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Effects of prepartum dietary cation-anion difference and source of vitamin D in dairy cows: Health and reproductive responses

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ABSTRACT

The objectives of the experiment were to evaluate the effects of feeding diets with distinct dietary cation-anion difference (DCAD) levels and supplemented with 2 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 would be potentiated by supplementing calcidiol compared with cholecalciferol. Cows at 252 d of gestation were blocked by parity (28 nulliparous and 52 parous cows) and milk yield within parous cows, and randomly assigned to 1 of 4 treatments arranged as a 2 × 2 factorial, with 2 levels of DCAD, positive (+130 mEq/kg) or negative (−130 mEq/kg), and 2 sources of vitamin D, cholecalciferol or calcidiol, fed at 3 mg for each 11 kg of diet dry matter. 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 dry matter. Blood was sampled 7 d before parturition, and at 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%), reduced the incidences 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 diet with calcidiol reduced morbidity by at least 60% compared with any of the other treatments. Cows with morbidity had lower blood ionized Ca and serum total Ca concentrations than healthy cows. Treatments did not affect the daily risk of hyperketonemia in the first 30 d of 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.

Key words: dairy cow, dietary cation-anion difference (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 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

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DMI 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 as 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 decrease the probability of pregnancy at first AI and increase 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 reduce the risk of milk fever in multiparous cows (Ender et al., 1971; Charbonneau et al., 2006; Lean et al., 2006), although the effects 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 feeding 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 calcium 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 the vitamin D status of cows but did not improve calcium status or reduce hypocalcemia (Weiss et al., 2015).

We hypothesized that feeding acidogenic diets would reduce the incidence of clinical and subclinical hypocalcemia and that 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 2 sources of vitamin D during the last 3 wk of gestation on health and reproduction in dairy cows.

MATERIALS AND METHODS

This is 1 of a series of 3 companion papers (Martinez et al., 2018; Rodney et al., 2018). 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 article, 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, 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. (2018) and Rodney et al. (2018). Eighty pregnant dry Holstein cows, 28 nulliparous and 52 parous, were enrolled in the experiment. For consistency of terminology throughout the manuscript, prepartum nulliparous animals that became primiparous postpartum were designated "nulliparous," and prepartum parous animals that became postpartum multiparous were designated "parous." Cows were fed once daily prepartum and twice daily postpartum and amounts offered and refused were measured daily. Description of the diets is presented in Table 1, and details of feed ingredients and chemical analyses are presented elsewhere (Martinez et al., 2018).

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, were assigned randomly to 1 of 4 treatments. Treatments were arranged as a factorial with 2 levels of DCAD, positive (+130 mEq/kg) or negative (−130 mEq/kg), and 2 sources of vitamin D (CH or CA) that were fed at 3 mg for each 11 kg of diet DM. The amount of vitamin D to be fed was based on the work of Wilkens

et al. (2012), who fed quantities above current guidelines established by the NRC (2001). The 4 treatments were positive DCAD with CH (**PCH**; 7 nulliparous, 5 in lactation 1, 6 in lactation 2, and 2 in lactation 3 or greater), positive DCAD with CA (**PCA**; 7 nulliparous, 6 in lactation 1, 4 in lactation 2, and 3 in lactation

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

	Prepartum diet[1]				
	Positive DCAD		Negative DCAD		Postpartum diet
Item	Cholecalciferol	Calcidiol	Cholecalciferol	Calcidiol	
Ingredient, % 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, heat treated[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]					
NE,[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
NFC,[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 ± 11	−129 ± 11	−124 ± 11	293 ± 28

[1]Prepartum cows at 252 d of gestation were fed diets with either a positive (+130 mEq/kg) or a negative (−130 mEq/kg) DCAD. Within each DCAD diet, cows were fed either 3 mg of cholecalciferol or 3 mg of calcidiol.

[2]Amino Plus (heat-treated solvent-extracted 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 LLC, Parsippany, NJ).

[5]Hy-D (a product containing 153 mg of calcidiol per kg; Division of Animal Nutrition and Health, DSM Nutritional Products LLC).

[6]Each kilogram contained (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 of Zn, 600 mg of Cu, 1,090 mg of Mn, 21 mg of Se, 75 mg of Co, 21 mg of 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 kilogram contained (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 of Zn, 230 mg of Cu, 416 mg of Mn, 7.2 mg of Se, 24 mg of Co, 13.6 mg of I, 110,000 IU of vitamin A, 33,000 IU of cholecalciferol, 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. Five samples of each ingredient were analyzed for chemical composition.

[9]Calculated based on the chemical analysis of dietary ingredients and using the NRC (2001) for a DMI 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)].

3), negative DCAD with CH (**NCH**; 7 nulliparous, 4 in lactation 1, 6 in lactation 2, and 3 in lactation 3 or greater), and negative DCAD with CA (**NCA**; 7 nulliparous, 4 in lactation 1, 5 in lactation 2, and 4 in 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.

Blood Samples

Blood was sampled 3 times per week from 265 d of gestation until calving, and on d 0, 1, 2, and 3 postpartum, 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 interassay coefficients of variation (**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 intra- and interassay 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, Idexx Laboratories, Westbrook, ME). The percentage of neutrophils exhibiting phagocytosis of labeled *Escherichia coli* and oxidative burst activities 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 an indicator of 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 DMI 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 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 or 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 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 >1.20 m*M* on at least one day on d 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 1 of the 4 treatments. Therefore, 7 blocks of 4 nulliparous cows each and 13 blocks of 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 PROC TRANSREG in SAS (SAS/STAT, SAS Institute Inc., Cary, NC) before final analyses. For transformed data, the least squares means were back transformed and the respective standard errors of the means were calculated (Jørgensen and Pedersen, 1998).

Continuous data were analyzed with the MIXED procedure of SAS (SAS Institute Inc.) and the statistical models included the fixed effects of DCAD (positive vs. negative), vitamin D (cholecalciferol vs. calcidiol), parity (nulliparous vs. parous), and the 2- and 3-way interactions between DCAD, vitamin D, and parity, and the random effect of block. For responses with repeated measures within the same experimental unit, 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 tCa for the pre- and postpartum periods separately with the MIXED procedure of SAS (SAS Institute Inc.) 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 (SAS Institute Inc.) 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 (SAS Institute Inc.). 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 calculated. 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 1 parous cow fed PCH was removed from the data analyses because 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. calcium borogluconate solution and an oral calcium drench but developed aspiration pneumonia. The cow was euthanized and removed prematurely from the experiment; she contributed data from enrollment to 2 DIM. The length of gestation (±SD) was 275 ± 4.4 d, and days on the prepartum diets did not differ with treatments and averaged 22.7 ± 5.3. All cows in the

Table 2. Effect of DCAD and source of vitamin D fed prepartum on leukocyte counts and neutrophil function prepartum[1]

	Positive DCAD		Negative DCAD			*P*-value[2]		
Variable	CH	CA	CH	CA	SEM	DCAD	Vitamin D	DCAD × vitamin D
Leukocytes, $\times 10^3/\mu L$								
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, % of PMN	72.2	68.1	73.8	71.9	3.9	0.19	0.12	0.93
Oxidative burst, % of 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 DCAD (positive vs. negative); vitamin D = effect of source of vitamin D (CH vs. CA); DCAD × vitamin D = interaction between DCAD and vitamin D.

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

experiment stayed on the prepartum diets for at least 14 d 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., 2018). Briefly, feeding CH increased ($P < 0.001$) the concentrations of vitamin D_3 in plasma prepartum (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 CA increased ($P < 0.001$) the concentrations of 25-hydroxyvitamin D_3 in plasma prepartum (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., 2018). At calving and on d 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 on those days (CH = 1.042 vs. CA = 1.035 ± 0.008 m*M*). Similarly, at calving and on d 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 on those days (CH = 2.085 vs. CA = 2.060 ± 0.02 m*M*).

Table 3. Effect of DCAD and source of vitamin D fed prepartum on leukocyte count and neutrophil function postpartum[1]

	Positive DCAD		Negative DCAD			*P*-value[2]		
Variable	CH	CA	CH	CA	SEM	DCAD	Vitamin D	DCAD × vitamin D
Leukocytes, $\times 10^3/\mu L$								
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, % of PMN	73.7	75.4	72.9	76.7	2.9	0.99	0.49	0.82
Oxidative burst, % of 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 DCAD (positive vs. negative); vitamin D = effect of source of vitamin D (CH vs. CA); DCAD × vitamin D = interaction between DCAD and 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 4. Effect of DCAD (positive or negative) fed prepartum and parity on neutrophil function pre- and postpartum[1]

	Nulliparous		Parous			*P*-value[2]		
Neutrophil function	Positive	Negative	Positive	Negative	SEM	DCAD	Parity	DCAD × parity
Prepartum								
Phagocytosis, % of PMN	71.2	74.2	69.1	71.5	4.0	0.19	0.46	0.72
Oxidative burst, % of 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, % of PMN	77.9	75.0	71.2	74.7	3.0	0.99	0.31	0.32
Oxidative burst, % of PMN	67.4	64.2	58.5[b]	67.6[a]	3.7	0.36	0.53	0.06
Phagocytosis MFI, 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

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

[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× 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 (indicator of intensity of oxidative burst produced per neutrophil) dyes.

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 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).

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 but not 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 and source of vitamin D were observed for 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 CA compared with CH (Table 3). Also, a tendency for an 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 but not nulliparous cows (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).

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% of PCA, 20.0% of NCH, and 25.0% of 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 CA compared with CH 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 (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 the incidence of metritis from 42.1 to 27.5%. Similar to what was observed for

Table 5. Effect of DCAD and source of vitamin D fed prepartum[1] on postpartum diseases

Variable	Incidence, % (no./no.)	Positive DCAD		Negative DCAD		*P*-value[2]		
		CH	CA	CH	CA	DCAD	Vitamin D	DCAD × vitamin D
Clinical disease								
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[4]								
Hypocalcemia tCa ≤2.0 m*M*	39.3 (31/79)	63.2	55.0	10.0	30.0	0.001	0.37	0.14
Hypocalcemia tCa <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); vitamin D = effect of source of vitamin D (CH vs. CA); DCAD × vitamin D = interaction between level of DCAD and source of vitamin D.

[3]Not estimable because of data separation.

[4]tCa = total calcium; iCa = ionized calcium.

retained placenta, feeding CA compared with CH 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 DCAD diet compared with the positive DCAD diet reduced ($P = 0.05$) morbidity from 56.4 to 35.0% (Table 5). A tendency for an interaction ($P = 0.08$) between level of DCAD and source of vitamin D was observed for morbidity because the benefit

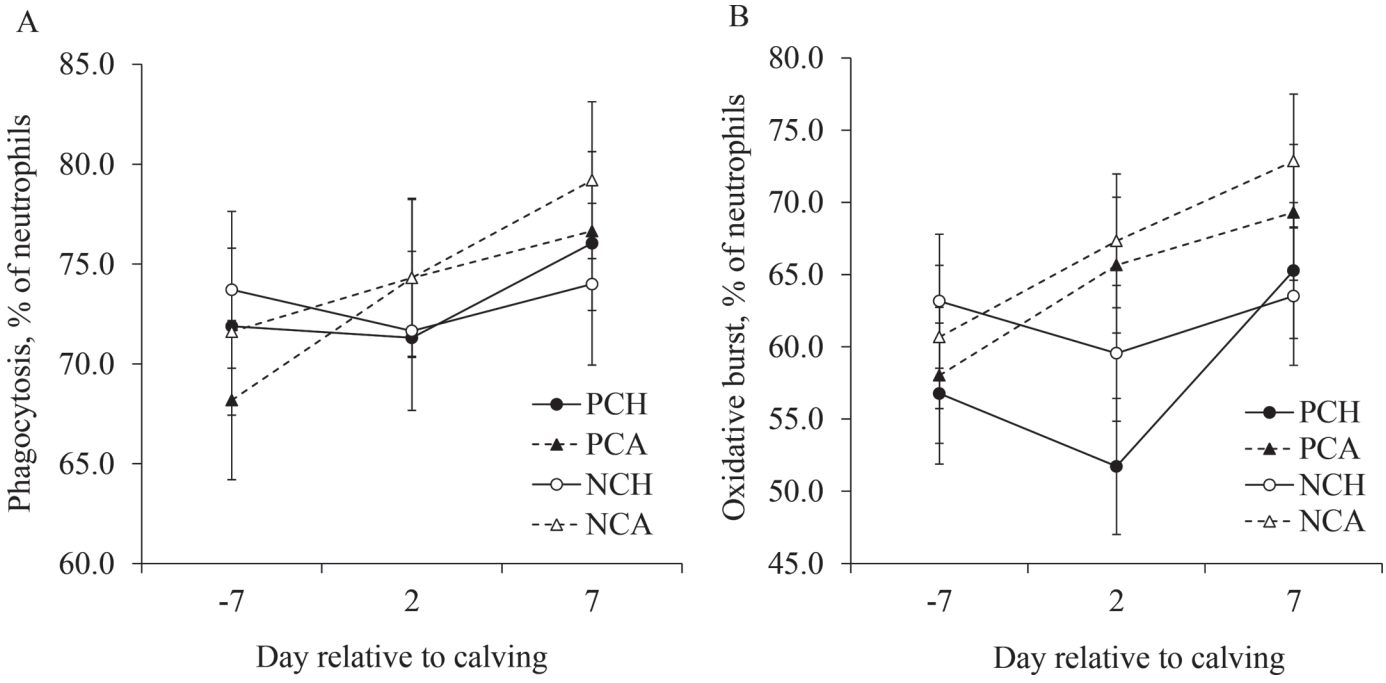

Figure 1. Percent of neutrophils with phagocytic (A) and oxidative burst (B) activities in cows fed prepartum diets containing either positive (P, +130 mEq/kg) or negative (N, −130 mEq/kg) DCAD and supplemented with either 3 mg of cholecalciferol (PCH and NCH) or 3 mg of calcidiol (PCA and NCA). For 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.

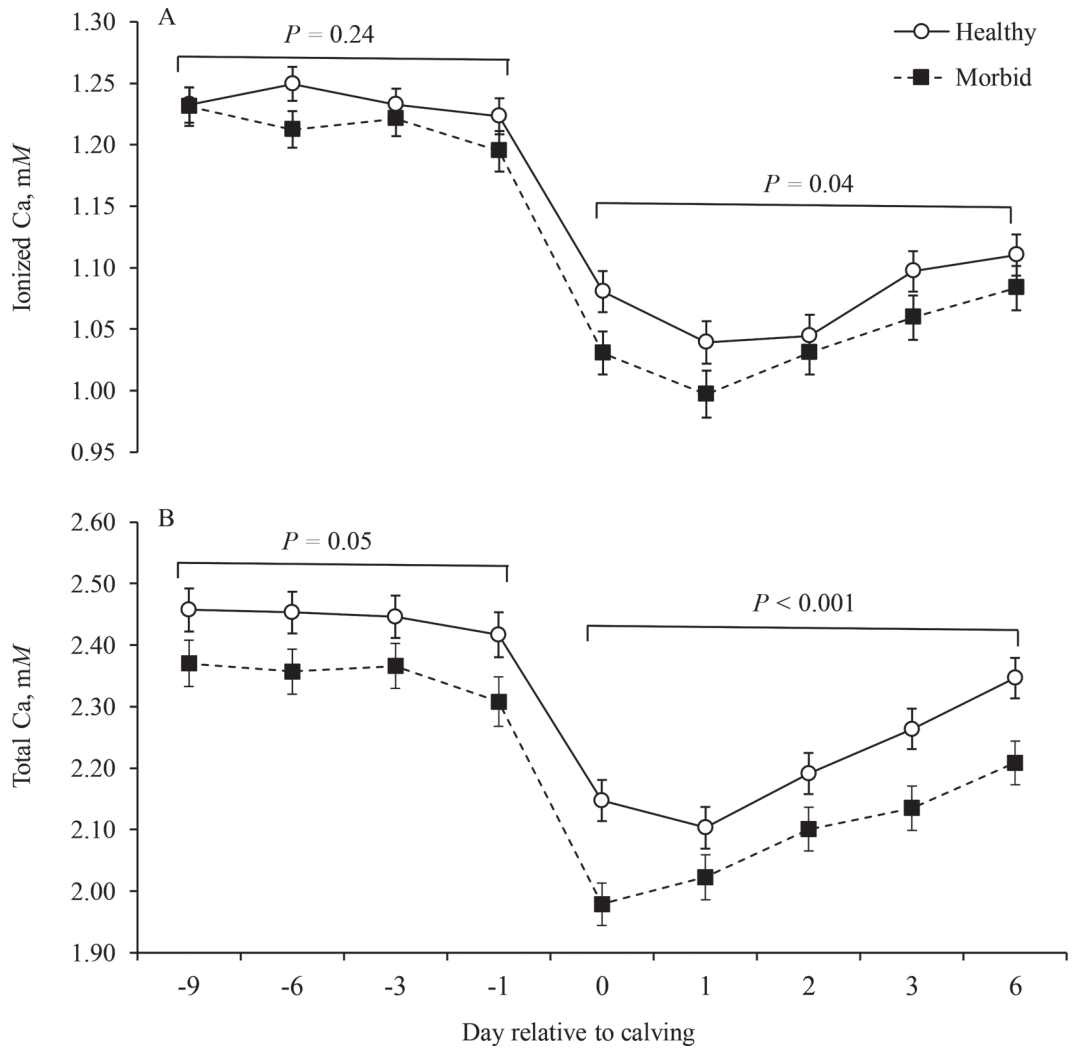

Figure 2. Concentrations of ionized Ca (iCa) in whole blood (A) and total Ca (tCa) in serum (B) of dairy cows according to morbidity. For iCa prepartum: effect of morbidity ($P = 0.24$) and interaction between morbidity and day ($P = 0.26$). For iCa postpartum: effect of morbidity ($P = 0.04$) and interaction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

of the negative DCAD diet was greater when combined with CA. Almost 27% of the cows were diagnosed with more than one clinical disease in the first 30 DIM. Feeding CA tended to reduce ($P = 0.06$) the proportion of cows with multiple diseases compared with feeding CH, 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 for 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 lower 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.

Subclinical Diseases

Incidence of subclinical hypocalcemia changed with the 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 subclini-

cal 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*, 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 when 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 d 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 m*M* 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 1 d 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 CA tended to increase ($P = 0.09$) the percentage of cows diagnosed with hyperketonemia compared with feeding CH (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.

Survival

Treatment did not affect survival of cows up to 305 DIM, and 77.2% of cows 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 DCAD diet compared with positive DCAD (adjusted HR = 0.93; 95% CI = 0.37 to 2.33) and for cows fed CH compared with CA (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 less ($P = 0.10$) for cows fed the positive compared with the negative DCAD diet (positive = 114.8 ± 35.7 vs. negative = 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 "do 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 CA 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%).

DISCUSSION

Feeding prepartum transition dairy cows a diet with negative DCAD eliminated clinical hypocalcemia and reduced the risk of subclinical hypocalcemia, whereas supplementing CA in place of CH 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 and CA resulted in the lowest morbidity. The use of acidogenic diets prepartum to reduce the risk of clinical hypocalcemia has been implemented for more than 4 decades, and the benefits are widely documented (Ender et al., 1971; Block, 1984; Lean et al., 2006). Less information is available on 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 lesser concentrations of iCa postpartum and tCa pre- and postpartum compared with healthy cows. One of the suggested mechanisms is that hypocalcemia interferes

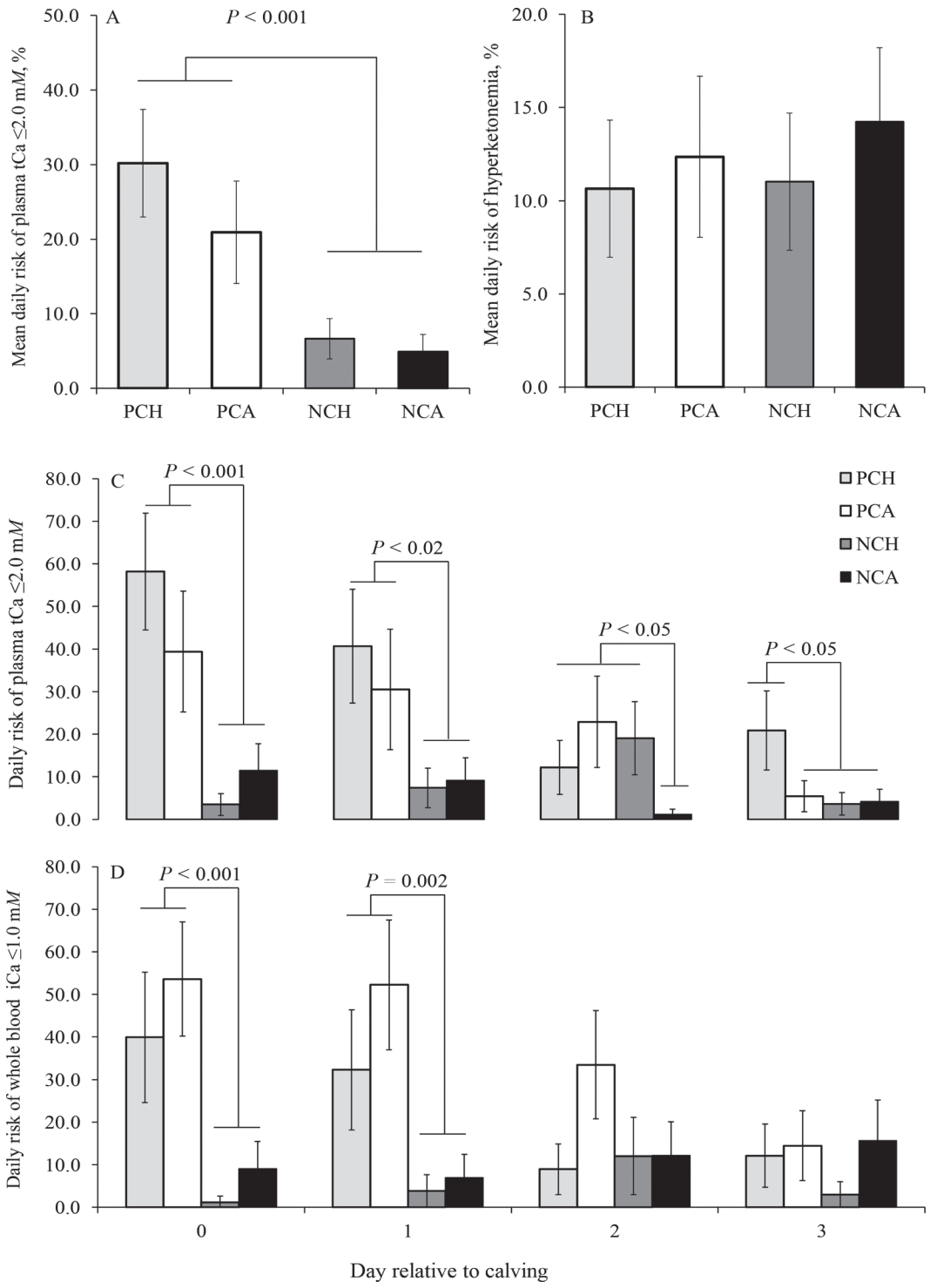

Figure 3. Mean daily risk of subclinical hypocalcemia based on serum total Ca (tCa) ≤2.0 m*M* from d 0 to 3 postpartum (A) and of hyperketonemia in the first 30 DIM (B), and the individual daily risks of subclinical hypocalcemia based on serum tCa ≤2.0 m*M* (C) or serum ionized Ca (iCa) ≤1.0 m*M* (D) 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). Panels 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.

with innate (Martinez et al., 2014) and potentially acquired immunity (Kimura et al., 2006), thereby 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., 2018) and had beneficial effects on measures of neutrophil function in parous but not nulliparous cows. The increase in percentage of neutrophils with killing activity and intensity of phagocytosis in parous cows 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 at 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 CA compared with CH 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 times 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 CH and CA, respectively (Rodney et al., 2018). 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 (2016) found that nutritionists in the United States typically supplement diets with 0.75 to 1.25 mg of vitamin D per cow, or 30,000 to 50,000 IU 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 (Hymøller 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 CH had concentrations of 25-hydroxyvitamin D_3 in plasma considered adequate based on current surveys (Nelson et al., 2016), it is plausible that the benefits to innate immunity and reduction in diseases observed in cows fed CA in the current experiment were not caused by inadequate vitamin D status in cows fed CH.

Calcidiol has beneficial effects on innate host defenses of cattle (Nelson et al., 2010, 2012; Merriman et al., 2015). Exposure of monocytes to pathogen-

Table 6. Cox's proportional hazard model for time to pregnancy in cows fed 2 levels of DCAD and 2 sources of vitamin D prepartum[1]

	Days to pregnancy[2]				
Variable	Median (95% CI)	Mean ± SEM	Pregnant, %	AHR[3] (95% CI)	*P*-value
DCAD[4]					
Positive	144 (79 to 179)	151 ± 13	76.5	Referent	—
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	Referent	—
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	Referent	—
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 DCAD and source of vitamin D was not significant and dropped from final model.

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 β defensins, which can disrupt bacterial cell membrane and cause bacterial death (Liu et al., 2006). Recently, Merriman et al. (2015) showed that culture of bovine monocytes treated with calcitriol showed 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 β-defensin gene 7 in macrophages isolated from the mammary gland (Merriman et al., 2015), suggesting that calcitriol stimulates defensive mechanisms in bovine immune cells. Cows fed CA in our experiment had increased concentrations of 1,25-dihydroxyvitamin D_3 in the last 9 d of gestation compared with cows fed CH (Rodney et al., 2018). 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 receive a subcutaneous dose of calcitriol immediately after calving (Vieira-Neto et al., 2017). Also, the high concentrations of 25-hydroxyvitamin D_3 from feeding CA might have acted directly on vitamin D receptor (Lou et al., 2010), thereby stimulating activity of immune cells independently of systemic changes in 1,25-dihydroxyvitamin D_3 or cellular synthesis of 1,25-dihydroxyvitamin D_3 and the resulting autocrine effects.

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 (Davies et al., 2004), and the function of cells of the innate immune system seems to play an important role in the release of the placenta (Gunnink, 1984a,b; 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 CA reduced the incidence of metritis, which might be linked with the reduction in retained placenta as the 2 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 (Elliott 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 CA stimulated defense mechanisms in the uterus that prevented metritis. The combined effect of CA on retained placenta and metritis explains the reduction in the proportion of cows diagnosed with multiple diseases in the first month of lactation.

We cannot disregard the fact that hypocalcemia has been shown to suppress gut and reproductive tract motility (Robalo 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 (Robalo 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 of CA enhancing neutrophil function worked to reduce morbidity in the first 30 DIM in dairy cows.

Although CA supplementation 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 CA had greater production of ECM with no differences in caloric intake, resulting in improved feed efficiency but worse net energy balance (Martinez et al., 2018), which explains the tendency for increased incidence of cows with BHB >1.20 m*M*. This tendency for an increased incidence of cows with hyperketonemia was not at the expense of production, reproduction, or health because cows fed CA produced more ECM (Martinez et al., 2018), 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, the similar daily risk indicates that cows fed CA 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 DMI 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 CA 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 the 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, all cows in the experiment had plasma concentrations 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 CA 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). It is well established that diseases have marked negative effects on fertility of dairy cattle, particularly those of an inflammatory nature (Ribeiro et al., 2016). Thus, the improved peripartum health with CA supplementation might explain the tendency for improved rate of pregnancy. Also, vitamin D receptor is expressed in numerous reproductive tissues in mammals (Dokoh et al., 1983; Stumpf et al., 1987). Therefore, another possibility is that metabolites of vitamin D such as the high concentrations of 25-hydroxyvitamin D_3 that remained postpartum in cows fed CA might directly stimulate vitamin D receptors in reproductive tissues (Lou et al., 2010), which might have resulted in positive effects on fertility. Nevertheless, additional work with larger numbers of cows is needed to elucidate whether the potential benefits to reproduction from CA are direct or indirect effects through improved postpartum health.

CONCLUSIONS

Feeding a diet containing a DCAD of −130 mEq/kg during the last 3 wk of gestation reduced the incidence of clinical and subclinical hypocalcemia and the risk of subclinical hypocalcemia in dairy cows in the first 3 d in lactation. These benefits were observed regardless of source of vitamin D supplemented or parity of the 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, the 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 CA 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 CA tended to increase the rate of pregnancy and reduce days open.

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