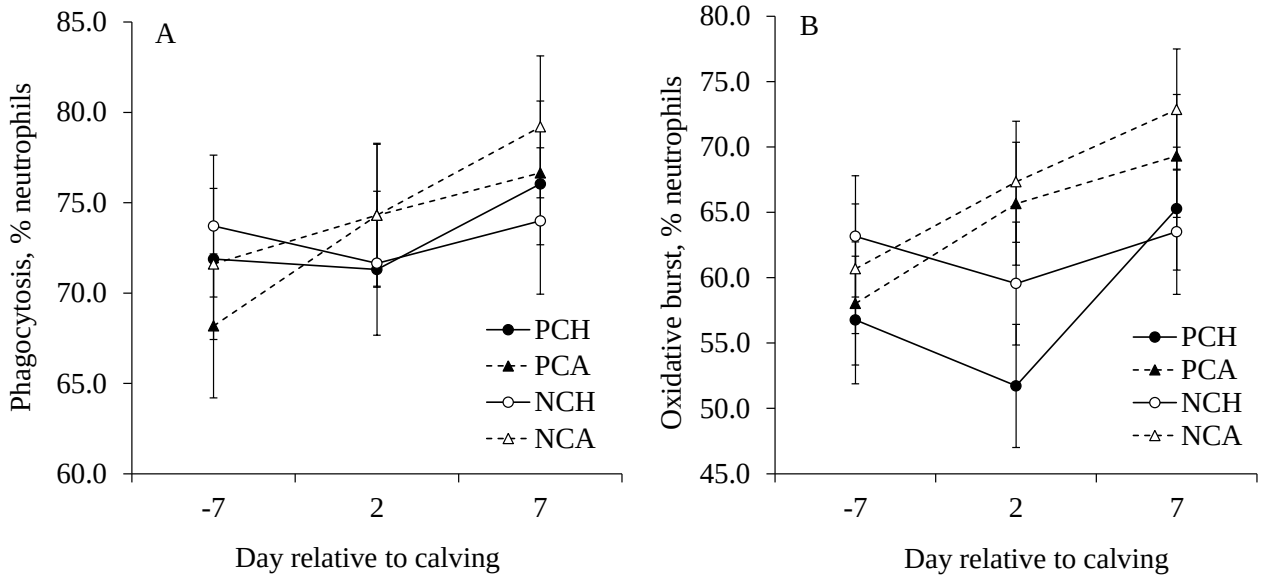

Figure 1. Percent of neutrophils with phagocytic (Panel A) and oxidative burst (Panel B) activities in cows fed prepartum diets containing either positive (P, +130 mEq/kg) or negative (N, -130 mEq/kg) DCAD and supplemented with either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). For phagocytosis, effects of level of DCAD (P = 0.47), source of vitamin D (P = 0.83), and interaction between level of DCAD and source of vitamin D (P = 0.91). For oxidative burst, effects of level of DCAD (P = 0.20), source of vitamin D (P = 0.05), and interaction between level of DCAD and source of vitamin D (P = 0.75). Error bars represent the SEM.

Table 4. Effect of dietary cation-anion difference (DCAD) fed prepartum and parity on neutrophil function pre- and postpartum

	Nulliparous		Parous			*P*-value[2]		
Neutrophil function	Positive	Negative	Positive	Negative	SEM	DCAD	Parity	DCAD x Parity
Prepartum								
Phagocytosis, % PMN	71.2	74.2	69.1	71.5	4.0	0.19	0.46	0.72
Oxidative burst, % PMN	58.6	62.7	56.6	61.5	4.7	0.31	0.60	0.71
Phagocytosis MFI[3], Log_{10}	4.90	4.87	4.82[b]	4.90[a]	0.04	0.41	0.72	0.07
Oxidative burst MFI, Log_{10}	4.54	4.62	4.56	4.65	0.06	0.07	0.69	0.91
Postpartum								
Phagocytosis, % PMN	77.9	75.0	71.2	74.7	3.0	0.99	0.31	0.32
Oxidative burst, % PMN	67.4	64.2	58.5[b]	67.6[a]	3.7	0.36	0.53	0.06
Phagocytosis MFI[3], Log_{10}	5.00	4.97	4.91	4.94	0.03	0.74	0.21	0.29
Oxidative burst MFI, Log_{10}	4.76	4.70	4.76	4.78	0.05	0.67	0.49	0.33

[1] Prepartum cows at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol or 3 mg of calcidiol.

[2] DCAD = effect of level of DCAD (positive vs. negative); Parity = effect of parity (nulliparous vs. parous); DCAD x parity = interaction between level of DCAD and parity.

[3] MFI = mean fluorescence intensity of the red (indicator of number of bacteria phagocytized per neutrophil) and green dyes (indicator of intensity of oxidative burst produced per neutrophil).

[a,b] Within parity, distinct superscripts within the same row indicate difference ($P < 0.05$).

Clinical Diseases and Morbidity

Prepartum level of DCAD or source of vitamin D did not influence dystocia, which affected 21.1% of PCH, 15.0% PCA, 20.0% NCH, and 25.0% NCA cows, resulting in an overall incidence of 20.3% in the experimental cows.

Prepartum DCAD did not affect the incidence of retained placenta, but feeding calcidiol compared with cholecalciferol reduced ($P < 0.01$) the incidence from 30.8 to 2.5% (Table 5). The 0% incidence of retained placenta in cows fed NCA resulted in no estimable interaction between level of DCAD and source of vitamin D.

All 9 cases of clinical hypocalcemia, or milk fever, affected parous cows fed the positive DCAD treatments (Table 5). Source of vitamin D did not affect the incidence of clinical hypocalcemia, but feeding the diets with negative DCAD reduced ($P < 0.01$) the incidence from 23.1 to 0%. Feeding a diet with negative DCAD only numerically reduced in incidence from 42.1 to 27.5%. Similar to what was observed for retained placenta, feeding calcidiol compared with cholecalciferol reduced ($P = 0.04$) the incidence of metritis by half, from 46.2 to 23.1%. No treatment effect was observed for the incidences of displaced abomasum and mastitis in the first 30 DIM (Table 5).

Feeding the negative compared with the positive DCAD diet reduced ($P = 0.05$) morbidity from 56.4 to 35.0% (Table 5). A tendency for interaction ($P = 0.08$) between level of DCAD and source of vitamin D was observed for morbidity because the benefit of the negative DCAD diet was greater when combined with calcidiol. Almost 27% of the cows were diagnosed with more than 1 clinical disease in the first 30 DIM. Feeding calcidiol tended to reduce ($P = 0.06$) the proportion of cows with multiple diseases compared with feeding cholecalciferol, and the lowest incidence of multiple diseases was observed in cows fed NCA.

Concentrations of iCa in whole blood prepartum in cows subsequently considered healthy or with morbidity did not differ (Figure 2A); however, whole blood concentrations of iCa from 0 to 6 DIM were less ($P = 0.04$) for cows with morbidity than healthy cows (1.040 ± 0.011 vs. 1.074 ± 0.011 m*M*). On the other hand, tCa concentrations in serum of cows with morbidity were less prepartum (2.350 ± 0.034 vs. 2.443 ± 0.032 m*M*; $P = 0.05$) and postpartum (2.089 ± 0.023 vs. 2.211 ± 0.022 m*M*; $P < 0.001$) compared with healthy cows.

Table 5. Effect of dietary cation-anion difference (DCAD) and source of vitamin D (VitD) fed prepartum[1] on postpartum diseases

		Positive DCAD		Negative DCAD		*P*-value[2]		
	Incidence, % (n/n)	CH	CA	CH	CA	DCAD	VitD	DCAD x VitD
Clinical diseases								
Retained placenta	16.5 (13/79)	31.6	5.0	30.0	0.0	0.61	<0.01	NE[3]
Hypocalcemia	11.4 (9/79)	15.8	30.0	0.0	0.0	<0.01	0.32	NE
Metritis	34.6 (27/78)	52.6	31.6	40.0	15.0	0.16	0.03	0.66
Displaced abomasum	5.1 (4/79)	0.0	10.5	10.0	0.0	0.96	1.00	NE
Mastitis	12.8 (10/78)	5.3	15.8	10.0	15.0	0.66	0.28	0.63
Morbidity	45.6 (36/79)	52.6	60.0	50.0	20.0	0.05	0.27	0.08
Multiple diseases	26.6 (21/79)	36.8	25.0	35.0	10.0	0.29	0.06	0.37
Subclinical diseases								
Hypocalcemia tCa ≤ 2.0 mM	39.3 (31/79)	63.2	55.0	10.0	30.0	0.001	0.37	0.14
Hypocalcemia iCa < 2.15 m*M*	54.4 (43/79)	80.0	65.0	30.0	45.0	0.004	0.96	0.18
Hypocalcemia iCa ≤ 1.0 m*M*	59.5 (47/79)	68.4	85.0	40.0	45.0	0.004	0.27	0.47
Hyperketonemia BHB > 1.2 m*M*	66.7 (52/78)	63.2	73.7	55.0	75.0	0.62	0.09	0.59
Left herd by 305 DIM	22.8 (18/79)	26.3	20.0	20.0	25.0	0.94	0.93	0.54

[1] Prepartum cows at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

[2] DCAD = effect of level of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between level of DCAD and source of vitamin D.

[3] Not estimable because of data separation.

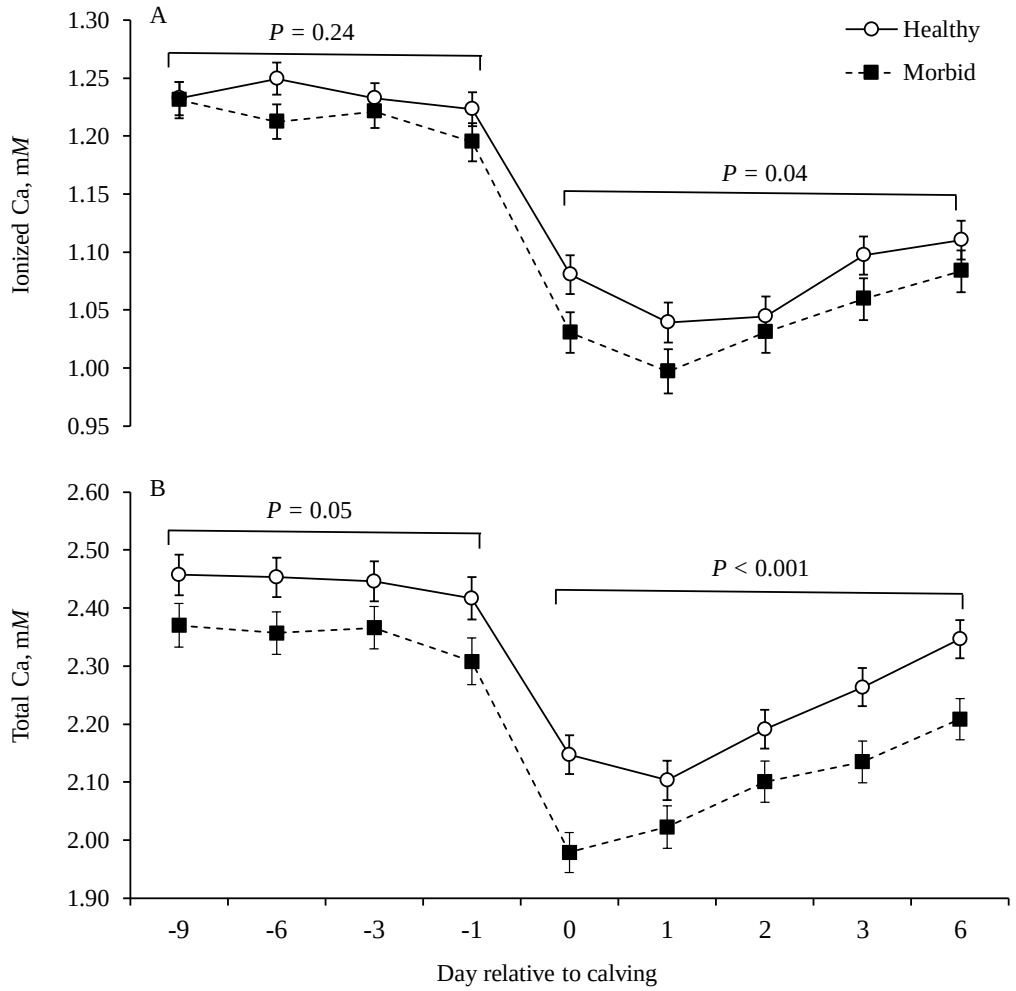

Figure 2. Concentrations of ionized Ca in whole blood (A) and total Ca in serum (B) of dairy cows according to morbidity. For ionized Ca prepartum, effect of morbidity (P = 0.24) and interaction between morbidity and day (P = 0.26). For ionized Ca postpartum, effect of morbidity (P = 0.04) and interaction between morbidity and day (P = 0.77). For total Ca prepartum, effect of morbidity (P = 0.05) and interaction between morbidity and day (P = 0.88). For total Ca postpartum, effect of morbidity (P < 0.001) and interaction between morbidity and day (P = 0.56). Error bars represent SEM.

Subclinical Diseases

Incidence of subclinical hypocalcemia changed with threshold selected and it was smallest when based on serum tCa ≤ 2.0 m*M*, followed by serum tCa < 2.15 m*M*, and then by whole blood iCa ≤ 1.0 m*M* (Table 5). Nevertheless, the effects of DCAD and vitamin D on incidence of subclinical hypocalcemia did not differ with threshold selected. Feeding a diet with negative DCAD markedly reduced ($P < 0.01$) the incidence of subclinical hypocalcemia from 0 to 3 DIM. No nulliparous cow was diagnosed with subclinical hypocalcemia when the threshold was tCa ≤ 2.0. Nevertheless, when the threshold was iCa ≤ 1.0 m*M*, then the diet with negative DCAD reduced the incidence of subclinical hypocalcemia in both nulliparous (positive = 42.9 vs. negative = 7.1%) and parous cows (positive = 96.0 vs. negative = 61.5%). Source of vitamin D did not affect the incidence of subclinical hypocalcemia irrespective of the threshold used and no interaction between DCAD and vitamin D was observed. Similar to the incidence, the daily risk of subclinical hypocalcemia based on tCa ≤ 2.0 m*M* from calving to 3 DIM decreased ($P < 0.001$) 5-fold with feeding the negative compared with the positive DCAD treatments (positive = 25.3 vs. negative = 5.7%; Figure 3A). The reduction in daily prevalence by feeding the diet with negative DCAD was observed on d 0 to 3 postpartum when the threshold was tCa ≤ 2.0 m*M* (Figure 3C) and on days 0 and 1 postpartum when the threshold was iCa ≤ 1.0 m*M* (Figure 3D). Moreover, the benefits of the negative DCAD in reducing the daily risk of subclinical hypocalcemia based on iCa ≤ 1.0 mM was observed in both nulliparous (positive = 9.3 vs. negative = 1.2%) and parous cows (positive = 58.9 vs. negative = 26.5%). No effects of source of vitamin D or interaction between level of DCAD and source of vitamin D were observed for the daily risk of subclinical hypocalcemia.

Hyperketonemia based on serum BHB concentrations above 1.20 m*M* in at least one day in the first 30 DIM affected 66.7% of the cows in the experiment (Table 5). Level of DCAD did not affect the incidence of hyperketonemia, but feeding calcidiol tended to increase ($P = 0.09$) the percentage of cows diagnosed with hyperketonemia compared with feeding cholecalciferol (59.0 vs. 74.4%). Nevertheless, treatments had no effect on the daily risk of hyperketonemia during the first 30 DIM (Figure 3B), which averaged 12% of the cows.

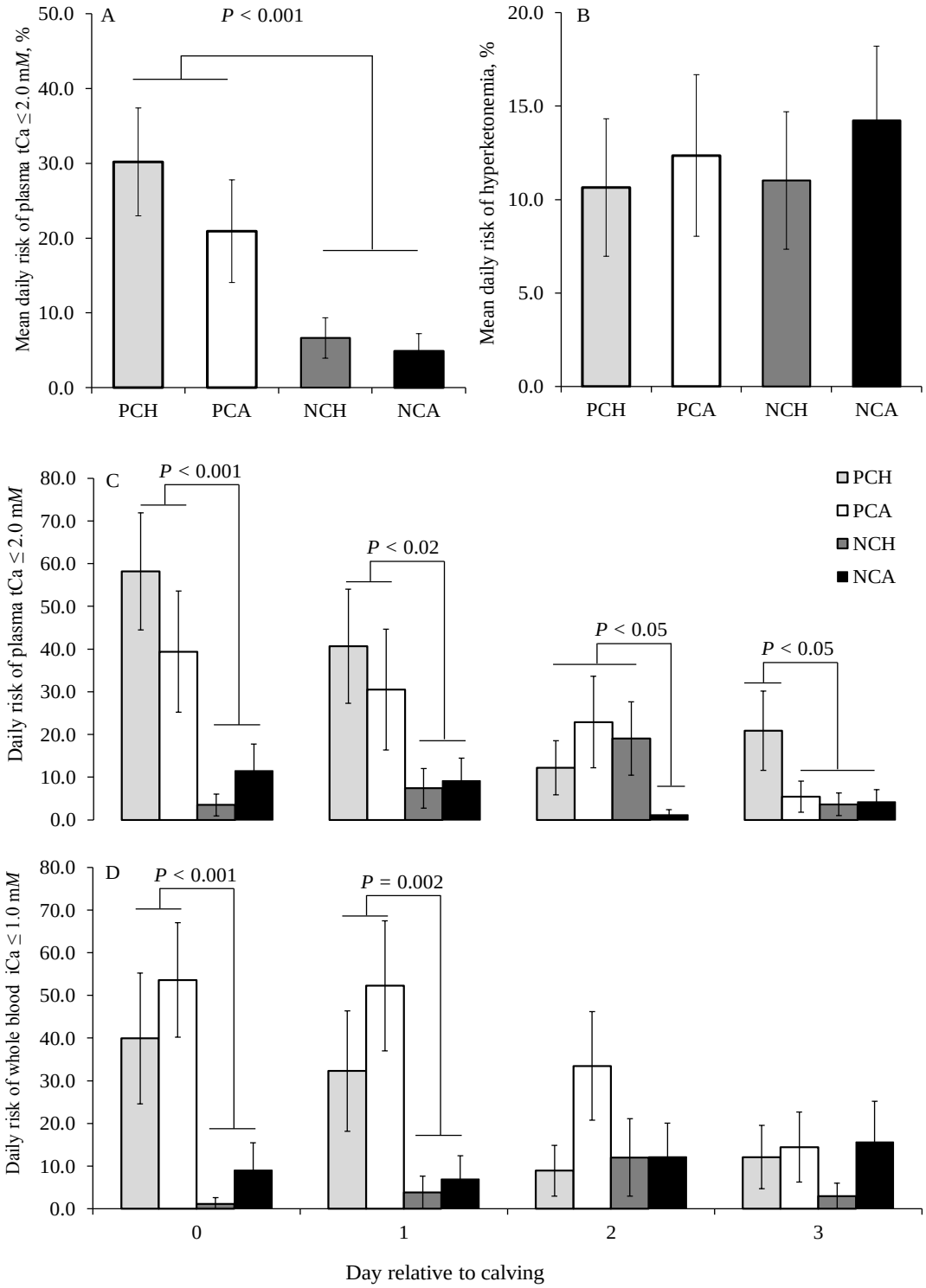

Figure 3. Mean daily risk of subclinical hypocalcemia based on serum tCa ≤ 2.0 m*M* from d 0 to 3 postpartum (A) and of hyperketonemia in the first 30 DIM (B), and the individual daily risks of subclinical hypocalcemia based on serum tCa ≤ 2.0 m*M* (C) or serum tCa ≤ 1.0 m*M* (D) in cows fed prepartum diets containing positive DCAD (+130 mEq/kg) with 3 mg of cholecalciferol (PCH) or 3 mg of calcidiol (PCA), or negative DCAD (-130 mEq/kg) with 3 mg of cholecalciferol (NCH) or 3 mg of calcidiol (NCA). Panel A and C, effects of DCAD ($P < 0.001$), vitamin D ($P = 0.38$), day ($P < 0.01$), and interaction between DCAD and vitamin D ($P = 0.78$). Panel B, effects of level of DCAD ($P = 0.77$), vitamin D ($P = 0.50$), and interaction between DCAD and vitamin D ($P = 0.86$). Panel D, effects of DCAD ($P = 0.005$), vitamin D ($P = 0.06$), day ($P < 0.58$), and interaction between DCAD and vitamin D ($P = 0.63$). Differences within day are depicted in panels C and D. Error bars represent the SEM for the adjusted proportions.

Survival

Treatment did not affect survival of cows up to 305 DIM and 77.2% of them remained in the herd at the end of the evaluation period (Table 5). The median days to leaving the herd could not be estimated because only 22.7% of the cows left the herd by culling or death, and the hazard of leaving the herd was only numerically smaller for cows fed the negative compared with positive DCAD (adjusted HR = 0.93; 95% CI = 0.37 to 2.33) and for cows fed cholecalciferol compared with calcidiol (adjusted HR = 0.95; 95% CI = 0.38 to 2.40). Nevertheless, the mean days to leaving the herd for those that left tended to be sooner (P = 0.10) for cows fed the positive compared with the negative DCAD (P = 114.8 ± 35.7 vs. N = 189.4 ± 29.8 d). No difference was observed for interval to leaving the herd with source of vitamin D.

Reproductive Performance

Of the 79 cows in the experiment, 74 received at least 1 AI and 73 had a pregnancy diagnosis performed. Five cows did not receive an insemination either because they were culled before the end of the voluntary waiting period (3 cows) or were coded as to not inseminate (2 cows). Because of timed AI, the interval postpartum to first AI did not differ with treatments and averaged 76.2 DIM. Pregnancy at first AI based on the diagnosis on d 70 after AI did not differ with level of DCAD (P = 0.17) or source of vitamin D (P = 0.85) and averaged 32.7, 34.7, 21.2, and 16.5% for PCH, PCA, NCH, and NCA, respectively. The median days to pregnancy did not differ with level of DCAD (Table 6), but feeding calcidiol tended (P = 0.10) to increase the rate of pregnancy by 55% and reduce the median days to pregnancy by 19. By 305 DIM, of the 79 cows that started the experiment, 76% of them became pregnant (PCA = 70.4%, PCH = 81.7%, NCH = 72.1%, NCA = 86.4%).

Table 6. Cox's proportional hazard model for time to pregnancy in cows fed two levels of dietary cation-anion difference (DCAD) and two sources of vitamin D fed prepartum[1]

	Days to pregnancy[2]				
Item	Median (95% CI)	Mean ± SEM	Pregnant, %	AHR[3] (95% CI)	*P*-value
DCAD[4]					
Positive	144 (79 to 179)	151 ± 13	76.5	Reference	---
Negative	150 (133 to 183)	165 ± 11	80.2	0.84 (0.50 to 1.39)	0.49
Vitamin D					
Cholecalciferol	163 (135 to 183)	166 ± 12	71.2	Reference	---
Calcidiol	144 (110 to 150)	150 ± 11	84.2	1.55 (0.92 to 2.61)	0.10
Parity					
Parous	163 (144 to 184)	172 ± 11	73.1	Reference	---
Nulliparous	114 (76 to 144)	130 ± 10	82.9	1.76 (1.03 to 3.02)	0.04

[1] Prepartum cows at 252 d of gestation were fed diets with either positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol or 3 mg of calcidiol.
[2] Pregnancy was based on the diagnosis on d 70 after each AI within the first 305 DIM.
[3] AHR = adjusted hazard ratio.
[4] Interaction between level of dietary cation-anion difference (DCAD) and source of vitamin D was not significant and dropped from final model.

DISCUSSION

Feeding prepartum transition dairy cows a diet with negative DCAD eliminated clinical hypocalcemia and reduced the risk of subclinical hypocalcemia, whereas supplementing calcidiol in place of cholecalciferol reduced the incidence of retained placenta and metritis, which resulted in a smaller proportion of cows with multiple diseases. Combining a diet with negative DCAD with calcidiol resulted in the smallest morbidity. The use of acidogenic diets prepartum to reduce the risk of clinical hypocalcemia has been implemented for more than four decades and the benefits are widely documented (Ender et al., 1971; Block, 1984; Lean et al., 2006). Less information is available quantifying the effect of diets with negative DCAD to prevent subclinical hypocalcemia in dairy cows. Oetzel et al. (1988) showed that feeding acidogenic diets greatly reduced the prevalence of subclinical hypocalcemia in early lactation.

Clinical and subclinical hypocalcemia are known to be associated with increased risk of other peripartum diseases in dairy cattle (Chapinal et al., 2011; Seifi et al., 2011; Martinez et al., 2012), and cows diagnosed with diseases in the current experiment had less concentrations of iCa postpartum and tCa pre- and postpartum compared with healthy cows. One of the suggested mechanisms is that hypocalcemia interferes with innate (Martinez et al., 2014) and potentially acquired immunity (Kimura et al., 2006), therefore, increasing the risk of diseases such as

retained placenta and metritis (Kimura et al., 2002; Martinez et al., 2012). As anticipated, prepartum feeding of a diet with negative DCAD improved concentrations of iCa and tCa during early postpartum (Rodney et al., 2017 – Chapter 6), and it had beneficial effects on measures of neutrophil function in parous cows, but not in nulliparous cows. The increase in percentage of neutrophils with killing activity and intensity of phagocytosis in parous cows when fed the diet with negative DCAD was likely caused by enhanced concentrations of blood iCa and serum tCa. Because neutrophil function is compromised during spontaneous (Martinez et al., 2012) or induced subclinical hypocalcemia (Martinez et al., 2014), and multiparous cows are more likely to suffer from hypocalcemia (Reinhardt et al., 2011), it is not surprising that the benefits of increased blood Ca on measures of immune function were observed in the cohort with increased risk for the disease. Nevertheless, and importantly, feeding a diet with negative DCAD prepartum reduced the risk of subclinical hypocalcemia in both, nulliparous and parous cows in the current experiment. On the other hand, one of the first reported functions of vitamin D on immune cells was that of increased differentiation of bone marrow myeloid cells (Koeffler et al., 1984). It is possible that 1,25-dihydroxyvitamin D_3 plays a role in hematopoiesis and favors leukocyte function at a time when cows undergo leucopenia because of neutropenia. This would have favored neutrophil function despite changes in blood concentrations of iCa.

Source of vitamin D also influenced postpartum health in dairy cows. Feeding calcidiol compared with cholecalciferol improved neutrophil oxidative burst and reduced the incidence of diseases typically linked with immune dysfunction, such as retained placenta and metritis. The amount of vitamin D supplemented was approximately 6-fold that recommended by the NRC (2001) for a 650-kg prepartum dairy cow, which resulted in concentrations of 25-hydroxyvitamin D_3 in plasma of approximately 60 and 240 ng/mL in cows fed cholecalciferol and calcidiol, respectively (Rodney et al., 2017 – Chapter 6). Such concentrations are considered more than adequate for dairy cattle (Nelson et al., 2016). Wilkens et al. (2012) showed that a combination of a diet with negative DCAD and 3 mg of calcidiol resulted in the greatest plasma iCa concentrations around calving and no adverse signs of excessive vitamin D feeding. A recent survey conducted by Nelson and colleagues (Nelson et al., 2016) found that nutritionists in the USA typically supplement diets with 0.75 to 1.25 mg of vitamin D. Perhaps, the larger amount supplemented is based on a consideration that rumen bacteria might deactivate vitamin D, allowing the ruminant to tolerate larger doses of oral vitamin D (Gardner et al., 1988), although recently that view has been challenged (Hymoller and Jensen, 2010). Nevertheless, when cows were fed 6 mg of calcidiol, it was not beneficial and numerically increased the incidence of

clinical hypocalcemia in dairy cows (Weiss et al., 2015). Because cows fed cholecalciferol had concentrations of 25-hydroxyvitamin D_3 in plasma considered adequate based on current surveys (Nelson et al., 2016), it is plausible to suggest that the benefits to innate immunity and reduction in diseases observed in cows fed calcidiol were not caused by inadequate vitamin D status in cows fed cholecalciferol.

Calcidiol has beneficial effects on innate host defenses of cattle (Merriman et al., 2015; Nelson et al., 2010; 2012). Exposure of monocytes to pathogen-associated molecular patterns in bacteria activates toll-like receptors that stimulate immune cells such as monocytes to induce expression of CYP27B1 to convert 25-hydroxyvitamin D_3 into the more active form of vitamin D, 1,25-dihydroxyvitamin D_3 (Nelson et al., 2010). Activation of toll-like receptors results in production of peptides with potent antimicrobial activity such as cathelicidin and beta defensins, which can disrupt bacterial cell membrane and causing bacterial death (Liu et al., 2006). Recently, Merriman et al. (2015) showed that culture of bovine monocytes treated with calcitriol increased expression of a cluster of antimicrobial peptides and the increase in antimicrobial peptide expression was dose-dependent. Moreover, the authors showed that intramammary infusion of calcitriol increased expression of beta-defensin gene 7 in macrophages isolated from the mammary gland (Merriman et al., 2015), thereby suggesting that calcitriol stimulates defensive mechanisms in bovine immune cells. Cows fed calcidiol had increased concentrations of 1,25-dihydroxyvitamin D_3 in the last 9 d of gestation compared with cows fed cholecalciferol (Rodney et al., 2017 – Chapter 6). Therefore, it is suggested that the increased blood concentrations of 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 stimulated innate immune cell function which is paramount for prevention of certain periparturient diseases in cattle. A similar positive response in neutrophils has been demonstrated when cows received a subcutaneous dose of calcitriol immediately after calving (Vieira-Neto et al., 2017).

Calcidiol increased the percentage of neutrophils with bacterial killing activity, which would likely favor elimination of the placenta and reduces the risk of establishment of bacterial infections in the uterus. The etiology of retained placenta in cows involves the inability of the maternal immune system to recognize the semiallogenic fetal tissues (Davis et al., 2004), and function of cells of the innate immune system seems to play an important role on the release of the placenta (Gunnink, 1984a, 1984b; Kimura et al., 2002) and subsequent risk of uterine diseases in dairy cattle (Hammon et al., 2006). Therefore, it is reasonable to suggest that dietary interventions that improve immune function are expected to reduce the risk of retained placenta

and metritis. In fact, feeding calcidiol reduced the incidence of metritis, which might be linked with the reduction in retained placenta as the two diseases are linked. However, calcidiol might have further contributed to reduction of metritis incidence by its effects on neutrophil function. After parturition, almost all cows have bacteria that contaminate the uterus (Elliot et al., 1968); however, a combination of pathogen type and the cow's immune defenses dictate whether the infection will resolve or persist leading to metritis. Because calcidiol improved function of immune cells and it has been shown to stimulate the production of antimicrobial peptides by leukocytes (Nelson et al., 2012), it is conceivable that the increase in plasma concentrations of 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 observed in cows supplemented with calcidiol stimulated defense mechanisms in the uterus that prevented metritis. The combined effect of calcidiol on retained placenta and metritis explains the reduction in the proportion of cows diagnosed with multiple diseases in the first month of lactation.

One cannot disregard that hypocalcemia has also been shown to suppress gut and reproductive tract motility (Silva and Noakes, 1984; Al-Eknah and Noakes, 1989; Martinez et al., 2014), which might predispose cows to other diseases. In particular, a reduction in uterine motility following calving could have contributed to less clearance of uterine contents and predispose cows to metritis. When ewes (Silva and Noakes, 1984) and cows (Al-Eknah and Noakes, 1989) were induced to have subclinical hypocalcemia, uterine motility, measured with surgically implanted balloon-tipped catheters, decreased. Perhaps the combined effect of negative DCAD improving blood concentrations of iCa and tCa and calcidiol enhancing neutrophil function worked additively to reduce morbidity in the first 30 DIM in dairy cows.

Although calcidiol reduced the risk of some common diseases associated with the immune system in dairy cows, it did not reduce the incidence of cows diagnosed with hyperketonemia. Nevertheless, the daily risk of hyperketonemia did not change with dietary treatments. Cows fed calcidiol had greater production of ECM with no differences in caloric intake resulting in improved feed efficiency but worse net energy balance (Martinez et al., 2017 – Chapter 7), which explains the tendency for increased incidence of cows with BHB greater than 1.20 m*M*. This tendency for increased incidence of cows with hyperketonemia were not at the expense of production, reproduction or health as cows fed calcidiol produced more ECM (Martinez et al., 2017 – Chapter 7), tended to have fewer days to pregnancy, and had reduced incidence of retained placenta and metritis. Because the estimate of daily risk considers not only incidence, but also duration and relapses of hyperketonemia, then the similar daily risk indicates that cows

fed calcidiol had either shorter duration or fewer relapses of hyperketonemia which offset the slight increase in incidence. Lean et al. (1994) showed that hyperketonemia did not influence DM intake and milk yield, unless the cows showed clinical signs of disease. Because hepatic ketogenesis is a means by which calorie-rich compounds can be transferred to peripheral tissues during periods of negative energy balance, an increase in blood concentration of BHB might not necessarily be a negative finding.

All cows in the experiment received their first AI during the summer months, between June and September in Florida, a period of intense heat stress that depresses fertility (Hansen, 2009). The hyperthermia associated with heat stress disrupts numerous aspects of reproduction in lactating dairy cows and probably explains the low pregnancy at first AI in all treatments. Feeding calcidiol tended to improve the rate of pregnancy and reduced the days to pregnancy during the 305-d lactation. There is limited data on the effect of vitamin D on fertility of cattle, but early work by Ward et al. (1971) showed that supplementation with 7.5 mg of cholecalciferol per week as an oral bolus starting 45 d prepartum reduced interval from calving to pregnancy from 134 to 97. These data suggest that cattle not supplemented with vitamin D show benefits to supplementation on reproduction; however, but all cows in the experiment had concentrations in plasma of 25-hydroxyvitamin D_3 of at least 40 ng/mL, which is considered adequate (Nelson et al., 2016). On the other hand, cows fed calcidiol had improved measures of neutrophil function and reduced incidence of inflammatory diseases that affect the reproductive tract, which are known to depress fertility (Ribeiro et al., 2016). Thus, it is possible that the improved peripartum health with calcidiol might explain the tendency for improved rate of pregnancy. It is well established that diseases have marked negative effects on fertility of dairy cattle, particularly those of inflammatory nature. Ribeiro et al. (2016) showed that inflammatory diseases such as those that affect the uterus reduce fertilization, embryo quality, conceptus development, and pregnancy. Conceptus from cows diagnosed with inflammatory diseases in early lactation had marked changes in the transcriptome, suggesting that inflammation in early lactation have long-lasting effects on the biology of pregnancy that ultimately impair the establishment and maintenance of pregnancy. Nevertheless, additional work with larger numbers of cows is needed to elucidate if the potential benefits to reproduction from calcidiol are direct effects or indirect though improved postpartum health.

CONCLUSIONS

Feeding a diet containing a DCAD of -130 mEq/kg during the last 3 weeks of gestation reduced the incidence clinical and subclinical hypocalcemia and the risk of subclinical hypocalcemia in dairy cows in the first 3 d in lactation, and these benefits were observed regardless of source of vitamin D supplemented or parity of cows. Calcidiol improved neutrophil oxidative burst activity postpartum in all cows and feeding a diet with negative DCAD improved the intensity of neutrophil phagocytosis in parous cows and intensity of oxidative burst in neutrophils in all cows during the prepartum period, and the percentage of neutrophils displaying oxidative burst postpartum in parous cows. Calcidiol reduced the incidence of retained placenta and metritis, and the percentage of cows with multiple diseases in the first month of lactation, which are likely related to the improved measures of immune function evaluated in neutrophils. The combination of negative DCAD and calcidiol fed prepartum was most effective in reducing morbidity in dairy cows in early lactation. Cows with morbidity had lesser concentrations of ionized and total Ca than healthy cows. Despite the benefits to health, altering the prepartum DCAD did not affect reproduction in dairy cows, but feeding calcidiol tended to increase the rate of pregnancy and reduce days open.

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CHAPTER NINE: ASSOCIATIONS AMONG BONE AND ENERGY METABOLISM IN COWS FED DIETS DIFFERING IN LEVEL OF DCAD AND SUPPLEMENTED WITH CALCIDIOL OR CHOLECALCIFEROL

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OVERVIEW OF CHAPTER NINE

Studies in mice and man have identified a pivotal role for skeleton, particularly through production of active forms of osteocalcin, in integrating energy metabolism. This Chapter examines a subset of the cows from the experiment presented in Chapters 6, 7 and 8 to explore in more detail, the relationships between bone and energy metabolism in cattle. We postulated a specific role for bone metabolism, and links to energy metabolism, in the adaption of dairy cows to lactation. In this Chapter, we aim to identify associations between bone markers and energy metabolites, and determine if these responses are influenced by dietary vitamin D, and cation-anion difference interventions applied before calving using time-series analysis.

ABSTRACT

Studies have identified an important role for bone-derived hormones in murine and bovine metabolism and the integrated responses to lactation. The study examined the hypothesis that interactions between bone and energy metabolism observed in other species are also present in dairy cattle and have feedback mechanisms evident over time. We also examined the role of the form of dietary vitamin D supplementation and manipulation of DCAD in these interactions. Associations among metabolites were examined in 32 Holstein cows blocked by parity and milk yield and randomly allocated to receive diets containing either calcidiol or cholecalciferol (3 mg/11 kg of DM) and positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD from 255 d of gestation to calving. Blood was sampled every 3 d, from approximately 9 d prepartum to 30 d postpartum, and plasma concentrations of vitamin D_3, 25-hydroxyvitamin D_3, adiponectin, C-terminal telopeptide of type 1 collagen (CTX-1), glucose, insulin-like growth factor (IGF) 1, insulin, undercarboxylated osteocalcin (uOC), and carboxylated osteocalcin (cOC) were determined. Feeding calcidiol, compared with cholecalciferol, increased plasma concentrations of 25-hydroxyvitamin D_3 pre- (264.2 ± 8.0 vs. 61.3 ± 8.0 ng/mL) and postpartum (170.8 ± 6.2 vs. 51.3 ± 6.2 ng/mL), but decreased concentrations of vitamin D_3 pre- (1.2 ± 0.6 vs. 14.5 ± 0.6 ng/mL) and postpartum (1.9 ± 0.4 vs. 3.2 ± 0.6 ng/mL). This effect was enhanced by feeding a positive DCAD prepartum. Prepartum, cows fed the diet with negative DCAD had reduced concentrations of vitamin D_3 and glucose compared with cows fed the diet with positive DCAD. The combination of negative DCAD and cholecalciferol reduced IGF-1 concentrations prepartum. There was no effect of DCAD treatment on postpartum metabolite concentrations. Nulliparous cows had increased concentrations of OC, CTX-1, IGF-1, glucose and insulin compared with multiparous cows. Time series analysis identified associations between metabolites on the same day, and over 3 d lags up to ± 12 d. Feedback between 25-hydroxyvitamin D_3 and vitamin D_3 in the negative lags suggests 25-hydroxyvitamin D_3 may exert feedback on vitamin D_3, but not vice versa. A physiological feedback mechanism between vitamin D_3 and IGF-1, with positive effect size (ES) on the same day and 3 d later, and negative ES 9 d later, was more pronounced in cholecalciferol supplemented cows and suggests an important role of IGF-1 integrating bone metabolism with energy and protein metabolic pathways. Evidence of feedback was also identified between uOC, and particularly cOC with IGF-1, with positive ES on the same day, but negative ES 6 d before and 6 d after. An association between uOC or cOC and IGF-1 has not been previously identified in cattle, and suggests that both uOC and cOC may have marked biological activity. The associations between

OC and insulin identified in murine experiments were not observed herein, although associations between OC and glucose were, and were similar to those between IGF-1 and glucose, confirming associations between glucose, OC and IGF-1. There is evidence in cattle of cross-talk between vitamin D, bone hormones and energy metabolism.

Keywords: dietary cation-anion difference, IGF-1, osteocalcin, vitamin D

INTRODUCTION

The interplay between bone and energy metabolism has been identified in murine models (Lee et al., 2007), and associations between bone markers and energy metabolism have been demonstrated in humans (Wolf, 2008). We have postulated a specific role for bone metabolism, and links to energy metabolism, in the adaption of dairy cows to lactation (Lean et al., 2014), and anticipated that such associations are influenced by prepartum dietary interventions. Such interventions have had profound and long-lasting effects on productivity, health and fertility of cows (Lean et al., 2014; Martinez et al., 2017b – Chapter 8). However, the mechanisms by which metabolism is changed to produce these responses are not necessarily clear. Dairy cattle have marked irreversible losses of minerals, lipids, amino acids, and lactose in milk with the onset of lactation. These losses occur simultaneously with increased metabolic demands for fetal growth, parturition, insufficient nutrient intake, and development of insensitivity to insulin (Bell, 1995; McNamara, 1991). Adaptations to lactation impose challenges to the cow and make the study of metabolic pathways related to bone and energy metabolism, and the ability to manipulate responses to these metabolic challenges, particularly pertinent in dairy cattle.

Lee et al. (2007) identified actions of the osteoblast-derived hormone osteocalcin (**OC**) that created a feedback loop between bone and energy metabolism. Osteocalcin is produced by mature osteoblasts and undergoes vitamin K-dependent gamma carboxylation resulting in carboxylated OC (**cOC**) that increases the affinity of the molecule for calcium (**Ca**) and hydroxyapatite in bones and plays a role in bone accretion in numerous species, including bovine (Van Mosel and Corlett, 1990). A smaller fraction of OC remains undercarboxylated (**uOC**) and interacts with a G-protein coupled receptor GPRC6A in numerous tissues (Wei and Karsenty, 2015). In a seminal paper, Lee et al. (2007) conducted a series of experiments that demonstrated that OC promoted pancreatic β-cell proliferation and insulin secretion, independently increased peripheral tissue insulin sensitivity, and stimulated adiponectin secretion by adipocytes. On the other hand, adiponectin has been shown to increase osteoblast proliferation and differentiation (Berner et al., 2004), bone deposition (Kanazawa et al., 2007), glucose uptake by skeletal muscle, and may suppress hepatic gluconeogenesis (Yamauchi et al., 2002). Insulin acts to directly inhibit osteoblast activity, thereby enhancing bone resorption (Lee et al., 2007). Associations have been made between obesity and Ca metabolism in cattle (Heuer et al., 1999, DeGaris et al., 2010) and induced subclinical hypocalcemia resulted in transient insulin

resistance based on increased blood glucose and reduced plasma insulin concentrations in dairy cattle (Martinez et al., 2014).

It's been hypothesized that dietary interventions in the prepartum period such as vitamin D and dietary cation-anion difference (**DCAD)** (Lean et al., 2014), particularly through influences on OC, have the potential to influence the feedback loop between bone and energy metabolism demonstrated by Lee et al. (2007). Diets with negative DCAD enhance the response to parathyroid hormone (**PTH**) in dairy cows, which increases synthesis of 1,25-dihydroxyvitamin D_3 in dairy cows (Goff et al., 2014). Vitamin D plays a crucial role in the absorption of minerals from the gastrointestinal tract, but is also essential for bone metabolism, regulating the activity of osteoblasts and osteoclasts (Tanaka and DeLuca, 1971). Vitamin D responsive elements have been identified in the OC gene *BGLAP* (Terpening et al., 1991), and 1,25-dihydroxyvitamin D_3 and other vitamin D metabolites can activate the gene and stimulate OC synthesis (Uchida et al., 1994). It is possible that dietary interventions that influence mineral and vitamin D metabolism also influence energy metabolism in transition dairy cows as part of the homeorhetic adaptations to lactation (Bauman and Currie, 1980).

Establishing associations with potential feedback loops between mineral-bone-energy metabolism in transition dairy cows can create novel hypothesis on the mechanisms by which nutrients and dietary interventions influence adaptations to lactation. This cross-talk and integration of metabolism has been elegantly demonstrated in murine models (Lee et al., 2007), and it is suggested to apply to humans (Wolf, 2008), and evidence exists in dairy cattle (Lean et al., 2014). It was hypothesized that associations between bone-derived compounds and those involved in energy metabolism would be evident and more pronounced on the same day than in lags of 3-d. The objectives were to use time series analysis to identify associations between plasma compounds involved in bone, mineral and energy metabolism in transition dairy cows fed diets differing in level of DCAD and source of supplemental vitamin D.

MATERIALS AND METHODS

Throughout this manuscript, sources of vitamin D fed to cows prepartum will be referred as cholecalciferol (**CH**) and calcidiol (**CA**), whereas measurements of concentrations of vitamin D metabolites in plasma will be referred as vitamin D_3 and 25-hydroxyvitamin D_3

Cows, Diets and Treatments

All procedures were approved by the University of Florida Institutional Animal Care and Use Committee (protocol number 201408331). This study is part of a series of experiments conducted to evaluate the effects of level of dietary DCAD and source of supplemental vitamin D fed prepartum on transition cow metabolism, production, and health (Martinez et al., 2017a; 2017b; Rodney et al., 2017 – Chapter 6, 7 and 8).

Pregnant Holstein cows, 20 parous and 12 nulliparous, were moved into the experimental pens at approximately 252 d of gestation to become acclimatized to the facilities and be trained in the use of the Calan Broadbent feeding system (American Calan Inc., Northwood, NH). For consistency of terminology, cows are referred to as either nulliparous, those that were nulliparous pre-partum and primiparous postpartum, or parous, those that had previously calved, throughout the manuscript. Cows were blocked by parity (0 vs. > 0) and, for parous cows, 305-d milk yield during the previous lactation. Within each block, cows were randomly assigned into one of four dietary treatments in a randomized complete block design. Measurements started at 255 d of gestation until 49 d postpartum.

Treatments were arranged in a 2 x 2 factorial, with two levels of DCAD [DCAD mEq/kg = (Na^+ + K^+) – (Cl^- + S^-)], positive (+130 mEq/kg) or negative (-130 mEq/kg) achieved by replacing soybean meal with a high protein acidogenic product (Bio-Chlor, Church & Dwight Co. Inc., Trenton, NJ), and two forms of vitamin D, CH (Rovimix D_3; a product containing 300 mg of cholecalciferol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ) or CA (Hy-D; a product containing 153 mg of calcidiol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC), both fed at 3 mg of vitamin D for each 11 kg of diet DM. Hence, the four treatments were positive DCAD and CH (**PCH**), positive DCAD and CA (**PCA**), negative DCAD and CH (**NCH**), and negative DCAD and CA (**NCA**). The 32 cows encompassed 8 blocks of 4 cows each that represented all 4 treatments within a block. The diets were isocaloric and isonitrogenous and only varied on DCAD and source of supplemental vitamin D. Diet composition is provided in Supplementary Table 1.

Cows were individually fed diets as TMR using a Calan Broadbent feeding system. Prepartum, treatments were applied to diets and offered once daily until calving. After calving, a common lactation diet was fed to all cows postpartum. Amounts offered and refused were measured for individual cows daily and feed allowances were calculated daily with the goal of 5% refusals.

Full details of experimental procedures, cow housing, sample collection, diet composition, and analysis of feeds can be found in Martinez et al., (2017a – Chapter 7).

Blood Sample Collection and Analysis

Blood was sampled from the coccygeal vessels 3 times weekly prepartum and every 3 d from the day of parturition until 30 DIM. Only the samples closest to or on d -9, -6, and -3 relative to calving and those taken on the day of and every 3 d from calving to 30 DIM were used for analyses. Plasma concentrations of vitamin D_3, 25-hydroxyvitamin D_3, uOC, cOC, C-terminal telopeptide of type 1 collagen (**CTX-1**), glucose, insulin, IGF-1, and adiponectin were analyzed according to the procedures outlined in Rodney et al. (2017 – Chapter 6) and Martinez et al. (2017a). Daily measurements of DMI was evaluated for the first 42 DIM, whereas daily milk yield and BW and weekly body condition (BCS) were collected for the first 49 DIM. Details are presented elsewhere (Martinez et al., 2017a – Chapter 7).

Statistical Analysis

The cows used in this study are a subset of those examined in Martinez et al. (2017a; 2017b – Chapters 7 and 8) and Rodney et al. (2017 – Chapter 6). Eight blocks of cows each containing a cow assigned to one of the four treatments (n = 4 cows/block), with 3 blocks of nulliparous and 5 of multiparous cows, were selected for inclusion in the study based on completeness of samples taken for that block over the study period.

Cow performance and concentrations of metabolites in plasma were analyzed by ANOVA with mixed models using the MIXED procedure of SAS (SAS ver. 9.4, SAS/STAT, SAS Institute Inc., Cary, NC). Normality of residuals and homogeneity of variance were examined for each continuous variable analyzed after fitting the statistical model. Responses that violated the assumptions of normality were subjected to power transformation according to the Box-Cox procedure (Box and Cox, 1964) using the PROC TRANSREG in SAS. For transformed data, the LSM and SEM were back transformed for presentation according to Jørgensen and Pedersen (1998). Concentrations of CTX-1 and insulin had to be log-transformed before analyses either because of heteroscedasticity or because residuals were not normally distributed.

Pre- and postpartum data were analyzed separately. The statistical models included the fixed effects of level of DCAD (positive vs. negative), source of vitamin D (CH vs. CA), interaction between DCAD and vitamin D, parity (nulliparous vs. parous), day of measurement, and the

interactions between DCAD and parity, vitamin D and parity, DCAD and day, vitamin D and day, parity and day, DCAD and vitamin D and parity, DCAD and vitamin D and day, DCAD and parity and day, vitamin D and parity and day, and DCAD and vitamin D and parity and day. Random effects included block and cow nested within DCAD and vitamin D. The repeated statement was included in all mixed models and day the specified repeated effect. The covariance structure was modeled based on spacing between measurements and selection was based on model fit with the smallest corrected Akaike's information criterion and the autoregressive 1 was selected. The Kenward-Roger method was used to compute the approximate denominator degrees of freedom for the F tests in the statistical models. When an interaction was significant, parwise comparisons were performed with the adjustment by the method of Tukey. Statistical significance was considered at $P \leq 0.05$, and tendency was considered at $0.05 < P \leq 0.10$.

A time series analysis was conducted using Stata (ver. 13 Intercooled Stata v.13, USA) to examine relationships between metabolites over time. The data from each cow and metabolite from 9 d before calving to 30 d after calving (n = 14 samples/cow) were de-trended separately to produce an approximately stationary series (Shumway, 1988). Spline trends were removed from each and the remaining data points effectively equate to residuals from these models. Cross-correlations were then performed using the XCORR procedure of Stata on these data from pairs of metabolites (*x* and *y*) for each lag (*m)* using the following model

$$\rho_{xy}^{T}(m) = \frac{R_{xy}^{T}(m)}{\sqrt{R_{x}^{T}(0)R_{y}^{T}(0)}}$$

where it is assumed that series *x* and *y* are stationary and are observed at time points $t = 0, 1, \ldots, T\text{-}1$ and the cross-covariance function is

$$R_{xy}^{T}(m) = T^{-1} \sum_{t=0}^{T-1-m} (x_{t+m} - x^{T})(y_{t} - y.^{T}), m \geq 0$$

and

$$R_{xy}^{T}(-m) = R_{yx}^{T}(m)$$

for negative lags (Shumway, 1988). Cross-correlation coefficients were transformed using Fisher's transformation. Finally, a random effects, pooled effect of estimate was produced using DerSimonian and Laird (DerSimonian and Laird, 1986) random effects meta-analytic methods with the METAN procedure of Stata, in which the transformed cross-correlations for each cow

were treated as a separate study as described by Hedges and Vevea (1998). The effect size (**ES**) of each observation then is given weight

$$\omega_i = 1/[se(\widehat{\theta_i})^2 + \hat{\tau}^2]$$

and the pooled ES is given by

$$\hat{\theta}_{DL} = (\sum \omega_i \hat{\theta}_i)/(\sum \omega_i)$$

and

$$se\{\hat{\theta}_{DL}\} = 1/\sqrt{\sum \omega_i}$$

(Palmer and Sterne, 2009). Effect sizes were not transformed back to correlations. Significant effects of vitamin D or DCAD treatments, or their interactions, were identified for each pair of metabolites at each lag using analysis of variance using the ANOVA procedure in Stata.

Interpretation of Time Series Results

Briefly, when the relationship between two metabolites, for a given treatment, is significantly different from 0, as determined by the 95% CI, it has been highlighted in the Tables by use of color shading. Light blue colored cells indicate a positive ES between the two metabolites, whereas red squares indicate a negative ES between the two metabolites. The relationship between each pair of metabolites was assessed as follows. The ES at lag zero reflects the pooled ES for the group of cows of the correlations between the two metabolites measured on the same day. The negative lags (ie -1, -2, -3, and -4) present the pooled ES of the cows for the cross correlations between the first metabolite with the second metabolite 3 to 12 d later. For example, in Table 4, the ES for Lag -3 between 25-hydroxyvitamin D_3, the first metabolite, and vitamin D_3 concentrations, the second metabolite, 9 d later is -0.360. Similarly, a positive lag (+1, +2, +3, and +4) shows the pooled ES for the cows of the cross-correlations between the first metabolite with the second metabolite 3 to 12 d before. For example, in Table 4, 25-hydroxyvitamin D_3, the first metabolite, and CTX-1 concentrations, the second metabolite, at lag +2, that is 6 d earlier, is 0.240 for cows fed PCH. The ES is also termed the 'standardized mean difference' and, as such, essentially represents a Z-distribution response. Consequently, an ES of 1 represents a difference of 1 SD in response for the two metabolites. Cohen (1988) suggested effect sizes as small (ES = 0.2), medium (ES = 0.5), or large (ES $\geq$ 0.8). Results are presented in tables and changes over time are illustrated in figures. In tables, superscripts indicate instances in which, across all four treatments, an effect ($P < 0.05$) of treatment influenced the ES between the pair of metabolites. The superscript "a" indicates an influence of vitamin D treatment; superscript "b" indicates an

influence of DCAD treatment; and superscript "c" indicates that the interaction of the vitamin D and DCAD treatments influencing the ES. There were few significant ES between metabolites 12 d before (+4) and after (-4) so results are not shown.

RESULTS

As these cows are a sub-population of those described in Martinez et al. (2017a; 2017b – Chapters 7 and 8) and Rodney et al. (2017 – Chapter 6), the results pertaining to the mixed model responses are consistent with those of Martinez et al. (2017a; 2017b – Chapters 7 and 8), but more conservative because of the smaller sample size from the original experiment.

Pre- and Postpartum Concentrations of Metabolites

Cows supplemented with CA had increased ($P < 0.001$) plasma concentrations of 25-hydroxyvitamin D_3 (61.3 vs. 264.2 ± 8.0 ng/mL) and reduced ($P < 0.001$) concentrations of vitamin D_3 (14.55 vs. 1.56 ± 0.56 ng/mL) prepartum compared with cows receiving CH (Table 1, Figure 1A and 1B). The interaction ($P < 0.001$) between DCAD and Vitamin D for plasma concentrations of vitamin D_3 prepartum indicates that differences were greater when cows were fed the diet with positive compared with the negative DCAD. Concentrations of vitamin D_3 postpartum were greater ($P = 0.001$) for cows fed CH than CA (Table 2), although they rapidly declined ($P < 0.001$) postpartum reaching those of cows fed CA by 12 DIM (Figure 1A). Concentration of 25-hydroxyvitamin D_3 remained greater for cows fed CA than CH postpartum (CH = 52.0 vs. CA = 177.4 ± 6.9 ng/mL), but they declined at a greater rate ($P < 0.001$) after calving in cows fed CA than CH (Figure 1B). There was no effect of DCAD or interactions between DCAD and vitamin D treatments on postpartum concentrations of vitamin D metabolites.

Table 1. Prepartum concentrations of metabolites in plasma of dairy cows fed prepartum diets differing in level of dietary cation-anion difference (DCAD) and source of vitamin D[1]

	Positive		Negative			Parity			*P*-value[2]			
Item[3]	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Vitamin D_3, ng/mL	18.92[a]	1.68[c]	12.12[b]	0.74[c]	0.79	8.67	8.06	0.73	< 0.001	< 0.001	< 0.001	0.53
$25OHD_3$, ng/mL	61.0	277.0	56.3	244.0	10.9	159.3	159.8	8.6	0.09	< 0.001	0.20	0.96
Adiponectin, µg/mL	29.2	18.9	24.5	19.3	7.3	26.2	19.8	7.8	0.73	0.24	0.69	0.54
uOC, ng/mL	2.40	3.71	2.98	3.62	0.77	4.05	2.31	0.73	0.73	0.19	0.64	0.10
cOC, ng/mL	57.1	57.2	53.2	53.9	5.5	75.3	35.4	6.6	0.38	0.92	0.93	0.003
CTX-1, ng/mL[4]	0.60	0.62	1.19	0.54	0.18	1.22	0.40	0.17	0.27	0.13	0.11	0.006
Glucose, m*M*	4.12	4.03	3.77	3.92	0.09	4.11	3.81	0.08	0.02	0.77	0.19	0.03
Insulin, ng/mL	0.59	0.61	0.47	0.60	0.08	0.62	0.52	0.07	0.41	0.33	0.48	0.32
IGF-1, ng/mL	108.0	94.8	83.7	103.9	7.2	103.6	91.6	6.3	0.28	0.62	0.02	0.18

[a,b,c] Within a row, different superscripts differ after adjustment by the method of Tukey ($P < 0.05$).

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (P; +130 mEq/kg) or negative (N; -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Blood was sampled on d -9, -6, and -3 relative to calving.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[3] $25OHD_3$ = 25-hydroxyvitamin D_3; uOC = undercarboxylated osteocalcin; cOC = carboxylated osteocalcin; CTX-1 = C-telopeptide of type 1 collagen, IGF-1 = insulin-like growth factor 1.

[4] Values were transformed into natural log before analysis and back transformed values are reported.

Table 2. Postpartum concentrations of metabolites in plasma of dairy cows fed prepartum diets differing in level of dietary cation-anion difference (DCAD) and source of vitamin D[1]

	Positive		Negative			Parity			*P*-value[2]			
Item[3]	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
Vitamin D_3, ng/mL	4.25	2.22	3.76	1.35	0.60	2.80	3.00	0.47	0.27	0.001	0.75	0.74
$25OHD_3$, ng/mL	51.0	185.7	52.9	169.0	9.7	107.0	122.4	7.7	0.45	0.001	0.35	0.13
Adiponectin, µg/mL	21.2	20.0	23.9	18.5	4.7	23.0	18.8	5.8	0.87	0.35	0.54	0.59
uOC, ng/mL	2.01	2.88	2.51	2.46	0.68	3.32	1.61	0.67	0.95	0.52	0.46	0.09
cOC, ng/mL	44.8	41.4	47.7	40.3	4.9	60.8	26.3	3.9	0.86	0.28	0.68	< 0.001
CTX-1, ng/mL[4]	1.55	2.07	1.64	1.40	0.27	1.83	1.48	0.20	0.31	0.69	0.18	0.20
Glucose, m*M*	3.91	3.75	3.66	3.68	0.14	4.12	3.38	0.15	0.19	0.58	0.46	0.009
Insulin, ng/mL[4]	0.28	0.29	0.32	0.30	0.05	0.49	0.18	0.07	0.67	0.88	0.81	0.001
IGF-1, ng/mL	56.8	50.4	49.0	47.4	5.2	68.1	33.7	4.1	0.31	0.44	0.65	< 0.001

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (P; +130 mEq/kg) or negative (N; -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Blood was sampled on d 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, and 30 postpartum.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

[3] uOC = undercarboxylated osteocalcin; cOC = carboxylated osteocalcin; CTX-1 = C-telopeptide of type 1 collagen, IGF-1 = insulin-like growth factor 1.

[4] Values were transformed into natural log before analysis and back transformed values are reported.

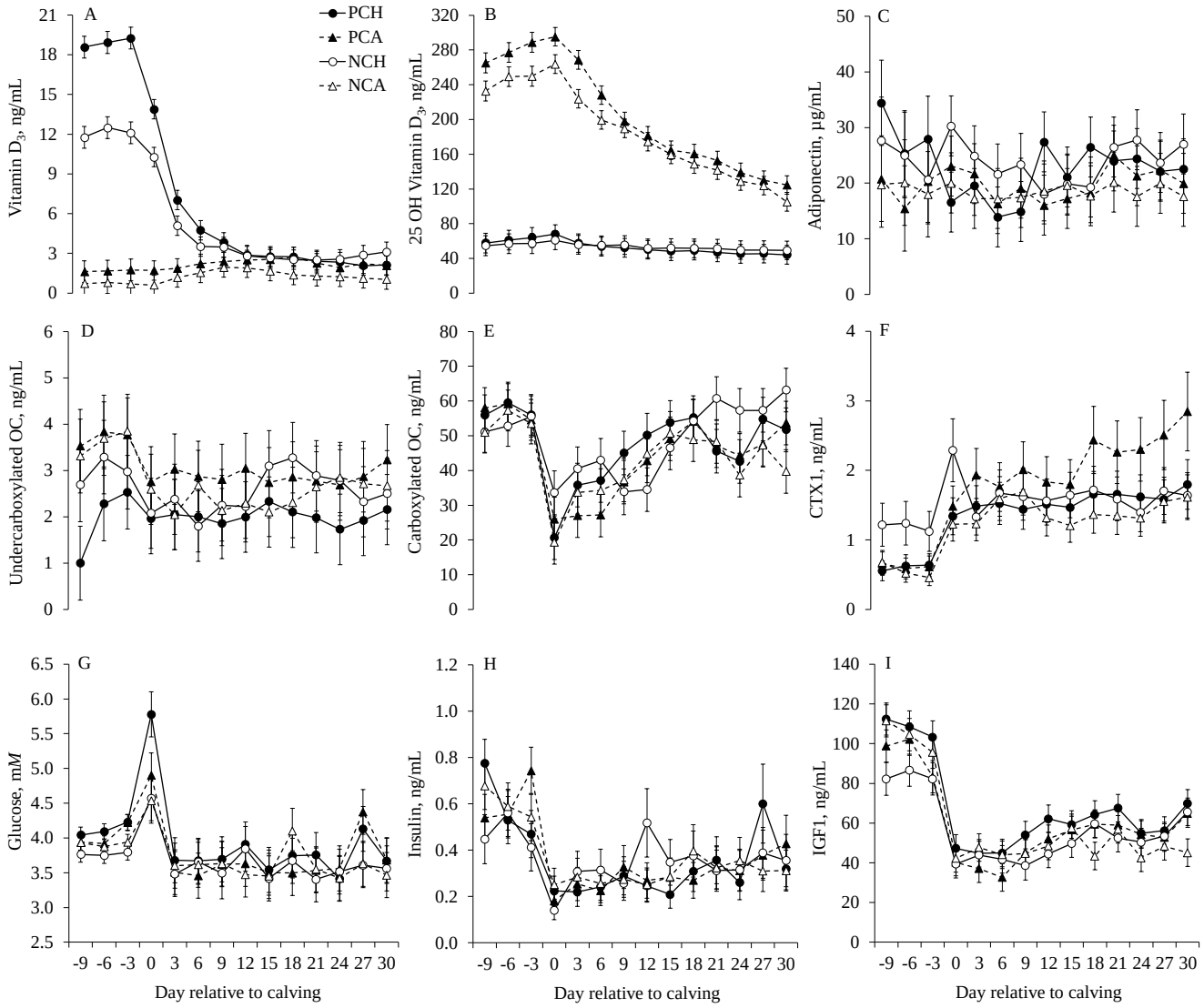

Figure 1. Plasma concentrations of vitamin D_3 (A), 25-hydroxyvitamin D_3 (B), adiponectin (C), undercarboxylated osteocalcin (OC; D), carboxylated OC (E), C-telopeptide of type 1 collagen (CTX-1; F), glucose (G), insulin (H), and insulin-like growth factor 1 (IGF-1; I) in plasma of cows from d -9 to 30 relative to calving. Prepartum cows starting at 252 d of gestation were fed diets with either positive (P; +130 mEq/kg) or negative (N: -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

Concentrations of adiponectin, uOC, cOC, and CTX-1 in plasma pre- and postpartum did not differ with treatments (Tables 1 and 2). Concentrations of adiponectin and uOC remained relatively stable throughout the transition period (Figure 1C and 1D), whereas those of cOC suffered dynamic changes during the transition period with an abrupt decline on the day of calving followed by a steady increase ($P < 0.001$) in the first 30 DIM in all four treatments

(Figure 1E). Concentrations of CTX-1 remained constant prepartum, and then increased immediately after calving and plateaued by 3 DIM (Figure 1F). Nulliparous cows had greater concentrations of cOC ($P < 0.01$), and tended to have greater concentrations of uOC($P \leq 0.10$) parous cows pre- and postpartum. Concentrations of CTX-1 prepartum, but not postpartum were greater ($P = 0.006$) in nulliparous than parous cows.

Level of DCAD altered ($P = 0.02$) prepartum concentration of glucose in plasma (positive = 4.07 vs. negative = 3.84 m*M*; Table 1), but not postpartum (Table 2). Source of vitamin D did not affect concentrations of glucose pre- or postpartum. Concentrations of glucose sharply increased on the day of calving and then declined by 3 DIM and remained relatively stable thereafter (Figure 1G). Concentrations of insulin pre- and postpartum did not differ with treatment, but those of IGF-1 prepartum were affected by the interaction ($P = 0.02$) between DCAD and vitamin D because within cows fed positive DCAD, those receiving PCH tended (P = 0.07) to have greater IGF-1 prepartum than those fed PCA. Concentration of insulin and IGF-1 decreased on the day of calving and then slowly increased ($P < 0.01$) with DIM in all four treatments (Figure 1H and 1I).

Production Performance

Intake of DM prepartum tended ($P = 0.10$) to be greater for cows fed positive than negative DCAD (positive = 11.9 vs. negative =11.0 ± 0.4 kg/d), but no differences were observed for source of vitamin D (Table 1). Treatment did not influence DMI postpartum. Parous cows ate more (P < 0.05) than nulliparous pre- and postpartum. Level of DCAD did not affect yields of milk or ECM, but cows fed CA produced more ($P < 0.05$) milk (CH = 30.1 vs. CA = 34.8 ± 1.5 kg/d) and ECM (CH = 35.0 vs. CA = 39.9 ± 1.7 kg/d) than cows fed CH (Table 3). Concentration of milk fat was greater (P = 0.01) for cows fed the negative compared with the positive DCAD (positive = 4.44 vs. negative = 4.94 ± 0.13%) resulting in increased ($P = 0.05$) milk fat yield (positive = 1.41 vs. negative = 1.63 ± 0.08 kg/d). Cows fed CA tended (P = 0.10) to have greater milk fat yield than those fed CH (CH = 1.43 vs. CA = 1.62 ± 0.08 kg/d). Concentration and yield of true protein did not differ with treatments. As expected, parous cows produced more fat and true protein than nulliparous cows (Table 3). Cows fed the diet with negative DCAD tended (P = 0.08) to be heavier postpartum than those fed the positive DCAD (608 vs. 571 ± 14 kg), and those fed NCA had the greatest BCS postpartum (Table 3). Nulliparous cows were lighter ($P < 0.001$) but had greater ($P = 0.006$) BCS than parous cows.

Table 3. Performance of dairy cows fed prepartum diets differing in level of dietary cation-anion difference (DCAD) and source of vitamin D[1]

	Positive		Negative			Parity			*P*-value[2]			
Item	CH	CA	CH	CA	SEM	Nulliparous	Parous	SEM	DCAD	VitD	DCAD x VitD	Parity
DMI, kg/d												
Prepartum	12.0	11.7	10.3	11.6	0.6	10.5	12.3	0.5	0.10	0.30	0.16	0.03
Postpartum	16.4	17.9	17.6	17.3	0.9	16.0	17.6	0.7	0.75	0.56	0.39	0.02
Milk, kg/d	27.9	35.3	32.3	34.3	2.0	25.7	34.8	1.5	0.39	0.02	0.17	< 0.001
ECM, kg/d	32.2	38.9	37.7	40.8	2.3	28.5	46.3	1.8	0.13	0.05	0.45	< 0.001
Fat												
%	4.50	4.37	4.93	4.94	0.18	4.46	4.92	0.14	0.01	0.75	0.71	0.02
Kg/d	1.28	1.54	1.57	1.69	0.11	1.11	1.93	0.08	0.05	0.10	0.55	< 0.001
True protein												
%	3.21	3.22	3.21	3.32	0.17	3.32	3.16	0.16	0.76	0.71	0.72	0.47
Kg/d	0.90	1.10	1.00	1.12	0.11	0.83	1.24	0.10	0.59	0.15	0.75	0.02
BW, kg	554.6	588.0	595.5	620.5	20.1	524.7	654.6	15.9	0.08	0.16	0.83	< 0.001
BCS, 1 to 5	3.19	3.07	3.06	3.40	0.13	3.37	2.99	0.10	0.44	0.38	0.08	0.006

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (P; +130 mEq/kg) or negative (N; -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA). Intake was measured from -21 to 42 DIM and other responses were measured for the first 49 DIM.

[2] DCAD = effect of DCAD (positive vs. negative); VitD = effect of source of vitamin D (cholecalciferol vs. calcidiol); DCAD x VitD = interaction between DCAD and vitamin D; Parity = effect of parity (nulliparous vs. parous).

Time Series Analysis

The most substantial effect sizes and 95% CI for cross-correlations between metabolites at lags 0 (on the same day) and ± 3, 6 and 9 d are presented in Table 4. Significant associations ($P < 0.05$) were observed between 25-hydroxyvitamin D_3 and vitamin D_3 cOC, (Figure 2a), uOC (Figure 2b), CTX-1, glucose, and IGF-1 (Figure 2c) at different lags, although for some of them, the association was present only in one treatment. For example, 25-hydroxyvitamin D_3 and vitamin D_3 associations were only observed for cows fed PCH, whereas for others such as 25-hydroxyvitamin D_3 and cOC or IGF-1, the association was observed in three of the four treatments. The pattern of ES for the association between 25-hydroxyvitamin D_3 and vitamin D_3 in the PCH group indicated positive associations on the same day and for 25-hydroxyvitamin D_3 3 d before, but a negative association for 25-hydroxyvitamin D_3 9 d before with vitamin D_3. Most of the associations for 25-hydroxyvitamin D_3 could be considered to be medium, > 0.25 or < -0.25 (Cohen 1988), although some, for example those of 25-hydroxyvitamin D_3 and IGF-1 9 d later are large (<-0.4) (Cohen 1988).

Significant associations were observed between vitamin D_3 and cOC, CTX-1, glucose, and IGF-1 (Figure 3). Large significant positive associations were present for IGF-1 and uOC and COC for all groups on the same day, and negative associations were present at negative and positive lags (Figure 4). The IGF-1 was positively associated with glucose 3 d before and negatively associated with glucose 9 d before (Table 4). Also, significant associations were observed between CTX-1 and glucose or insulin; and between glucose and insulin. The most consistent and significant of these findings are provided and discussed below.

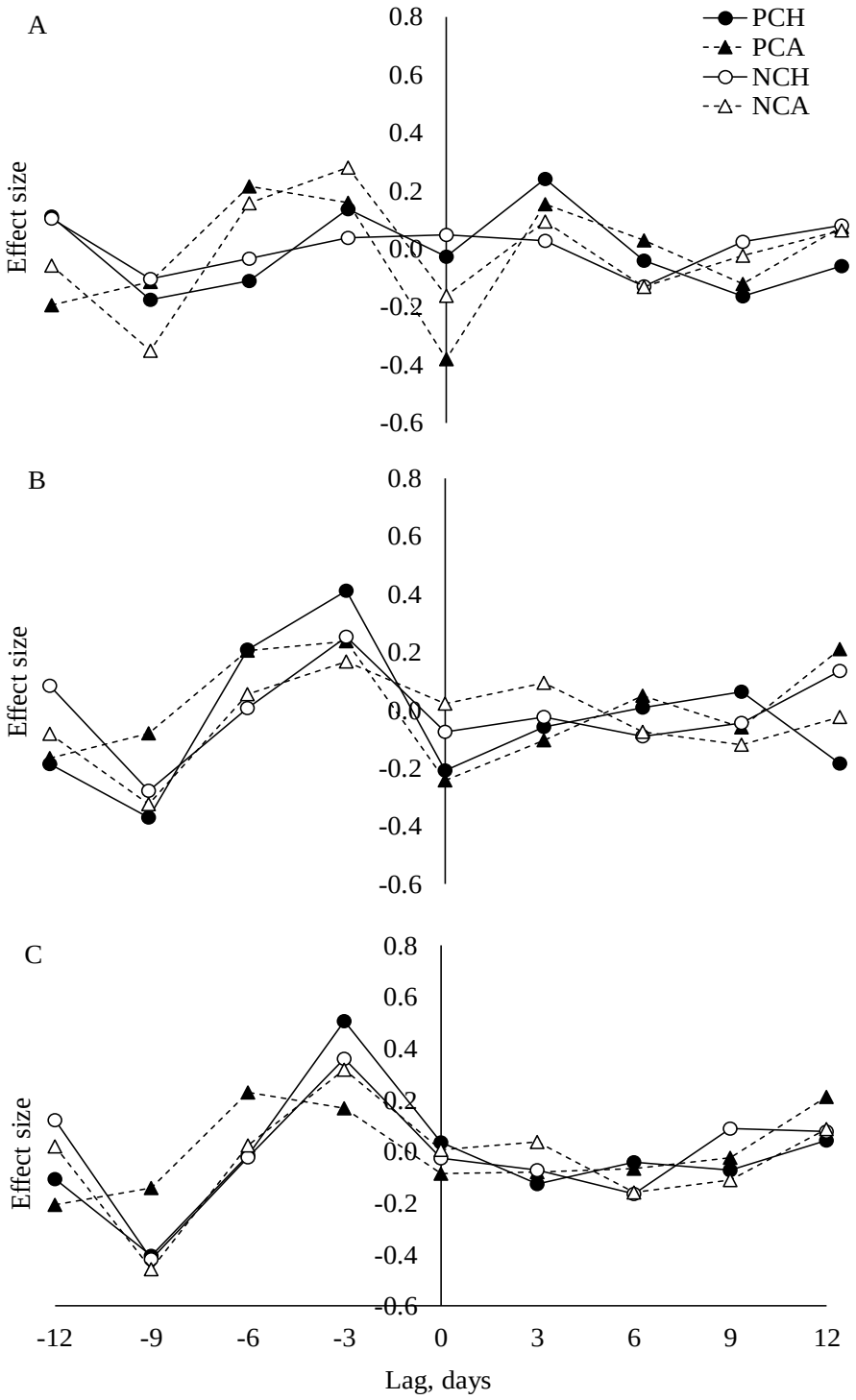

Figure 2. Effect size of the association of 25-hydroxyvitamin D_3 with undercarboxylated osteocalcin (A), carboxylated osteocalcin (B), and insulin-like growth factor 1 according to treatment and 3 d lags. Prepartum, cows starting at 252 d of gestation were fed diets with either positive (P; +130 mEq/kg) or negative (N; -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

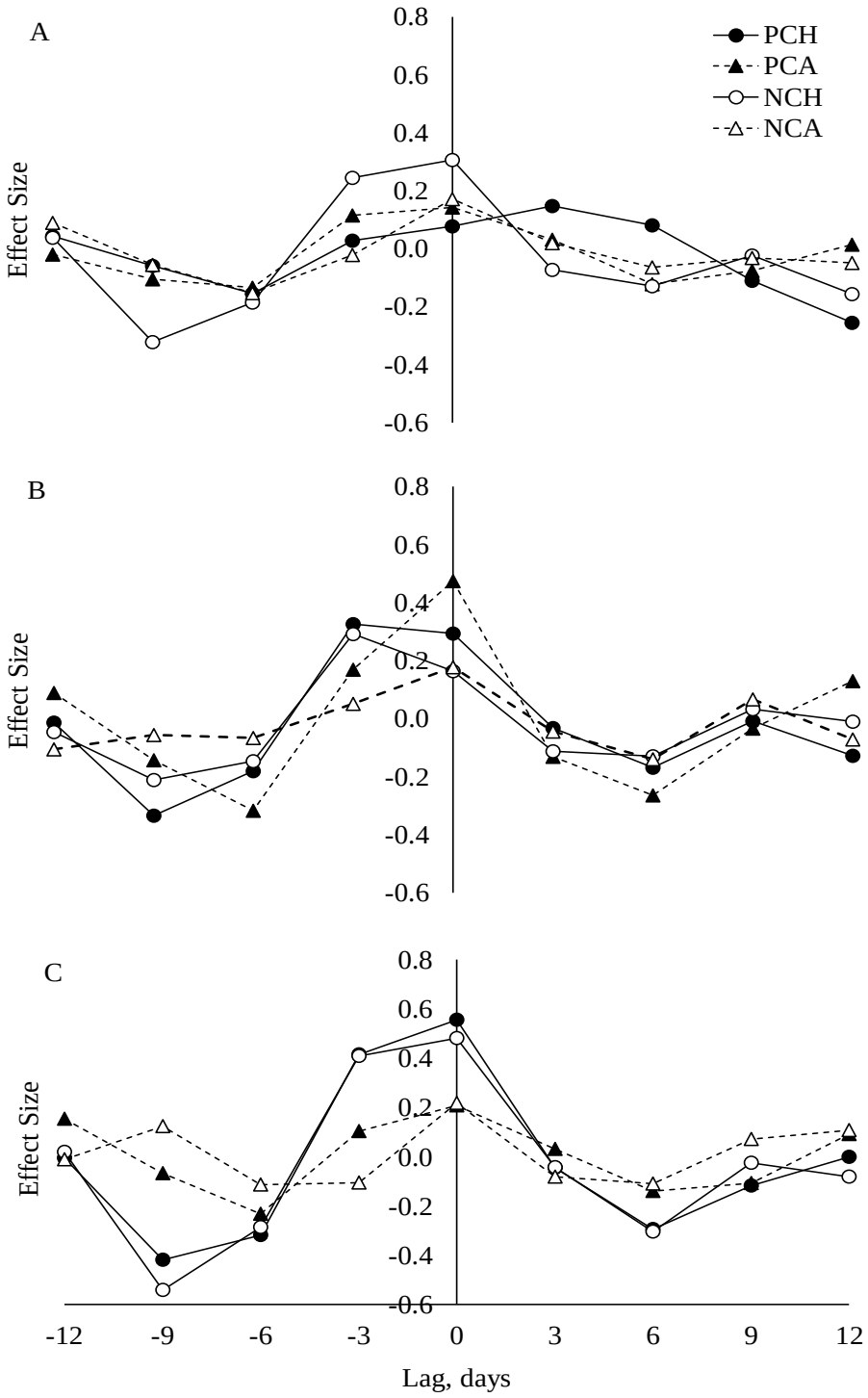

Figure 3. Effect size of the association of vitamin D_3 with undercarboxylated osteocalcin (A), carboxylated osteocalcin (B), and insulin-like growth factor 1 according to treatment and 3 d lags. Prepartum, cows starting at 252 d of gestation were fed diets with either positive (P; +130 mEq/kg) or negative (N; -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

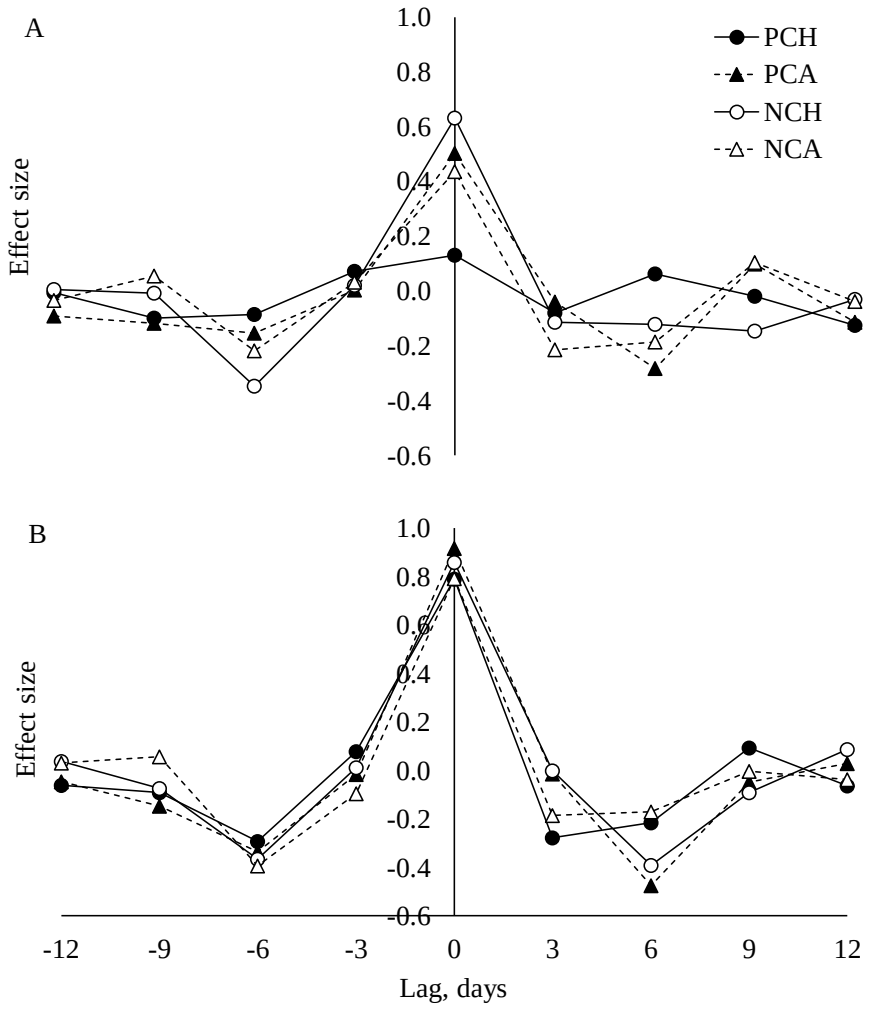

Figure 4. Effect size of the association between insulin-like growth factor (IGF) 1 with undercarboxylated osteocalcin (A) and carboxylated osteocalcin (B) according to treatment and 3 d lags. Prepartum, cows starting at 252 d of gestation were fed diets with either positive (P; +130 mEq/kg) or negative (N; -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA).

Table 4. Time-series analysis of relationships between blood concentrations of vitamin D, bone, and energy metabolites over 3-d lags up to ± 9 d, according to treatment[1]

Metabolite[2]			Lag -3 (9 d later)				Lag -2 (6 d later)				Lag -1 (3 d later)				Lag 0 (same day)				Lag +1 (3 d before)				Lag +2 (6 d before)				Lag +3 (9 d before)			
First	Second		PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA
25OHD$_3$	Vit. D$_3$	ES	-0.380	0.022	-0.068	-0.104	-0.223	0.086	-0.105	0.048	0.300	0.086	-0.001	0.002	0.536[b]	-0.041	0.409	0.044	-0.192	-0.223	-0.142	0.025	-0.191	0.023	-0.171	-0.019	-0.106	0.184	0.011	-0.096
		95% CI	-0.652	-0.187	-0.411	-0.382	-0.454	-0.123	-0.430	-0.161	0.034	-0.129	-0.220	-0.207	0.237	-0.448	-0.053	-0.236	-0.401	-0.608	-0.351	-0.184	-0.400	-0.302	-0.380	-0.298	-0.315	-0.046	-0.231	-0.384
			-0.109	0.231	0.276	0.174	0.008	0.295	0.220	0.257	0.566	0.302	0.217	0.211	0.835	0.366	0.871	0.323	0.017	0.163	0.067	0.234	0.018	0.347	0.038	0.260	0.103	0.415	0.253	0.191
25OHD$_3$	uOC	ES	-0.176	-0.114	-0.103	-0.352	-0.111	0.215	-0.033	0.157	0.137	0.159	0.038	0.280	-0.027[b]	-0.381	0.049	-0.162	0.241	0.155	0.028	0.094	-0.041	0.030	-0.130	-0.131	-0.164	-0.121	0.024	-0.024
		95% CI	-0.450	-0.335	-0.447	-0.561	-0.414	-0.084	-0.242	-0.067	-0.208	-0.350	-0.171	0.017	-0.282	-0.673	-0.160	-0.371	-0.033	-0.087	-0.216	-0.181	-0.378	-0.179	-0.339	-0.340	-0.373	-0.330	-0.294	-0.233
			0.097	0.107	0.240	-0.143	0.191	0.515	0.176	0.381	0.481	0.668	0.247	0.543	0.227	-0.088	0.257	0.047	0.515	0.397	0.273	0.369	0.295	0.239	0.079	0.078	0.045	0.088	0.343	0.185
25OHD$_3$	cOC	ES	-0.371	-0.081	-0.279	-0.325	0.208	0.205	0.006	0.054	0.412	0.237	0.252	0.168	-0.209	-0.242	-0.075	0.022	-0.058	-0.104	-0.024	0.093	0.008	0.049	-0.090	-0.075	0.063	-0.060	-0.044	-0.120
		95% CI	-0.643	-0.298	-0.490	-0.534	-0.008	-0.091	-0.203	-0.174	0.134	-0.071	-0.036	-0.041	-0.535	-0.515	-0.350	-0.187	-0.266	-0.356	-0.312	-0.116	-0.201	-0.164	-0.299	-0.284	-0.146	-0.269	-0.266	-0.329
			-0.100	0.137	-0.067	-0.116	0.424	0.502	0.215	0.281	0.690	0.545	0.541	0.377	0.117	0.031	0.200	0.231	0.151	0.147	0.264	0.302	0.217	0.262	0.119	0.134	0.272	0.149	0.179	0.089
25OHD$_3$	CTX-1	ES	0.219	0.024	0.192	0.389	0.050	-0.184	-0.105	-0.191	-0.137	0.026	-0.175	-0.061	-0.081	0.234	0.282	0.096	-0.100	-0.098	-0.072	-0.261	0.246	-0.040	0.005	0.128	-0.035	0.041	-0.025	0.136
		95% CI	0.011	-0.185	-0.035	0.140	-0.158	-0.442	-0.363	-0.400	-0.347	-0.183	-0.384	-0.278	-0.420	-0.134	0.018	-0.216	-0.524	-0.468	-0.388	-0.541	0.038	-0.310	-0.204	-0.164	-0.244	-0.250	-0.234	-0.073
			0.428	0.233	0.420	0.638	0.259	0.074	0.153	0.018	0.072	0.235	0.034	0.156	0.257	0.602	0.547	0.407	0.325	0.273	0.245	0.018	0.455	0.230	0.214	0.420	0.174	0.331	0.183	0.345
25OHD$_3$	Glucose	ES	-0.023[a]	-0.103	0.234	0.059	-0.246	-0.138	-0.222	-0.276	-0.214[b]	0.271	-0.120	0.048	0.796	0.158	0.210	0.162	-0.076	-0.191	0.048	-0.063	-0.217	-0.117	-0.020	0.053	-0.009	0.169	-0.125	-0.063
		95% CI	-0.232	-0.312	-0.016	-0.150	-0.465	-0.347	-0.531	-0.497	-0.423	-0.031	-0.495	-0.161	0.496	-0.334	-0.068	-0.099	-0.285	-0.442	-0.231	-0.272	-0.426	-0.476	-0.229	-0.215	-0.218	-0.095	-0.334	-0.324
			0.186	0.106	0.484	0.268	-0.027	0.071	0.087	-0.056	-0.005	0.572	0.256	0.257	1.097	0.650	0.488	0.424	0.133	0.060	0.327	0.146	-0.008	0.242	0.189	0.321	0.200	0.432	0.084	0.198
25OHD$_3$	IGF-1	ES	-0.406	-0.141	-0.418	-0.458	-0.015	0.229	-0.022	0.024	0.506	0.168	0.359	0.317	0.034	-0.086	-0.027	0.006	-0.127	-0.081	-0.073	0.037	-0.042	-0.067	-0.165	-0.160	-0.072	-0.025	0.088	-0.112
		95% CI	-0.615	-0.363	-0.727	-0.707	-0.332	-0.066	-0.231	-0.184	0.243	-0.200	0.077	0.108	-0.310	-0.333	-0.235	-0.203	-0.336	-0.290	-0.282	-0.172	-0.251	-0.276	-0.374	-0.390	-0.281	-0.234	-0.121	-0.321
			-0.197	0.082	-0.109	-0.209	0.302	0.524	0.186	0.233	0.769	0.536	0.641	0.526	0.379	0.161	0.182	0.215	0.081	0.127	0.136	0.246	0.167	0.142	0.043	0.069	0.137	0.184	0.297	0.097
Vit. D$_3$	cOC	ES	-0.335	-0.144	-0.212	-0.056	-0.181	-0.318	-0.147	-0.066	0.325	0.169	0.292	0.051	0.294	0.474	0.163	0.177	-0.032	-0.131	-0.112	-0.044	-0.169	-0.264	-0.129	-0.140	-0.008	-0.034	0.033	0.066
		95% CI	-0.633	-0.413	-0.488	-0.265	-0.389	-0.527	-0.356	-0.275	0.072	-0.040	0.066	-0.158	-0.123	0.265	-0.046	-0.032	-0.241	-0.340	-0.399	-0.253	-0.449	-0.499	-0.338	-0.349	-0.217	-0.314	-0.241	-0.239
			-0.038	0.124	0.064	0.153	0.028	-0.109	0.062	0.143	0.578	0.378	0.517	0.260	0.712	0.683	0.372	0.386	0.177	0.078	0.174	0.165	0.110	-0.029	0.080	0.069	0.201	0.246	0.308	0.371
Vit. D$_3$	CTX-1	ES	0.271	0.147	0.149	0.043	0.197	-0.075	-0.010	0.034	-0.281	-0.171	-0.242	-0.052	-0.342	-0.084	0.013	-0.156	0.025	0.165	0.226	0.047	0.391	0.072	0.112	0.130	0.092	-0.026	-0.197	-0.134
		95% CI	0.062	-0.086	-0.060	-0.333	-0.012	-0.427	-0.235	-0.201	-0.490	-0.432	-0.451	-0.399	-0.572	-0.507	-0.215	-0.524	-0.184	-0.104	-0.004	-0.228	0.154	-0.137	-0.096	-0.106	-0.117	-0.275	-0.406	-0.343
			0.480	0.380	0.358	0.420	0.406	0.277	0.216	0.268	-0.072	0.089	-0.034	0.296	-0.112	0.338	0.240	0.211	0.234	0.433	0.456	0.323	0.628	0.281	0.321	0.366	0.300	0.223	0.012	0.075
Vit. D$_3$	Glucose	ES	-0.036	0.115	0.126	0.051	-0.241	0.083	-0.166	-0.048	-0.236[c]	-0.304	-0.342	0.151	0.453[b]	0.189	0.408	-0.231	0.412[b]	0.094	0.217	0.022	-0.196	-0.119	-0.157	-0.010	-0.368[b]	-0.030	-0.235	0.133
		95% CI	-0.244	-0.094	-0.083	-0.198	-0.450	-0.261	-0.375	-0.257	-0.445	-0.692	-0.613	-0.058	0.164	-0.235	0.160	-0.492	0.203	-0.115	-0.019	-0.220	-0.405	-0.328	-0.451	-0.219	-0.577	-0.239	-0.453	-0.179
			0.173	0.324	0.335	0.300	-0.032	0.426	0.043	0.161	-0.027	0.084	-0.072	0.360	0.742	0.612	0.656	0.031	0.621	0.303	0.452	0.264	0.013	0.090	0.137	0.199	-0.159	0.179	-0.017	0.445
Vit. D$_3$	IGF-1	ES	-0.419[b]	-0.068	-0.541	0.124	-0.319	-0.232	-0.287	-0.113	0.415[b]	0.103	0.409	-0.106	0.556[b]	0.207	0.481	0.218	-0.045	0.031	-0.044	-0.082	-0.295	-0.140	-0.305	-0.110	-0.117	-0.108	-0.026	0.071
		95% CI	-0.628	-0.354	-0.828	-0.103	-0.536	-0.457	-0.496	-0.322	0.121	-0.106	0.200	-0.315	0.199	-0.044	0.181	0.009	-0.288	-0.217	-0.283	-0.291	-0.504	-0.349	-0.514	-0.319	-0.326	-0.364	-0.235	-0.138
			-0.210	0.218	-0.255	0.351	-0.101	-0.008	-0.078	0.096	0.709	0.312	0.618	0.103	0.913	0.458	0.782	0.427	0.197	0.280	0.195	0.127	-0.086	0.069	-0.096	0.099	0.092	0.149	0.183	0.280
CTX-1	Glucose	ES	-0.056	0.059	-0.120	0.158	0.136	0.031	-0.053	-0.043	0.156	0.099	0.042	-0.048	-0.105	0.049	0.462	0.105	-0.274	-0.316	-0.374	-0.237	0.005	-0.062	0.029	0.039	0.353	0.420	0.033	0.275
		95% CI	-0.265	-0.171	-0.329	-0.064	-0.073	-0.237	-0.262	-0.252	-0.223	-0.171	-0.254	-0.257	-0.345	-0.396	-0.074	-0.134	-0.483	-0.577	-0.649	-0.490	-0.204	-0.317	-0.216	-0.170	0.131	0.211	-0.303	-0.038
			0.153	0.289	0.088	0.380	0.345	0.299	0.156	0.166	0.534	0.370	0.338	0.161	0.135	0.494	0.997	0.344	-0.065	-0.055	-0.098	0.016	0.214	0.192	0.274	0.248	0.574	0.629	0.369	0.588
CTX-1	Insulin	ES	-0.206[b]	0.022	-0.184	-0.007	-0.040[a]	0.319	-0.067	-0.109	0.270[b]	0.046	0.366	0.078	-0.114[c]	-0.466	-0.240	-0.137	0.023	0.035	-0.104	0.086	-0.067	0.153	0.081	0.117	0.128	0.216	0.009	-0.017
		95% CI	-0.415	-0.209	-0.392	-0.216	-0.299	0.110	-0.288	-0.318	-0.019	-0.163	0.157	-0.168	-0.323	-0.688	-0.472	-0.345	-0.239	-0.176	-0.313	-0.201	-0.276	-0.106	-0.128	-0.119	-0.164	-0.030	-0.312	-0.312
			0.003	0.253	0.025	0.201	0.218	0.527	0.153	0.100	0.559	0.255	0.575	0.324	0.095	-0.245	-0.008	0.072	0.284	0.247	0.105	0.372	0.141	0.412	0.290	0.353	0.419	0.461	0.330	0.278
Glucose	Insulin	ES	0.092	-0.279	-0.087	-0.153	-0.118	-0.054	0.085	0.016	-0.074[a]	0.305	-0.283	-0.175	0.262	0.062	0.629	0.477	-0.095	-0.067	-0.161	0.067	0.011	-0.142	-0.199	-0.212	0.030	0.073	-0.003	0.018
		95% CI	-0.117	-0.488	-0.329	-0.432	-0.394	-0.419	-0.258	-0.279	-0.422	-0.032	-0.595	-0.384	-0.259	-0.456	0.029	-0.131	-0.434	-0.276	-0.590	-0.215	-0.241	-0.432	-0.515	-0.469	-0.179	-0.182	-0.212	-0.191
			0.301	-0.070	0.155	0.126	0.157	0.310	0.428	0.312	0.273	0.642	0.029	0.034	0.783	0.579	1.229	1.084	0.244	0.142	0.267	0.349	0.263	0.148	0.117	0.045	0.239	0.329	0.206	0.227

Table 4 Continued. Time-series analysis of relationships between blood concentrations of vitamin D, bone, and energy metabolites over 3-d lags up to ± 9 d, according to treatment[1]

Metabolite[2]			Lag -3 (9 d later)				Lag -2 (6 d later)				Lag -1 (3 d later)				Lag 0 (same day)				Lag +1 (3 d before)				Lag +2 (6 d before)				Lag +3 (9 d before)			
First	Second		PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA	PCH	PCA	NCH	NCA
IGF-1	uOC	ES	-0.098	-0.117	-0.008	0.054	-0.086	-0.153	-0.348	-0.219	0.072	0.004	0.021	0.033	0.131	0.503	0.632	0.437	-0.079	-0.040	-0.113	-0.214	0.063	-0.283	-0.121	-0.186	-0.020	0.098	-0.147	0.105
		95% CI	-0.307	-0.326	-0.217	-0.155	-0.380	-0.435	-0.557	-0.428	-0.148	-0.205	-0.197	-0.220	-0.215	0.248	0.276	0.188	-0.336	-0.249	-0.350	-0.616	-0.146	-0.491	-0.336	-0.484	-0.229	-0.137	-0.356	-0.175
			0.111	0.092	0.201	0.263	0.209	0.128	-0.139	-0.010	0.292	0.213	0.239	0.285	0.476	0.758	0.989	0.687	0.177	0.169	0.124	0.188	0.272	-0.074	0.094	0.113	0.189	0.332	0.062	0.385
IGF-1	cOC	ES	-0.092	-0.147	-0.074	0.056	-0.293	-0.335	-0.366	-0.394	0.078	-0.016	0.012	-0.096	0.790	0.918	0.859	0.794	-0.277[c]	-0.014	-0.000	-0.186	-0.216[c]	-0.474	-0.391	-0.170	0.093	-0.047	-0.090	-0.002
		95% CI	-0.301	-0.356	-0.283	-0.153	-0.502	-0.599	-0.575	-0.603	-0.131	-0.225	-0.197	-0.305	0.422	0.709	0.395	0.585	-0.486	-0.230	-0.220	-0.420	-0.425	-0.683	-0.600	-0.379	-0.116	-0.258	-0.329	-0.211
			0.117	0.062	0.135	0.265	-0.084	-0.070	-0.157	-0.186	0.287	0.193	0.221	0.113	1.158	1.127	1.323	1.003	-0.068	0.201	0.220	0.047	-0.007	-0.265	-0.182	0.039	0.302	0.164	0.148	0.207
IGF-1	CTX-1	ES	-0.050	0.182	-0.054	-0.036	0.174	0.055	0.027	0.141	-0.074	-0.104	0.016	0.108	-0.224	-0.273	-0.173	-0.358	0.082	-0.018	0.282	0.107	0.132[a]	0.389	-0.007	-0.041	-0.052	-0.004	-0.122	-0.135
		95% CI	-0.276	-0.027	-0.263	-0.245	-0.035	-0.167	-0.182	-0.068	-0.283	-0.313	-0.193	-0.101	-0.433	-0.482	-0.382	-0.611	-0.131	-0.237	-0.075	-0.102	-0.083	0.180	-0.216	-0.334	-0.261	-0.250	-0.366	-0.375
			0.175	0.391	0.155	0.173	0.383	0.277	0.236	0.350	0.135	0.105	0.225	0.317	-0.015	-0.064	0.036	-0.106	0.295	0.201	0.639	0.316	0.347	0.598	0.202	0.251	0.156	0.242	0.122	0.106
IGF-1	Glucose	ES	-0.015	0.015	-0.062	-0.021	-0.079	-0.068	-0.015	-0.004	-0.025	-0.052	-0.036	-0.032	-0.169	-0.046	-0.004	-0.110	0.576[a]	0.460	0.221	0.275	-0.019	-0.008	0.018	0.073	-0.265	-0.553	-0.257	-0.322
		95% CI	-0.224	-0.194	-0.271	-0.229	-0.288	-0.277	-0.248	-0.213	-0.252	-0.261	-0.245	-0.241	-0.535	-0.271	-0.243	-0.329	0.257	0.251	0.012	0.059	-0.228	-0.229	-0.192	-0.136	-0.486	-0.762	-0.626	-0.531
			0.194	0.224	0.147	0.188	0.130	0.141	0.219	0.205	0.202	0.157	0.173	0.177	0.197	0.178	0.236	0.109	0.896	0.669	0.430	0.490	0.190	0.214	0.229	0.282	-0.044	-0.344	0.111	-0.113

[1] Prepartum cows starting at 252 d of gestation were fed diets with either positive (P; +130 mEq/kg) or negative (N; -130 mEq/kg) DCAD and containing either 3 mg of cholecalciferol (CH) or 3 mg of calcidiol (CA) resulting in four treatments, PCH, PCA, NCH and NCA. Blood was sampled and analyzed every 3 d from d -9 prepartum to 30 DIM.

A relationship between two metabolites, for a given treatment, is significantly different from 0 (as determined by the 95% CI) when shaded with blue or red. Light blue cells indicate a positive relationship between the two metabolites, whereas light red cells indicate a negative, or inverse, relationship between the two metabolites. The effect size (ES) at lag 0 reflects the pooled ES for the cows of the correlations between the two metabolites measured on the same day. The negative lags (i. e. -1, -2, and -3) present the pooled ES of the cows for the cross correlations between the first metabolite with the second metabolite 3 to 12 d later. Effects of treatment on relationships are indicated by superscripts. Effect size was calculated based on correlation coefficients of detrended data and have not been back-transformed.

Superscripts indicate instances in which treatment significantly influenced the relationship between the pair of metabolites; [a] indicates an influence of DCAD on the relationship between two metabolites, [b] indicates an influence of vitamin D on the relationship between the pair metabolites, and [c] indicates that the interaction of the vitamin D and DCAD influenced on the relationship between the pair of metabolites. [d] 95% CI upper (top) and lower (bottom) are presented for each pair of metabolites, at each lag, for each treatment.

[2] $25OHD_3$ = 25-hydroxyvitamin D_3; Vit. D_3 = vitamin D_3; uOC = undercarboxylated osteocalcin; cOC = carboxylated osteocalcin; CTX-1 = C-telopeptide of type 1 collagen; IGF-1 = insulin-like growth factor 1.

DISCUSSION

The intent of the study was to identify if interactions between bone and energy metabolism in dairy cattle during the transition period were present, and to determine if these are influenced by dietary vitamin D and DCAD interventions fed before calving. Improved understanding of these metabolic pathways may be important in dairy cow management because of the need for cows to adapt to extreme increases in energy, nutrient and mineral demands at the onset of lactation. Where cows adapt poorly an increased incidence of metabolic disease, poor fertility and decreased production occur. We used time series analyses to examine relationships between metabolites over periods as long as ± 12 d and identify how these could be altered by dietary vitamin D and DCAD treatment.

In this experiment, there is a proposed hierarchy of effects with a presumption that the form of vitamin D supplementation and DCAD of diet would influence responses; firstly those associated with vitamin D metabolism and, subsequently, those related to metabolites influenced by these. Notwithstanding the treatment effects, the fundamental associations between metabolites would underpin responses observed over time. Supplementation with CH or CA increased blood concentrations of the respective metabolite, as is consistent with previous studies (Taylor et al., 2008, Wilkens et al., 2012, Weiss et al., 2015). This effect was enhanced for cows that were fed positive DCAD diets before calving which may, in part, reflect an increased DMI, and hence, intake of supplement; however, the effect appears to be larger than the increase in DMI would explain. It is possible that the slow clearance of 25-hydroxyvitamin D_3 from the body may produce a cumulative effect resulting in markedly increased concentrations.

The amounts of vitamin D treatments fed produced blood vitamin D_3 and 25-hydroxyvitamin D_3 concentrations that exceed the concentrations that could be expected under normal conditions (50-80ng/mL 25-hydroxyvitamin D_3, (Horst et al., 1994)). Cows produce the majority of vitamin D_3 in the skin in response to UV exposure. This vitamin D is predominantly bound to vitamin D binding protein, and some to albumin, for transport in the blood (Bikle et al., 1986). However, oral sources of vitamin D are taken up with chylomicron lipid (Haddad et al., 1993). It is possible that these different presentations of vitamin D may affect metabolism, especially in the liver, as vitamin D, or 25-hydroxyvitamin D_3, bound to lipid may assume a similar clearance pattern to that of fat.

A positive ES for 25-hydroxyvitamin D_3 and vitamin D_3 was observed on the same day (ES = 0.54) and 3 d later (ES = 0.30) for only the PCH cows (Table 4). The PCH cows were fed CH in pharmacological doses and the positive ES on the same day, with a similar, but non-significant ES for the NCH group supports the hydroxylation of 25-hydroxyvitamin D_3 to vitamin D_3 in the liver and elsewhere, primarily through the 25-hydroxylase action of CYP2R1. Horst and Reinhardt (1983) discuss evidence for a product-based inhibition of 25-hydroxylase action in chickens and rats (Hughes et al., 1977) and provide evidence of lagged relationships between vitamin D_3 and 25-hydroxyvitamin D_3 in cattle. A negative ES was observed between these metabolites, again only in the PCH group 9 d later (ES = -0.38), suggesting that 25-hydroxyvitamin D_3 may exert feedback on vitamin D_3, but not vice versa, at least in response to the PCH treatment or possibly differences in affinity to the vitamin D binding protein. The latter finding supports those findings and understandings of Horst and Reinhardt (1983). It is unclear why these findings were only significant in the PCH group, however, it is clear (Martinez et al., 2017 a,b; Rodney et al., 2017 – Chapters 6, 7 and 8) that the PCH group were the least successfully adapted in the transition period in this experiment, suggesting a biological basis for the findings.

For several relationships including either vitamin D metabolite (vitamin D_3 or 25-hydroxyvitamin D_3) a significant effect of vitamin D treatment was observed, which may have reflected the substantial increase in supply of the respective vitamin D metabolite through the dietary interventions. Such instances include the associations between 25-hydroxyvitamin D_3 and IGF-1 3 d later (ES = 0.51, 0.17 NS, 0.36, and 0.32 (NS indicates effect was not significant); between 25-hydroxyvitamin D_3 and IGF-1 9 d later (ES = -0.41, -0.14 NS, -0.42, and -0.46); for vitamin D_3 and IGF-1 on the same day (ES = 0.56, 0.21, 0.48, and 0.22 NS); and vitamin D_3 and IGF-1 3 d later (ES = 0.42, 0.10 (NS), 0.41, and -0.11 (NS) for PCH, PCA, NCH, and NCA groups, respectively. A physiological feedback mechanism is characterized by alternating positive and negative influences between two metabolic pathways and indicated by such alternating positive and negative ES between metabolites. Such physiological feedback mechanisms have been identified for energy metabolites and bone hormones in murine models (Lee et al., 2007; Clemens and Karsenty 2011) and are evident here between both 25-hydroxyvitamin D_3 and IGF-1 (Figure 2c) and for vitamin D_3 and IGF-1 (Figure 3c). The vitamin D_3 and IGF-1 responses were influenced by vitamin D treatment, as the ES were further from zero in CH supplemented groups, whereas the vitamin D_3 and IGF-1 ES responses were present in all but the PCA group and of similar magnitude. Further differences were observed

between the 25-hydroxyvitamin D_3 and vitamin D_3 relationships with IGF-1 with calcidiol having a 3 d lagged positive association with IGF-1, followed 6 d later with a negative association, whereas there is an immediate positive association with vitamin Dl and IGF-1 with evidence of negative feedback at lags of 6 and 9 d. The actions of 25-hydroxyvitamin D_3 and IGF-1 are related in human studies of cancer (Tuohimaa 2008) and diabetes (Hyppönen et al., 2008; Kamycheva et al., 2012) and 25-hydroxyvitamin D_3, growth hormone and IGF-1 act synergistically to increase and integrate bone growth in man (Giustina et al., 2008). Studies show that mice without a vitamin D receptor have 30% lower IGF-1 than mice with that receptor (Song et al., 2003). Interestingly, IGF-1 upregulates 1α-hydroxylase enzyme activity in the kidney, resulting in increased production of 1,25-dihydroxyvitamin D_3 (Wei et al., 1997) and less production of 24,25-dihydroxyvitamin D_3 (Tuohimaa 2008). Further, 25-hydroxyvitamin D_3 also exerts positive feedback to increase the number of intracellular IGF-1 receptors (Ogata et al., 2000). Vitamin D treatment increased both 25-hydroxyvitamin D_3 and IGF-1 concentrations in man (Ameri et al., 2013).

Blood concentrations of IGF-1 decreased in all groups at the onset of lactation, as previously observed (Ronge et al., 1988, Vega et al., 1991, Moore et al., 2000). Before calving, cows fed the NCH diet had lower blood IGF-1 concentrations than cows fed the other treatments (61 vs 73 to 81 ng/mL for NCH and other treatment, respectively). Contrastingly, Moore et al. (2000) did not identify a significant response of DCAD on blood IGF-1 concentrations. IGF-1 is important for cellular growth and differentiation, stimulates mammary cell proliferation (McGrath et al., 1991), and mediates the action of growth hormone (**GH**) on mammogenesis (Ruan et al., 1995, Plath-Gabler et al., 2001). Mammary cell apoptosis in both mice and cattle is inhibited by IGF-1. (Neuenschwander et al., 1996, Accorsi et al., 2002). Further, and importantly, IGF-1 stimulates blood flow to the mammary gland, mammary tissue DNA synthesis and milk production (Baumrucker, 1986).

The increased mineral demand associated with lactation may have been, at least partly, addressed in cows on all dietary treatments through increased bone break down and decreased bone remodeling, as identified by decreased cOC and uOC, and increased CTX-1 at the onset of lactation (Table 1; Rodney et al., 2017 – Chapter 6). Liesegang et al. (2000) reported a decrease in OC concentrations in early lactation that returned to prepartum levels by 30 DIM, a similar pattern to that observed in this study for cOC (Figure 1E). Liesegang et al. (2000) suggested that higher producing cows mobilize more Ca from bone. However, in the present experiment, blood

concentrations of OC, cOC and CTX-1 did not differ between treatments, despite CA supplemented cows producing 3.8 L more milk per day (Table 1). Neither CA nor CH supplementation was associated with blood OC concentration, when fed as a single 15mg dose (Taylor et al., 2008) although calcitriol injection elevated plasma total OC, and uOC concentrations in non-pregnant, non-lactating cows (Kim et al., 2011) and blood CTX-1 concentrations in sheep (Wilkens et al., 2010). The metabolic acidosis resulting from a negative DCAD diet may have been expected to influence bone metabolism indicators as a result of increased bone resorption and decreased osteogenesis (Wu et al., 2008), but only prepartum CTX-1 concentrations tended to be increased in the cows receiving the NCH treatment prepartum (P = 0.068), and other studies found no effect of negative DCAD diets on markers of bone metabolism in cattle (Moore et al., 2000). In the larger dataset examined (Rodney et al., 2017 – Chapter 6), there was no effect of DCAD or vitamin D treatment on PTH, OC, uOC or CTX-1. However, in that study (Rodney et al., 2017 – Chapter 6), the ratio of cOC to CTX-1, which is an index of bone turnover, had interactions ($P < 0.05$) between DCAD and day and vitamin D. Cows fed the diet with positive DCAD had a greater ($P < 0.01$) ratio on the day before calving than those fed the diet with negative DCAD and cows fed CA had greater ($P < 0.01$) ratio on the day before calving than those fed CH.

When examined over time, blood 25-hydroxyvitamin D_3 and cOC concentrations were associated at negative lags (Table 4). The cross correlation of 25-hydroxyvitamin D_3 and cOC 9 d later was negative (ES < -0.27) in all but the PCA group (ES = - 0.081 NS), and with a positive ES 3 d later (ES > 0.16), although this was significant only in the PCH group (ES = 0.412). These findings indicate that 25-hydroxyvitamin D_3 exerts a negative feedback on cOC concentrations, an action consistent with a role of vitamin D in skeletal and energy metabolism. Lips (2006), in a review of 1,25-dihydroxyvitamin D_3 noted it's actions to upregulate or down-regulate OC production in a process that can take place over days.

The ES for 25-hydroxyvitamin D_3 and glucose on the same day were positive (ES > 0.16), but only significant for the PCH group (ES = 0.80). In the PCH group there was also a significant relationship of 25-hydroxyvitamin D_3 3 d before (ES = -0.21) and 6 d before with glucose (ES = -0.25), the latter of which NCA was also significant; (ES = -0.28, all other groups had ES > -0.14). The ES between vitamin D_3 and glucose was positive on the same day (ES = 0.45, 0.19 (NS), 0.41, and 0.23 (NS) for PCH, PCA, NCH, and NCA respectively) and negative 3 d before (ES = -0.24, -0.30 (NS), -0.34, and 0.15 (NS) for PCH, PCA, NCH, and NCA groups,

respectively) for CA groups. These findings show a positive association between either 25-hydroxyvitamin D_3 and vitamin D_3 with glucose on the same day, but not significantly for all groups and evidence of negative feedback at lags of 3 and 6 d. The results are very consistent with the small positive or neutral effects on blood glucose concentrations in humans supplemented with vitamin D (George et al., 2012).

An association between OC and IGF-1 had not been identified in cattle. Large positive ES were present between IGF-1 and both uOC (ES > 0.79 for all groups) and cOC (ES > 0.43 for all but PCH) on the same day (Figures 4A and B). Feedback was identified for uOC, and particularly, cOC, with IGF-1 with negative feedback observed 6 d later (ES < -0.29 for IGF-1 and cOC and ES < -0.21 for N DCAD groups for IGF-1 and uOC) as well as 6 d before for IGF-1 and cOC, in all but the NCA group (ES < 0.21, ES = -0.170 for NCA). The IGF-1 is essential for bone growth and maintenance of bone density (Ogata et al., 2000), increasing osteoblast proliferation and differentiation (Yeh et al., 1997, Li et al., 2009), and stimulating an increase in the concentration of OC in the osteoblasts (Li et al., 2009). There was little evidence of a treatment effect perturbing the associations between IGF-1 and OC.

The conversion of uOC to cOC involves the vitamin K-dependant carboxylation of glutamic residues to gamma-carboxyglutamic acid (Gla), which have high affinity for bone. An increased concentration of uOC in mice, or selective inhibition of the *Esp* gene which in turn inhibits carboxylation, increased insulin production and sensitivity (Lee et al., 2007). These observations suggested that uOC is the biologically active metabolite. Similar patterns of significance (ES negative 6 d later, positive on the same day, negative 3-6 d before) were present between IGF-1 and both forms of OC, however these were greater for cOC. This and the findings for 25-hydroxyvitamin D_3 and cOC, suggests that, at least in cattle, both uOC and cOC may have marked biological activity.

A negative ES existed between IGF-1 and glucose 9 d before (ES < -0.26) in all but NCH groups (ES = -0.26 NS), however significant positive ES betweenIGF-1 and glucose 3 d before were present in all groups. This effect was stronger in groups fed positive DCAD diets (ES = 0.58 and 0.46 for PCH and PCA treatments vs 0.22 and 0.28 for NCH and NCA, respectively). A positive ES was identified between IGF-1 and insulin on the same day in PCA and NCH groups (ES = 0.29 and 0.35, respectively). Feeding positive DCAD diets resulted in increased glucose concentrations prepartum, possibly due to increased intake or decreased disposal of glucose or

increased insulin resistance. Associations between IGF-1 and glucose are logical and reflect the stimulatory effect of glucose on production mediated through IGF-1. A negative feedback for this relationship is consistent with the tight homeostatic control of blood glucose.

Insulin secretion and sensitivity are stimulated by OC (Lee et al., 2007, Wolf, 2008; Clemens and Karsenty 2011), though this effect was not identified in this study. However, the association between uOC and cOC and glucose concentrations identified in mice showing greater uptake of glucose in peripheral tissues and increased insulin sensitivity (Lee et al., 2007) was supported. There was a negative ES for glucose with both uOC and cOC 9 d before in the CA groups (ES < -0.34), a positive ES for uOC and glucose 3 d before in the CA groups (ES > 0.25), and cOC and glucose 3 d before in the positive DCAD groups (ES > 0.32). These associations are similar to those for IGF-1 and glucose, confirming strong associations between glucose, IGF-1 and OC as part of the homeorhetic control of metabolism. Further, the negative association of CTX-1 with glucose concentrations 3 d before (ES <-0.27) in all but NCA (ES = -0.24 NS) highlights the anabolic signals associated with increased blood glucose concentrations. Similarly, there was evidence also that CTX-1 and insulin were negatively associated on the same day in the NCH and PCA groups, and positive associations were present for CTX-1 3 or 6 d before with insulin in the NCH and PCA groups, respectively. These findings are consistent with strong associations positive found between CTX-1 and OC and CTX-1 and insulin in pregnant women (Winhofer et al., 2012). Further, concentrations of CTX-1 are higher in women with gestational diabetes (Winhofer et al., 2012), a condition characterized by insulin resistance and with a similar insulin resistance late gestation and early lactation (Bell 1995; McNamara 1991). Clemens and Karsenty (2011) discuss the evidence that insulin is involved in the regulation of bone resorption through a forward regulating loop that includes OC, which under acidic conditions decarboxylates to uOC and increases insulin sensitivity of peripheral tissues and increases insulin production from pancreatic β-cells.

Vitamin D was associated with bone mobilization, as a positive ES was identified between 25-hydroxyvitamin D_3 and CTX-1 9 d later in PCH and NCA groups (ES = 0.22 and 0.39, respectively), and a negative effect of vitamin D_3 and CTX-1 3 d later in CH groups (ES < -0.24). A negative ES was identified between IGF-1 and CTX-1 on the same day in all but NCH (ES < -0.22 and = -0.17 for NCH). This effect was coupled with a positive association between IGF-1 and both uOC and cOC on the same day, indicating that IGF-1 may preserve bone mass, a

finding consistent with Ogata et al., (2000) and the very substantial role of growth hormone and IGF-1 in bone development (Giustina et al., 2008).

A negative association was also identified for CTX-1 with glucose 3 d before in all groups but NCA (ES < - 0.27, and ES = -0.24 for NCA), and for CTX-1 and insulin on the same day in NCH and PCA groups (ES = -0.24 and -0.47, respectively). The feedback mechanism that exists between glucose and insulin is well established, but was not observed here, as effect sizes between the two metabolites were generally close to zero. It is possible that the timing of this feedback between glucose and insulin differed from the 3 d lags examined in this study. It is very unlikely that any particular lag will be optimal to assess all relationships between metabolites. A 3 d lag was suitable for this type of analysis (Lean et al., 1992a, Lean et al., 2014), but it may also be beneficial to examine these relationships using more frequent sampling, particularly over the metabolically volatile time of calving. It is also possible that the constant gluconeogenic state of cows and lack of insulin sensitivity in the immediate periparturient period (Baldwin and Smith, 1979) may have limited expression of this effect. Notwithstanding the latter suggestion, in the larger study (Martinez et al., 2017a – Chapter 7), cows fed the diet with positive DCAD had greater prepartum concentrations of glucose, insulin and IGF-1 compared with cows fed the diet with negative DCAD and the increases in insulin and IGF-1 with the positive compared with the negative DCAD diet were more exacerbated in cows supplemented with CA. These findings suggest the possibility that some degree of insulin sensitivity may be restored in cows fed a negative DCAD diet.

Younger, nulliparous cows had increased concentrations of bone and energy related metabolites (uOC, cOC, CTX-1, IGF-1, glucose, and insulin) in their blood when compared with multiparous cows, a finding consistent with Sato et al. (2011) and Taylor et al. (2008), who found that younger cows had increased serum OC concentrations. This finding possibly reflects a need to accrete and remodel bone to a greater extent than older cows, or less irreversible loss of glucose and minerals to milk production. Prepartum, plasma CTX-1 concentrations were higher in nulliparous than multiparous cows, although these were similar between parities in lactation. Moore et al. (2000) found plasma hydroxyapatite concentrations were higher in heifers than cows, but other studies found no relationship between parity and bone metabolism (Block, 1984). The IGF-1 concentrations were also higher in nulliparous than multiparous cows, both before and after calving, a finding which is consistent with Moore et al. (2000).

Relationships were not identified between adiponectin and OC (results not shown), despite the stimulatory action of OC on adiponectin (Tschritter et al., 2003). Adiponectin is also associated with increased proliferation and differentiation of osteoblasts (Berner et al., 2004) resulting in increased bone deposition (Kanazawa et al., 2007). Nor were any clear associations present between adiponectin and indicators of energy metabolism. These associations may have been expected as adiponectin increases glucose uptake by skeletal muscle and may suppress hepatic gluconeogenesis (Yamauchi et al., 2002), and a positive correlation between plasma insulin and adiponectin has been observed (Singh et al., 2014).

CONCLUSIONS

This study examines homeostatic and homeorhetic relationships between vitamin D, bone and energy metabolism in dairy cattle. The hypothesis that homeorhetic relationships exist is only rarely specifically tested by examining relationships between variables over time. This study provides support that relationships between bone and energy metabolism observed in man and mice also exist in cattle. Further, there was strong evidence that active forms of vitamin D and CA were part of the integrated cross-talk between bone and energy metabolism. In particular, the pattern of strong positive ES between vitamin D_3 and IFG1, and between OC and IGF-1 on the same day and positive and negative ES at other lags provides evidence for a feedback mechanism between these. The relationship between OC and IGF-1 had not been identified in cattle. As these mechanisms were observed with both uOC and cOC it suggested, at least in cattle, that both forms of OC may have biological activity. The linkage between OC and insulin identified in murine studies was not observed here, although associations between OC and glucose were seen that were similar to those of IGF-1 and glucose, confirming strong associations between glucose and these hormones. These mechanisms may be an important part of the physiological process by which pre-calving interventions upregulate and integrate metabolism and have positive and long-lasting effects on production and health.

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Supplementary Table 1. Dietary ingredients and nutrient composition of diets fed pre- and postpartum

	Prepartum diets[1]				
	Positive DCAD		Negative DCAD		
Item	Cholecalciferol	Calcidiol	Cholecalciferol	Calcidiol	Lactation diet
Ingredients, % of DM					
Corn silage	61.80	61.80	61.80	61.80	25.8
Bermuda hay	9.10	9.10	9.10	9.10	7.5
Brewer's grains, wet	---	---	---	---	8.6
Corn grain, finely ground	---	---	---	---	25.9
Citrus pulp	9.10	9.10	9.10	9.10	5.2
Soybean hulls	---	---	---	---	8.6
Whole cottonseed	6.40	6.40	6.40	6.40	3.4
Soybean meal, solvent extract	---	---	4.50	4.40	8.2
Soybean meal, cooker-processing[2]	11.18	11.08	---	---	3.3
Acidogenic supplement[3]	---	---	7.25	7.25	--
Cholecalciferol mixture[4]	0.08	---	0.08	---	--
Calcidiol mixture[5]	---	0.18	---	0.18	--
MgO + NaCl	0.54	0.54	---	---	---
Prepartum mineral[6]	1.80	1.80	1.80	1.80	--
Postpartum protein and mineral[7]	---	---	---	---	3.5
DM, %	55.4 ± 1.0	55.6 ± 1.0	55.4 ± 1.0	55.4 ± 1.0	69.5 ± 0.6
Nutrients, DM basis (± SD)[8]					
Net energy,[9] Mcal/kg	1.65	1.65	1.65	1.65	1.67
OM, %	94.0 ± 0.4	93.9 ± 0.4	94.2 ± 0.4	94.1 ± 0.4	94.0 ± 0.1
CP, %	13.5 ± 0.3	12.9 ± 0.3	13.5 ± 0.3	13.4 ± 0.3	15.7 ± 0.6
Starch, %	20.2 ± 0.2	20.1 ± 0.2	20.8 ± 0.2	20.9 ± 0.2	27.6 ± 1.0
Non-fibrous carbohydrates,[10] %	38.7 ± 1.1	38.1 ± 1.1	38.3 ± 1.1	38.5 ± 1.1	40.8 ± 1.2
NDF, %	37.8 ± 0.6	39.0 ± 0.6	38.3 ± 0.6	38.2 ± 0.6	33.3 ± 0.5
NDF from forage, %	30.8 ± 0.7	30.8 ± 0.7	30.8 ± 0.7	30.8 ± 0.7	15.8 ± 0.4
Fatty acids, %	3.28 ± 0.03	3.33 ± 0.03	3.45 ± 0.03	3.37 ± 0.03	3.93 ± 0.22
Ca, %	0.61 ± 0.08	0.62 ± 0.08	0.54 ± 0.08	0.55 ± 0.08	0.59 ± 0.03
P, %	0.32 ± 0.01	0.31 ± 0.01	0.33 ± 0.01	0.32 ± 0.01	0.36 ± 0.01
Mg, %	0.39 ± 0.02	0.37 ± 0.02	0.38 ± 0.02	0.39 ± 0.02	0.27 ± 0.01
K, %	1.22 ± 0.08	1.19 ± 0.08	1.15 ± 0.08	1.15 ± 0.08	1.15 ± 0.06
Na, %	0.20 ± 0.01	0.20 ±0.01	0.16 ± 0.01	0.16 ± 0.01	0.46 ± 0.04
Cl, %	0.54 ± 0.04	0.55 ± 0.04	0.94 ± 0.04	0.90 ± 0.04	0.30 ± 0.01
S, %	0.17 ± 0.004	0.16 ± 0.004	0.37 ± 0.004	0.36 ± 0.004	0.18 ± 0.01
DCAD,[11] mEq/kg	145 ± 11	130 ± 119	-129 ± 11	-124 ± 11	293 ± 28

[1] Prepartum cows starting at 252 d of gestation were fed diets with either a positive (+130 mEq/kg) or a negative (-130 mEq/kg) dietary cation-anion difference (DCAD). Within each DCAD diet, cows were fed either 3 mg of cholecalciferol or 3 mg of calcidiol.
[2] Amino Plus (cooker-processing soybean meal; Ag Processing Inc., Emmetsburg, IA).
[3] Bio-Chlor (a fermentation product containing dried condensed extracted glutamic acid fermentation product, dried condensed corn fermentation solubles, processed grain by-products, and magnesium chloride; Arm & Hammer Animal Nutrition, Princeton, NJ).
[4] Rovimix D_3 (a product containing 300 mg of cholecalciferol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ).
[5] Hy-D (a product containing 153 mg of calcidiol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products, LCC, Parsippany, NJ).
[6] Each kg contains (DM basis) 10.3% Ca, 0.7% P, 4.0% Mg, 0.9% K, 0.25% S, 1.8% Na, 2.7% Cl, 1,750 mg Zn, 600 mg Cu, 1,090 mg Mn, 21 mg Se, 75 mg Co, 21 mg I, 260,000 IU of vitamin A, and 7,500 IU of vitamin E.
[7] A supplement containing 30% blood meal enriched with rumen-protected lysine and methionine (LysAAMet, Perdue Ag Solutions, LLC, Salisbury, MD). Each kg contains (DM basis) 26.4% CP, 5.1% Ca, 1.6% P, 4.1% Mg, 6.8% K, 0.3% S, 10.7% Na, 2.5% Cl, 665 mg Zn, 230 mg Cu, 416 mg Mn, 7.2 mg Se, 24 mg Co, 13.6 mg I, 110,000 IU of vitamin A, 33,000 IU of cholecalciferol (0.825 mg), 1,100 IU of vitamin E, and 460 mg of monensin (Rumensin 90, Elanco Animal Health, Eli Lilly and Co, Indianapolis, IN).
[8] Samples collected weekly and composited monthly for chemical analyses.
[9] Calculated based on the chemical analysis of dietary ingredients and using the NRC (2001) for a DM intake of 12.0 kg/d prepartum and 18 kg/d postpartum.
[10] Calculated using the equation DM – [(CP + NDF + fat + ash – (NDF insoluble protein)].
[11] Calculated using the equation [(mEq of Na + mEq of K) - (mEq of Cl + mEq of S)]

CHAPTER TEN: SUMMARY AND GENERAL CONCLUSION

This thesis compiles a collection of research that was undertaken to better understand the influence of nutritional intervention during the peri-parturient period (3 to 4 weeks before and after calving) on dairy cow fertility, health and production. It addresses a key question; *how can relatively short interventions, applied during the pre-partum or early lactation period have long lasting effects on fertility, health and productivity?*

The transition period is a time of substantial and sudden metabolic change, and how successfully cows perform at this time is vital to their future productivity. Different experimental designs, techniques and statistical approaches were used to examine the effects of nutritionally perturbing metabolism during this pivotal period. Specific metabolic pathways, including those related to mineral, energy, and protein metabolism were examined to provide evidence supporting the hypothesis that nutritional management during the transition period can have substantial effects on reproductive and productive success and health outcomes.

A systematic review and meta-analysis of the effects of diet on fertility provided insight into the limitations of published literature on this topic. Of the papers identified in the review, many were not suitable for quantitative analysis and only 39 papers containing 118 individual diets were able to be used. A negative binomial model including the effect of study was used, in which each diet represented a single observation. This allowed the effects of overall diet on reproduction to be examined, rather than differences between specific interventions as has been employed in previous meta-analyses on nutrition and reproduction. The later technique was used in Chapter 2 to assess of calving to pregnancy interval, and in the fats meta-analysis presented in Chapter 3.

The systemic review (Chapter 2) supported previously identified beneficial effects of increasing metabolizable energy balance (IRR = 1.004) on proportion pregnant. Proportion pregnant was also increased by increasing duodenal c14:0 availability (IRR = 1.008), and fatty acid intake (IRR = 1.00). Potential roles for specific carbohydrate fractions were identified, including the effect of increased starch intake to increase the proportion of cows pregnant to service (IRR = 1.06) and the effect of dietary intake (IRR = 0.81) or percentage (IRR = 0.96) of sugar to reduce the proportion of cows pregnant to service. This variation between starch and sugar may possibly have been because of a slower fermentation rate for starches than sugars, and hence less risk of ruminal disturbance. Milk protein yield was also associated with a lesser proportion of cows

pregnant (IRR = 0.99). Univariably, increased metabolizable protein balance, and milk fat yield or percentage increased and decreased proportion pregnant, respectively.

The effectiveness of fat interventions in the early post-partum period to improve reproductive success was specifically examined in Chapter 3 using a random effects meta-analysis utilizing the Kapp-Hartung method. Feeding fats during transition may be an essential part of an integrated response to the challenges of controlling tissue mobilization in early lactation and limiting the amount of readily fermentable carbohydrate fed, and fats provide specific precursors for reproductive hormones. The analysis included 17 studies containing 26 comparisons and identified a 27% overall increase in pregnancy to service was observed (RR = 1.27; 95% Confidence interval Knapp Hartung 1.09 to 1.45) and results were relatively consistent (I^2 = 19.9%). A strong indication of a reduction in calving to pregnancy interval was also identified, which was consistent across studies (I^2 = 0.0%) supporting a conclusion that overall, the inclusion of fats does improve fertility. The magnitude of these responses to fat interventions varied among types of fat, possibly reflecting different roles, or provision of different fatty acids, from the different fat types. Further exploration of the factors contributing to proportion pregnant using bivariate meta-regression identified variables that reflected changes in diet composition or animal response resulting from inclusion of the fat interventions in the experimental diets fed. Increased fermentable neutral detergent fibre and soluble fibre (coefficient = 1.17 and 2.18, respectively) intakes increased the proportion of cows pregnant while increased milk yield of the treatment group decreased this measure. Unexpectedly, the estimated energy costs of urea production (coefficient = 0.53) also had a positive association with proportion pregnant. This study did not identify any effects of fat intervention on milk yield or composition, although other larger studies have found inclusion of fat in the early lactation diet to increase milk yield.

Despite these new insights into key areas of the transition diet, the complexities of transition diets were also highlighted. For example, despite anticipating the importance of individual amino acids and fatty acids to be identified, these proved highly challenging to assess due to limitations of the model to accurately predict these, and substantial collinearity among individual fatty acids. The lower than expected number of suitable diets and the complexity of responses of the cattle to these diets strongly suggests that there is a need for further focused field studies exploring the roles of nutrition, and particularly fats, on reproduction, and interactions among dietary components. Research in this field would also benefit from the development of guidelines to assist study design in this area of research.

The meta-analytic analyses presented here clearly demonstrate the potential for early-lactation nutritional interventions to influence reproductive outcomes. However, nutrition during the period before calving was also expected to be important. Existing data from a study on the influence of genetic merit and dietary protein degradability was utilized to explore the influence of pre-partum nutrient intakes on reproductive performance, as this had not been previously examined (Chapter 4). A diet high in rumen undegradable protein (RUP) increased proportion of first services that resulted in pregnancy from 41 to 58%, suggested a positive effect of increased metabolisable protein (**MP**) on fertility. However, the effects of increasing dietary RUP on fertility may be curvilinear as increased pre-calving metabolizable protein balance decreased the proportion of first services that resulted in pregnancy when evaluated in a model containing casein %, milk protein yield, diet, and genetic merit. Prepartum metabolizable protein balance was important to production and reproductive outcomes, while surprisingly, metabolizable energy balance was not. There was support for the hypothesis that low milk protein percentage in early lactation may be an indicator for reduced pregnancy, as the hazard of pregnancy in the first 150 d of lactation was 28% lower in cows producing milk with the lowest quartile of protein percentage when compared with cows with milk in upper three quartiles. Early lactation milk yield, pre-partum MP balance, and glucose and calcium concentrations were higher in the cows producing lower milk protein percentage while pre-partum urea was higher in this group. Milk casein % was also positively associated with improved pregnancy at first service (OR = 9.86 ± 10.261).

As part of this investigation, we also took the opportunity to examine milk casein and casein variants, as there are rarely reported. Yields (kg/d) for casein variants were 0.49 and 0.45 of alpha casein, 0.38 and 0.34 of beta casein, 0.07 and 0.06 for kappa casein, and 0.10 and 0.09 of gamma casein for high and low RUP diets, respectively. Increased RUP increased milk (36.3 to 39.7 kg/d), casein (0.95 to 1.04 kg/d) and milk protein (1.13 to 1.26 kg/d) yields and higher genetic merit for milk soilds increased milk protein (1.15 to 1.23 kg.d) and gamma casein (0.09 to 0.10 kg/d) yields and tended to increase milk casein yield. This study also aimed to identify pre-calving and early lactation variables that predict production outcomes. The effects of indicator variables that may predict productive and reproductive outcomes were largely consistent, confirming the importance of some well determined causal factors such as body weight (**BW**) and disease, and also identifying some less well explored indicators including cholesterol and alpha amino nitrogen that are worthy of further exploration.

Studies have identified a profound role for bone in murine and bovine metabolism and the integrated responses to lactation. A focus of this thesis was the evaluation of vitamin D interventions to improve dairy cow performance, and build understanding of vitamin D and related metabolism in dairy cattle. To evaluate the usefulness of calcidiol supplementation to alter mineral, bone, and energy metabolism, two preliminary randomized controlled experiments were conducted in mid-lactation cows (Chapter 5). Mid-lactation cows were used in this study to allow effects to be assessed without the metabolic complications brought about by the onset of lactation. Calcidiol supplementation was effective in increasing blood and milk calcidiol concentrations in a dose response manner, and plasma calcidiol concentration in the group receiving 0.5 mg calcidiol/day was increased from 32 ng/mL to 52 and 67 ng/mL in the 2 experiments. Further short-term effects (over the 30 days of the study) on the concentrations of other vitamin D metabolites were also observed in calcidiol supplemented cows. Calcidiol supplementation increased plasma phosphate concentrations in a curvilinear manner, and blood insulin concentration from 3.36 to 3.86 mIU/mL when cows were supplemented with 0.5 mg calcidiol/day. Time-series analysis was used to identify associations between blood calcidiol and mineral or metabolites concentrations over lags at long at ± 9 days. Positive, but limited, associations between calcidiol and indicators of energy (insulin, non-esterified fatty acids, beta-hydroxybutyrate, cholesterol), bone (osteocalcin), and mineral metabolism were identified.

Feeding calcidiol and negative dietary cation-anion difference (**DCAD**) diets before calving have improved milk production, health and reproduction in dairy cattle. A randomized controlled experiment (Chapters 6, 7 and 8) was conducted to examine the effects of pre-partum calcidiol or cholecalciferol (3 mg/11 kg of the diet) supplementation and positive (+130 mEq/kg) or negative (-130 mEq/kg) DCAD diets on dairy cow metabolism, production, health and reproductive outcomes. Supplementation (from 255 d gestation until parturition) of calcidiol in peri-parturient cows had positive and prolonged effects on vitamin D and mineral, in particular calcium (**Ca**), metabolism. Calcidiol supplementation increased calcidiol, 3-epi 25-OH-D_3, calcitriol, 24,25-$(OH)_2$-D_3, and 25-OH-D_2 concentrations in plasma pre-partum and concentrations in plasma of all these other than calcitriol post-partum when compared with cholecalciferol supplementation. Cows fed a negative DCAD diet pre-partum had lower concentrations of calcidiol, cholecalciferol, 3-epi 25-OH-D_3, 24,25-$(OH)_2$-D_3 and higher concentrations of calcitriol and 25-OH-D_2 in plasma prepartum, and concentrations of all of these metabolites were lower except 25-OH-D_2 post-partum. Plasma total Ca concentration was increased in calcidiol supplemented cows before and after calving when compared with those fed cholecalciferol. These responses

persisted into lactation, despite the intervention being confined to the pre-partum period. Key metabolites such as calcidiol and Ca, differed in their response to DCAD treatment between pre- and post- partum periods, recognizing the requirements of cattle differ throughout the different stages of transition of lactation, and they need to be managed to reflect this.

Feeding a negative DCAD diet during the last 21 d of gestation reduced DM intake prepartum only in parous cows. The reduced dry matter intake prepartum in cows fed the diet with negative DCAD resulted in reduced concentrations of glucose, insulin and insulin-like growth factor 1 (**IGF-1**) in plasma prepartum. Nevertheless, cows fed the negative DCAD remained in positive energy balance until 2 d before calving. Cows fed the negative DCAD produced numerically more energy corrected milk (ECM) (1.1 kg/d). Feeding calcidiol in place of cholecalciferol prepartum at 3 mg/d improved yields of colostrum, milk, 3.5% fat corrected milk (FCM), and ECM with a numerical increase in DMI. Colostrum protein output, including IgG was enhanced by calcidiol. Because of the increased production, cows fed calcidiol were under more negative energy balance, although changes in BW and BCS did not differ among treatments. The greatest productions of 3.5% FCM and ECM were observed in cows fed the negative DCAD combined with calcidiol. Improvements in production in cows fed calcidiol were attributed to differences in morbidity, although the changes in colostrum yield and composition leads us to speculate that increases in 25-hydroxyvitamin D_3 in plasma pre- and postpartum in cows fed calcidiol might have influenced mammary biology.

Calcidiol supplementation was associated with not only substantially increased production but also improved health outcomes, with reduced incidence of retained placenta and metritis in the supplemented cows. Negative DCAD diets increased ionized Ca levels both before and after parturition, resulting in elimination of clinical hypocalcemia (0 vs. 23.%) and drastically reduction the incidence (94.5 vs. 71.1%) of subclinical hypocalcemia. These effects on health were further supported by improved immune cell function, particularly in parous cows. The integration of pre-partum calcidiol supplementation and acidogenic diet reduced morbidity by at least 60% compared with any of the other treatments. Despite the changes in cow health, manipulating the prepartum DCAD did not influence reproduction, but feeding calcidiol tended to increase the rate of pregnancy by 55% and reduced the median days open by 19. These results, coupled with positive metabolic, health and productivity outcomes, indicate that productivity and health may be synergistically improved when diets are properly integrated.

Building on the work presented in Chapters 6, 7, and 8, the hypothesis that interactions between bone and energy metabolism observed in other species are present in dairy cattle and have feedback over time was examined using time-series analysis (Chapter 9). This Chapter also aimed to identify how such interactions are altered by vitamin D supplementation and manipulation of DCAD. Feeding calcidiol, compared with cholecalciferol, increased blood concentrations of calcidiol pre- (264.2 ± 7.96 vs 61.3 ± 7.96 ng/mL) and post-partum (170.8 ± 6.21 vs 51.3 ± 6.21 ng/mL) and decreased blood concentrations of cholecalciferol (1.2 ± 0.56 vs 14.5 ± 0.56 ng/mL pre-partum and 1.9 ± 0.36 vs 3.2 ± 0.56 ng/mL post-partum for calcidiol and cholecalciferol respectively). This effect was enhanced by feeding a positive DCAD pre-partum. Before calving, cows fed the negative DCAD diets had decreased concentrations of cholecalciferol and glucose when compared with cows fed the positive DCAD diet. The combination of negative DCAD and cholecalciferol reduced IGF-1 concentrations pre-partum. There was no effect of DCAD treatments on post-partum metabolite concentrations. Nulliparous cows had increased concentrations of osteocalcin (**OC**), C-terminal telopeptide of type 1 collagen (CTX-1), IGF-1, glucose and insulin when compared with mulitparous cows.

Time series analysis identified associations between metabolites on the same day, and over 3 day lags up to ± 12 d. Feedback between calcidiol and cholecalciferol in the negative lags suggests calcidiol may exert feedback on cholecalciferol, but not vice versa. The importance of IGF-1 was highlighted with the identification of a physiological feedback mechanism between cholecalciferol and IGF-1 (positive ES on the same day and 3 d later, and negative ES 9 d later) that was more pronounced in cholecalciferol supplemented cows. Evidence of feedback was also identified between undercarboxylated (**uOC**), and particularly carboxylated (**cOC**) OC with IGF-1 (positive ES on the same day, and negative ES 6 d before and 6 d later). This had not been previously identified in cattle, and the suggested that at least in cattle, both uOC and cOC have marked biological activity. The linkages between OC and insulin identified in murine studies was not observed here, although associations between OC and glucose were seen that were similar to those of IGF-1 and glucose, confirming strong associations between glucose and these hormones.

Through the collection of research presented, this thesis is able to demonstrate that nutritional interventions applied during the peri-parturient period do have homeostatic, and also homeorhetic effects on metabolism, and can substantially influence reproductive, productive and health outcomes of dairy cows well into lactation. This work highlighted the complexity and

interrelated nature of transition nutrition, and importantly showed that productivity does not have to come at the cost of fertility and health where diets are properly integrated. The complex and broad nature of nutrition research means that although the work presented in this thesis addressed multiple areas of the diet and provided several meaningful new findings, the optimal transition diet is still to be determined. The need for more high quality studies focused on transition nutiriton and fertility, the examination of time series relationships using lags shorter than three days, and further exploration of the links identified between bone and energy metabolsm were just a few of the areas highlighted as worthy of continued research.

The insights gathered regarding fat, protein, and vitamin D interventions and the relationships of metabolites underlying these, gives us tools to move closer to identifying the components of optimal transition diets and understanding the mechanisms by which these relatively short term interventions can have powerful, and long lasting effects of dairy cow fertility, productivity and health.