Effect of 25-hydroxyvitamin D₃ during prepartum transition and lactation on production, reproduction, and health of lactating dairy cows

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ABSTRACT

We hypothesized that feeding 25-hydroxyvitamin D₃ [25-(OH)D₃] during lactation and prepartum in conjunction with negative dietary cation-anion difference diets would improve milk production, increase the probability of pregnancy, and reduce the incidence of postcalving diseases. Cows from 4 dairies with prepartum transition diets negative in dietary cation-anion difference were used in 2 randomized cohort experiments. In Experiment 1 (Exp. 1), cows were assigned to control [CON; n = 645; no 25-(OH)D₃] or treatment [TRT; n = 537; 2 mg/d of 25-(OH)D₃ from ~21 d prepartum to parturition and 1 mg/d in lactation] groups at ~21 d prepartum. Cows were monitored for weekly milk yield, milk composition every 60 d, and health and reproductive measures. In Experiment 2 (Exp. 2), cows (n = 2,064; median 147 d in milk) were assigned to 4 groups and monitored for the same measures as in Exp. 1 to the end of that lactation (L1), the subsequent transition (~21 d prepartum to parturition), and the next lactation (L2). Groups were as follows, with the amount of 25-(OH)D₃ fed (mg/d) indicated in parentheses for L1, transition, and L2, respectively: (A) control-control (CON-CON; 0–0–0), (B) treatment-treatment (TRT-TRT; 1–2–1), (C) control-treatment (CON-TRT; 0–2–1), and (D) treatment-control (TRT-CON; 1–0–0). For L1, a total of 1,032 cows entered the control groups A or C and a total of 1,032 cows in groups B or D. The number of cows in groups A to D that entered L2 was 521, 523, 273, and 248, respectively. Blood calcium, phosphorus, and 25-(OH)D₃ concentrations were measured from 17 cows/group at 5 times. In Exp. 1, TRT cows had 0.2 lower log somatic cell count than CON cows (4.21 ± 0.045 vs. 4.01 ± 0.050 , respectively) and multiparous TRT cows had $41 \pm 23\%$ higher probability of pregnancy/day than multiparous CON cows, resulting in a 22-d median decrease in time

to pregnancy. Primiparous TRT cows had 1.67 ± 0.40 times greater odds of mastitis/day than primiparous CON cows. In Exp. 2 TRT-TRT cows had between 16 and 29% lower probability to be bred/day than other groups. Multiparous CON-CON and TRT-CON cows had $20 \pm 8\%$ and $30 \pm 17\%$ greater probability of pregnancy, respectively, than multiparous TRT-TRT cows. Serum calcium concentrations were not affected by group, but phosphorus and 25-(OH)D₃ concentrations were highest in the TRT-TRT cows. The study provides further insights into the use of 25(OH)D₃ in transition and lactation.

Key words: calcium, calcidiol, negative dietary cation-anion difference, subclinical hypocalcemia

INTRODUCTION

Calcium (Ca) metabolism in the periparturient period plays an important role in the health and energy status of dairy cattle during this period and into the subsequent lactation. The increased lactational demand for Ca, which can be up to 80 g/d (Horst et al., 1994), may only be satisfied by increasing absorption of dietary Ca from the rumen or intestines, increasing Ca mobilization from tissue, especially bone reserves of Ca, and renal conservation of Ca, as circulating blood reserves are limited (DeGaris and Lean, 2008). The vitamin D signaling pathway is essential in these processes (Lund and DeLuca, 1966; Fraser and Kodicek, 1970). The mechanisms for Ca replenishment are relatively inactive during a cow's dry period, and the cow's intestine and bone adapt to lactation (Horst et al., 1994). When the homeostatic and homeorhetic mechanisms that control Ca metabolism do not respond fast enough, clinical hypocalcemia occurs. When the vitamin D cascade is triggered, vitamin D₃ (cholecalciferol), the inactive form of the vitamin is hydroxylated by 25-hydroxylases to the circulating form, 25-(OH)D₃ (also referred to as calcidiol) in the liver, which is then hydroxylated by 1 α -hydroxylase to the active form, 1,25-dihydroxyvitamin D₃ (calcitriol) in the kidney (DeLuca, 1980; Goff et al., 1991), resulting in an increase in blood Ca.

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Most prepartum diets for dairy cows are supplemented with vitamin D₃ (Martinez et al., 2018a). The recommended dose is 25,000 IU of vitamin D₃/d for a 680-kg close-up cow (NRC, 2001). In practice, cows in most US dairy herds receive 1.5 to 2.5 times the recommended dose (Nelson et al., 2016; Wilkens et al., 2020), whereas in Europe supplementation is restricted to 4,000 IU/kg EFSA (2012).

Calcidiol or 25-(OH)D₃ can be delivered in feed as a relatively inexpensive supplement. It can also be injected or given as an intramammary or slow-release rumen bolus. It has a longer half-life, ranging from 14 to 34 d in blood circulation in cattle (Wilkens et al., 2013) than calcitriol; mean half-life in humans ranges from 3.5 to 25.9 h (Levine et al., 1985; Brandi et al., 2002). Wilkens et al. (2012) demonstrated improvements in peripartum Ca metabolism in cows on low DCAD prepartum diets supplemented with 3 mg/d of 25-(OH)D₃, or 120,000 IU. Supplementing a -130 mEq/kg DCAD prepartum diet with 3 mg/d of 25-(OH)D₃ versus vitamin D₃ increased FCM by 4.4 kg and reduced the risk of retained fetal membranes from 30 to 0% and metritis from 40 to 15% (Martinez et al., 2018a,b). Feeding 25-(OH)D₃, compared with vitamin D₃, also tended to increase the probability of pregnancy by 55% and reduced the median days open by 19 (Martinez et al., 2018a), albeit with a relatively small number of cows.

Studies on oral 25-(OH)D₃ supplementation only during lactation are limited. However, Poindexter et al. (2020) found that feeding 3 mg/d of 25-(OH)D₃ in lactation for 56 d decreased the severity of mastitis in *Streptococcus uberis*-challenged cows. Rodney et al. (2018a) found that milk yield, milk composition, BW, and BCS of mid-lactation cows were not affected by supplementation with 0, 0.5, 1, 2, or 4 mg/d of 25-(OH)D₃ for 30 d.

There is a need to investigate lactation, reproduction, and health responses to long-term feeding of 25-(OH)D₃ during lactation, because continued supplementation may provide further benefit to use prepartum. Calcidiol concentration accumulates in the blood (Weiss et al., 2015; Rodney et al., 2018a) and may potentially reach a plateau (Poindexter et al., 2020). It is not known precisely how long it takes to reach this plateau or the dose rate required to maintain this plateau in a lactating cow. Feeding 5.4 mg/d of 25-(OH)D₃ with a negative DCAD diet to dairy cows for the last 13 d prepartum resulted in the highest incidence of clinical hypocalcemia compared with diets feeding 18,000 IU/d of vitamin D₃ with either positive or negative DCAD (Weiss et al., 2015), suggesting that feeding an excess of 25-(OH)D₃ may not be beneficial.

The objective of this study, which comprised 2 experiments, was to evaluate milk performance, reproduction,

and health of dairy cows fed 25-(OH)D₃ during ~21 d prepartum to parturition (transition), and in lactation. Experiment 1 evaluated supplementation of 25-(OH)D₃ from transition to the end of the subsequent lactation [control (CON) and treatment (TRT) groups]. Experiment 2, which was planned a priori and spanned 2 partial lactations had two aims: (1) determine whether benefits of 25-(OH)D₃ could occur independently of 25-(OH)D₃ supplementation in transition; and (2) evaluate the effects of extended supplementation of 25-(OH)D₃ across 2 partial lactations (CON-CON, TRT-TRT, CON-TRT, and TRT-CON groups). We intended that 50% of the cows in this experiment would swap treatment groups at the commencement of transition to give further insights into responses to extended supplementation. All transition diets were designed to be negative in DCAD. We hypothesized that feeding 25-(OH)D₃ during lactation and in the prepartum period, in conjunction with negative DCAD diets, would improve milk production, increase the probability of pregnancy, and reduce the incidence of postcalving diseases.

MATERIALS AND METHODS

This study was approved by the Scibus Animal Care and Use Committee (Scibus Project number 1215–1217).

Experimental Design

A total of 3,246 Holstein, Jersey, Holstein cross, or Jersey cross female cattle were enrolled in 1 of 2 concurrent randomized cohort experiments (Exp. 1 and Exp. 2) from 4 commercial dairies, with herd sizes between 500 and 620 lactating cows at peak. Three of the four dairies were in Australia, and one was on the North Island of New Zealand. All dairies milked cows twice daily and fed some pasture, depending on the season. Cows from all dairies were housed on pasture for the full duration of the study. Details of the dairies are summarized in Table 1. We considered that a geographical spread of the dairies would help to improve the external validity of the study. The study was conducted from August 2016 to June 2019.

Dairy Selection Criteria

The dairies were selected for use in the study on the basis that they had good record keeping, which was determined by reviewing previous records, which suggested that they would be capable of maintaining the attention to detail consistent with successful study conduct. Specifically, the dairies enrolled met the following criteria: they had a rotary milking parlor; had a herd size ≥500 lactating cows; had the ability to record

Table 1. Summary of dairies enrolled and baseline blood concentrations

Descriptor	Dairy 1	Dairy 2	Dairy 3	Dairy 4
Region	Waikato, North Island	Western Districts, Vic	Western Districts, Vic	South Coast, NSW
Country	New Zealand	Australia	Australia	Australia
Predominant breed	Holstein × Jersey	Holstein	Holstein	Holstein
Lactation herd size	620	500	620	600
Farm feeding type	PMR ¹	PMR	In-parlor feeding with some silage	PMR
Calving pattern	Split (autumn and spring)	Split (autumn and spring)	Split (autumn and spring)	Year-round
Housing system	Nonhoused	Nonhoused	Nonhoused	Nonhoused
Milk volume meters	YieldSense	YieldSense	DeLaval	ELI Innovation
Herd management software	(Jaotec Systems)	On 25% of milking stalls		
	Identity	Identity		
	(Jaotec Systems)			
	Inline meters	Inline meters	DeLaval	iDairy
Type of milk component and cell count measurement			Herd Recording Agency	(Farm Automation Australia)
Milk component measurement	YieldSense	YieldSense		Herd Recording Agency
system or agency	On 100% of milking stalls	On 25% of milking stalls	National Herd Development	Dairy Express
Milk cell count measurement	CellSense	CellSense	National Herd Development	Dairy Express
system or agency	(Jaotec Systems)	On 25% of milking stalls		
Pellet dispenser	On 100% of milking stalls			
Pellet production	PPP Industries Ltd.	Feedtech Feeding Systems	Feedtech Feeding Systems	Northern Feed Systems
Body weight scales	Intergrain	Pristine Animal Nutrition	Pristine Animal Nutrition	Pristine Animal Nutrition
Laboratory for sera analyses	Gallagher XDS5000	Tru-Test Limited XR 3000	Tru-Test Limited XR 3000	Gallagher XDS5000
	IDEXX Laboratories	University Veterinary Teaching Hospital Camden	University Veterinary Teaching Hospital Camden	University Veterinary Teaching Hospital Camden
Baseline blood measures (mean ± SD)				
Plasma 25-(OH)D ₃ (ng/mL)				
Primiparous	—	32.0 ± 9.9	48.0 ± 11.8	54.6 ± 14.2
Multiparous	—	27.4 ± 10.2	47.7 ± 12.3	47.1 ± 14.1
Serum Ca (mM)				
Primiparous	—	2.27 ± 0.18	2.35 ± 0.10	2.35 ± 0.11
Multiparous	—	2.16 ± 0.39	2.33 ± 0.10	2.24 ± 0.16
Serum P (mM)				
Primiparous	—	2.11 ± 0.31	2.03 ± 0.24	2.28 ± 0.34
Multiparous	—	1.95 ± 0.39	1.92 ± 0.32	2.05 ± 0.36

¹PMR = partial mixed ration.

daily milk, health and reproductive data, and milk solids production on a regular basis; had cows with clear identification, history, and pregnancy status; were willing to bring transition cows into the milking parlor once daily to receive treatment; had facilities suitable for weighing and blood sampling a subgroup of cows. A study monitor was available in the region to assist with protocol compliance.

Cow Eligibility Selection Criteria

Cows were eligible to enter the study if they met the following criteria: they were Holstein, Jersey, or a Holstein or Jersey cross (based on herd records); they were uniquely identified with at least one ear tag; they had not aborted in the current lactation (for Exp. 2 or in the dry period for Exp. 1); they had a BCS between 2.0 and 4.25 on a 5-point scale (Edmonson et al., 1989); and they had complete biographical, reproductive, and health records that made physiological sense (i.e., cows without previous calving dates were not enrolled). Cows that had clinical diseases or did not have 4 functioning quarters entered the study if the intention was to breed them and keep them in the herd.

Experiment 1

The aim of Exp. 1 was to evaluate the performance, health and, reproduction of cattle, fed daily with 25-(OH)D₃ from ~21 d prepartum through to the end of that lactation (Figure 1A). The prepartum diets were negative in DCAD. A total of 1,182 cows ranging from nulliparous to 11 lactations at the start of transition (target of 21 d before estimated calving date) were enrolled at approximately 21 d prepartum. Of these cattle, 64.1% were nulliparous as the majority of the cattle from each of the 4 dairies were enrolled in Exp. 2 and thus unavailable for Exp. 1. Of the remainder, 5.9% were primiparous, 7.5% were in second lactation, 6.1% were in third lactation, and 16.3% were in ≥4 lactation.

Cows were randomly allocated using the *ralloc* function in Stata version 14.1 (StataCorp LP) to 1 of 2 treatment groups based on their status of being nulliparous or parous and estimated days to calving if this information was available. An accurate estimated calving date was not known for several of the nulliparous cattle because these had often been bull bred, and early pregnancy diagnosis was not performed. The mean ± SD prepartum transition interval for the nulliparous cows was 14 ± 13.7 d, the median was 10 d, and the range was 0 to 89 d. Throughout the text, nulliparous cows are referred to as “primiparous” because all outcome

variables refer to measures taken after parturition. The mean ± SD prepartum transition interval for the multiparous cattle was 13 ± 10.9 d, the median was 12 d, and the range was 0 to 87 d. Treatment groups were (1) CON [n = 645; no 25-(OH)D₃] and (2) TRT [n = 537; 2 mg/d of 25-(OH)D₃ ~21 d prepartum to parturition and 1 mg/d in lactation] and were not blinded, except on dairy 1. Cows were terminated from the study in the lactation after enrollment on the date they were dried off, died, or were sold, or the final calendar date of the study, whichever occurred first.

Experiment 2

The aim of Exp. 2 was to evaluate the performance of cattle over part of 2 lactations, following the presence or absence of oral administration of 25-(OH)D₃ from mid-lactation, during the precalving transition period (~21 d prepartum to parturition), and through to the end of the subsequent lactation. A total of 2,064 cows entered the study during lactation, with a median of 147 DIM at entry, mean of 170, and ranging from 0 to 726 DIM. Late lactation cows, ≥400 DIM (5% of enrollment), were included because they are part of the herd structure of commercial dairy operations.

Cows were randomly allocated using the *ralloc* function in Stata version 14.1 (StataCorp LP) to 1 of 4 treatment groups based on their status of being primiparous or multiparous and DIM on the day of study commencement. We intended that 50% of the cows would switch treatment groups when entering the transition period (~21 d prepartum) after completing a partial lactation in the study (**L1**) and far-off dry period and then remain in the study for the subsequent lactation (**L2**). No cattle received treatment during the far-off dry period. We defined the transition period as ~21 d prepartum to parturition (mean ± SD 17 ± 9.5, median 16, and range 0 to 87 d). Figure 1B shows a timeline of events. Treatment groups were as follows, with the amount (mg/d) of 25-(OH)D₃ fed indicated in parentheses for L1, transition, and L2, respectively: (A) control-control (**CON-CON**; 0–0–0), (B) treatment-treatment (**TRT-TRT**; 1–2–1), (C) control-treatment (**CON-TRT**; 0–2–1), and (D) treatment-control (**TRT-CON**; 1–0–0). For L1 of the study, 1,032 cows entered the control groups A or C, and 1,032 cows entered groups B or D. We anticipated that not all of the cows enrolled would enter the second lactation of the study due to culling, death, or pregnancy failure. The number of cows that entered L2 of the study for treatment groups A to D was 521, 523, 273, and 248, respectively. We intended that these groups would be close to even in cow numbers, but a failure to switch

Figure 1. Example timeline of events for (A) cows that entered experiment (Exp.) 1 at 21 d prepartum as either control cows (CON) that were given no 25-(OH)D₃ throughout the study or treatment (TRT) cows that were given 2 mg/d of 25-(OH)D₃ during transition (~21 d prepartum to calving) followed by 1 mg/d of 25-(OH)D₃ in lactation up to 305 d, and (B) for a cow that entered Exp. 2 at 100 DIM. Note, cows entered at various DIM, but the median was 147 DIM. Cows were randomized into 1 of 4 treatment groups (A to D). We intended that 50% of the cows would switch treatment groups when entering the transition period (~21 d prepartum) after completing a partial lactation in the study (L1) and far-off dry period and remain in the study for the subsequent lactation (L2) up to 400 DIM. No cattle received treatment during the far-off dry period. We defined the transition period as ~21 d prepartum to parturition. Treatment groups were as follows, with the mg/d of 25-(OH)D₃ fed indicated in brackets for L1, transition, and L2, respectively: (A) control-control (CON-CON; 0–0–0); (B) treatment-treatment (TRT-TRT; 1–2–1); (C) control-treatment (CON-TRT; 0–2–1); and (D) treatment-control (TRT-CON; 1–0–0).

treatment groups from CON to TRT occurred for 27 and 68% of cows originally allocated to the CON-TRT group from dairies 3 and 4, respectively. A failure to switch from TRT to CON occurred for 45 and 72% of cows originally allocated to TRT-CON from dairies 3

and 4, respectively. Table 2 shows the target amount (mg/d) of 25-(OH)D₃ for each physiological stage for each treatment group. Cows were terminated from their treatment group in L1 on the date they were dried off, died, or were sold, depending on which occurred first.

Cows were terminated from their L2 treatment on the date they were dried off, died, or were sold, or the final date of the study, whichever occurred first.

Subgroups of cattle were selected for additional measurements, including plasma 25-(OH)D₃ concentration, to provide an indication of possible cross contamination between treatment groups and to monitor blood 25-(OH)D₃, Ca, and P concentrations, BW, and BCS responses to treatment. The intention was to select a population of 15 cows from each of the 4 treatment groups and collect measurements at the following 5 time points from the same cows: (0) before study commencement (baseline), (1) 100 d posttreatment in L1, (2) 200 d posttreatment in L1, (3) 100 d postpartum in L2, and (4) 200 d postpartum in L2 (Figure 1B). To account for the possibility that cows may not be drafted correctly or be available for sampling, a total of 17 cattle, 5 primiparous and 12 multiparous cows, were selected before each sampling. When possible, the same 17 cows from each treatment group were sampled at each sampling day. Cows that were not pregnant, had aborted, had died, had been culled, or did not calve when anticipated were replaced with other eligible cows. The largest number of cows that required replacement was between sample points 0 and 1 because pregnancy diagnosis had not been performed for all cows.

The subgroup population was selected primarily based on estimated days to calving and was balanced between treatment groups for not only estimated days to calving, but also DIM and parity. In addition, cows also had to be Holstein (except dairy 1), have 4 functioning quarters, be less than fifth parity at enrollment, and not received antibiotics in the last 30 d. Exceptions were the inclusion of crossbreds (5.1% of samples) or older cows (8.9% of samples) to balance treatment groups and DIM.

The measurements collected from the subgroup cows were BW, 1 to 5 BCS (Edmonson et al., 1989), serum Ca, serum P, plasma 25-(OH)D₃, and their mean milk yields over the 7 d before blood sampling (dairies 3 and 4) or 10 d before blood sampling (dairies 1 and 2) collected, using on-farm milk meters. For dairies 1 and 2, the last 10-d mean milk protein and fat percentages

were also collected. Blood was collected from the coccygeal vein or artery into silicon-coated collection tubes for serum separation and EDTA coated collection tubes for plasma separation (Becton, Dickinson and Co.).

The EDTA samples were centrifuged at $1,110 \times g$ at room temperature for 15 min to separate plasma, which was pipetted into 2×1.5 -mL aliquots and frozen at -20°C until shipment on dry ice to the laboratory at DSM Nutritional Products for 25-(OH)D₃ analysis. To measure 25-(OH)D₃ concentration, the proteins were removed from the plasma samples with a double volume of acetonitrile, which contained deuterated 25-hydroxycholecalciferol as internal standard. After centrifugation, the supernatant was injected into a reverse phase UPLC system (Agilent 1290; Agilent Technologies) coupled with MS detector (API 4000; ABSciex). To assess the daily and long-term laboratory performance of the methods (both in plasma and the treatment pellets), dedicated standard and quality-control samples were analyzed daily with unknown samples to ensure the accuracy and precision of the method. Data acquisition of extracted ion chromatograms, integration, and quantification were performed using Analyst software (ABSciex).

For dairies 1 and 4, the silicon-coated blood tubes for serum separation were kept on ice or with ice bricks and transported to IDEXX Laboratories (Hamilton, New Zealand) and The University Veterinary Teaching Hospital Camden (Camden, NSW, Australia), respectively, for Ca and P analysis. Serum Ca and P concentrations were measured by IDEXX Laboratories using Beckman Coulter reagents Calcium Arsenazo OSR61117 and Inorganic Phosphorus OSR6122 on a Beckman Coulter AU680 Analyzer. For dairies 2 and 3, the silicon-coated blood tubes were centrifuged at $1,110 \times g$ at room temperature for 15 min to separate the serum, which was pipetted into 2×1.5 -mL aliquots and frozen at -20°C for later Ca and P analysis at The University Veterinary Teaching Hospital Camden. Serum Ca and P concentrations were measured by The University Veterinary Teaching Hospital Camden using Thermo Fisher Scientific Oy kits 981367/981772 and 981891/0, respectively, according to manufacturer's

Table 2. Target dose of active 25-(OH)D₃, indicated in parentheses (in mg/d), at each physiological stage for each treatment group in Exp. 2

Group	Treatment 1	Far-off dry	Treatment 2	
	Lactation 1		Transition	Lactation 2
1. Control-control (CON-CON)	CON (0)	0	CON (0)	CON (0)
2. Treatment-treatment (TRT-TRT)	TRT (1)	0	TRT (2)	TRT (1)
3. Control-treatment (CON-TRT)	CON (0)	0	TRT (2)	TRT (1)
4. Treatment-control (TRT-CON)	TRT (1)	0	CON (0)	CON (0)

protocols on a Konelab 20XTi analyzer (Thermo Fisher Scientific Oy).

Treatment Administration

Custom manufactured pellets were used to deliver a premix containing 25-(OH)D₃ (1.25% Rovimix, DSM Nutritional Products). The pellet consisted of 74.4% wheat middlings, 21% lime, 1% oil, and 3.6% 25-(OH)D₃ premix. Pellet manufacturer details for each dairy are in Table 1. During transition, each cow was brought through the milking parlor once daily, and approximately 60 g of pellet was delivered to the ration of each treatment cow, using a pellet dispenser. Approximately 30 g of pellet was delivered to each treatment cow during 1 milking/day. The release of pellets was controlled through the herd management software program of each dairy, based on electronic identification of cows as they entered the milking parlor. The pellets were delivered via a separate feedline at the same time as the transition or lactation mix, allowing pellets to be mixed with the entire feed drop. If the electronic tag of a cow could not be read or the cow went more than once around the parlor, the cow was given a default drop of feed that contained no treatment pellets. Video surveillance above the exit position on the rotary milking parlor (GoPro Hero 5) was used to monitor feed residuals and possible cross contamination. Feed residuals in the feeders in each milking stall were individually scored on a 0-to-5 scale. The pellet dispensers were calibrated at study commencement and validated every 2 wk by averaging the weight of 5 simulated pellet dispenses when the milking parlor was not being operated between a.m. and p.m. milkings. The dispensers were recalibrated if the average was more than $\pm 10\%$ of the target weight. The number of bags of pellets added to the pellet silos was recorded to reconcile against the number of cows on treatment to ensure that the correct amount of pellet was being administered. Multiple batches of pellets were manufactured throughout the trial to ensure pellets were fresh. The 25-(OH)D₃ content of each batch of pellets was tested at DSM Nutritional Products. In brief, after addition of the deuterated internal standard, pellet samples were saponified, followed by a liquid-liquid extraction with methyl *tert*-butyl ether. The extract was evaporated under nitrogen and then analyzed using a reverse phase HPLC system (Agilent 1260; Agilent Technologies) coupled with an MS detector (API 4000; ABSciex). Data acquisition of extracted ion chromatograms, integration, and quantification were performed by Analyst software (ABSciex). The mean $\pm$ SD of 25-(OH)D₃ concentration analyzed was 31.24 ± 4.40 mg/kg. The target was 50 mg/kg; therefore, the amount of pellets supplemented was increased

so the fed amount was approximately one-third higher than the initially intended rate to compensate for approximately a one-third loss (i.e., increased from 20 to 30 g/d). The exception was on dairy 1, located in New Zealand, which commenced the study first, where the initial amounts of 20 g/d in transition and 40 g/d in lactation were given. The cows in this herd were Holstein and Jersey crossbreds; hence, they had lower BW. Dairy 1 also had a pellet dispenser installed in the milking parlor, so a control pellet consisting of 74% wheat middlings, 25% limestone, and 1% oil was fed to the control cattle to act as a placebo, and the farm owner and study monitor were blinded to the treatment groups. On the dairies in Australia, it was not feasible to install 2 pellet dispensers in each milking parlor, and because such a small amount of pellet was being delivered, we decided not to use a placebo for these herds.

Diet

Each dairy fed a different diet, which varied throughout the study due to season and drought. It was our intention to test the efficacy of 25-(OH)D₃ under different commercial dairy conditions. We did not expect that the dairies would have identical diets but the pre-calving diets were designed to acidify the diet and deliver optimal levels of macromineral and microminerals, energy, and protein as described by DeGaris and Lean (2008). Bio-Chlor (Arm & Hammer Animal Nutrition) was included in the transition ration for each dairy at a dose of 700 or 750 g/d, and a negative DCAD transition diet was formulated.

Composition of the lactation ration at the start of the study and representative transition rations for the 4 dairies along with diet analysis performed in CPM Dairy Ration Analyzer (version 3.10; Cornell-Penn-Miner, Cornell University) are provided in Tables 3 and 4, respectively. Dairy 1 in New Zealand fed a partial mixed ration (**PMR**) that consisted of maize silage and several by-products, depending on the season, and had limited in-parlor feeding, typically 300 g of canola meal per milking. Dairies 2 and 4 also fed a season-dependent PMR, largely based on maize and grass silage and including concentrates fed in the milking parlor. Dairy 3 was pasture-based with 7 to 10 kg of DM concentrate supplemented in the parlor. Some grass silage or hay was fed in the field when required, and brassicas were grazed in summer through autumn. Pastures were predominantly ryegrass, including annual (*Lolium multiflorum*), Italian (*Lolium multiflorum*), and perennial (*Lolium perenne*) for all dairies and kikuyu (*Pennisetum clandestinum*) for dairy 4. Concentrate delivery was predominantly in the milking parlor, and concentrates were fed at a flat rate at all farms with

Table 3. Diet ingredients for control rations from each dairy¹

Ingredient (kg of DM/d)	Dairy 1		Dairy 2		Dairy 3		Dairy 4	
	Lactating	Transition: spring 2016	Transition: autumn 2017	Lactating	Transition: spring 2017	Transition: autumn 2018	Lactating	Transition: autumn 2018
Almond hulls						1.21		0.05
Ammonium chloride								
Barley grain ground							1.7	1.48
Barley straw	0.3	1.9	1.5	4.04	1.2			
Bio-Chlor ²		0.7	0.7		0.66	0.85		0.32
Bioplex Hi Five ³		0.01	0.01					0.03
Calcium chloride								
Calcium sulfate dihydrate		0.07	0.07			0.04		
Canola meal	0.3	1.35	0.5	0.85	0.41		0.51	0.44
Canola oil				0.01				0.11
Corn gluten	4	0.9	3					
Corn grain							0.5	
Dicalcium phosphate							0.05	
Dried distillers grain							2.6	1.34
Fat (tallow)							0.013	0.05
Fusion Dyad ⁴		0.01	0.01					
Grass hay	1.87							
Kiwi fruit	2.28	2.4		0.16	1.8×10^4		2.54	9.12
Levucell SC ⁵				1.3×10^4	0.05	0.07		
Lutrell Pure ⁶					0.04	0.03		
Lime	0.2	0.06	0.05				0.18	0.25
Magnesium chloride		0.1	0.1					0.01
Magnesium oxide	0.03	0.03	0.03	0.03	0.04	0.05	0.03	0.04
Magnesium sulfate		0.08	0.1		0.06	0.08	0.04	0.13
Maize silage	6	3.7	4	2.5	3	1.15		2.52
Megalac ⁷							0.24	
Molasses							2.73	0.11
Oat hay					4	4.49		
Oil					0.01	0.02		
Palm kernel extract		0.87						
Pasture	8.1		3	10.5	2		10	9.75
Premix ⁸							0.08	
RFT premix ⁹								
RT premix ¹⁰				0.007	0.005	0.006		
RTT premix ¹¹								0.004
Ryegrass silage								
Salt				2	1			
Sodium bicarbonate	0.04			0.05			0.05	0.02
Sugar							0.22	
Urea				0.03	0.02	0.03		
Vetch hay				1		0.45		
Wheat crushed				4.33	1.2	2.8	10.68	3.0
Wheat mill run					0.5	0.06	7.22	0.66
Wheat hay							23.01	4.52
Total (kg of DM/d)	21.25	14.05	13.07	25.51	14.20	11.34	21.02	22.26
								12.50

¹The transition rations are representative of various seasons, whereas the lactating diets are those at the start of the study, unless otherwise stated. Note for treatment cows 60 g/d of a pellet consisting of 3.6% 25-(OH)D₃ premix (1.25% Rovimix, DSM Nutritional Products), 74.4% wheat middlings, 21% lime, and 1% oil was added to transition rations (40 g/d for dairy 1) and 30 g/d was added to the lactation ration (20 g/d for dairy 1), equating to approximately 2 and 1 mg/d of 25-(OH)D₃, respectively.

²Bio-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).

³Bioplex Hi Five (a combined blend of Cu, Zn, Mn, Co, and Se; Alltech).

⁴Fusion Dyad (a broad-spectrum mycotoxin binder; Nutritech, Mangere).

⁵Levucell SC (yeast strain *Saccharomyces cerevisiae* CNCM I-1077; Lallemand Animal Nutrition).

⁶Lutrell Pure (a CLA product containing 20% CLA isomers comprising 10% *cis-9,trans-11-18:2* and 10% *trans-10,cis-12-18:2*; BASF Australia).

⁷Megalac (a source of rumen-bypass fat; Arm & Hammer Animal Nutrition).

⁸Premix (Rabar Animal Nutrition, Beaudesert) containing vitamins, minerals, trace elements, biotin, and Rovimix D3-500 (DSM Nutritional Products).

⁹RT premix (a blend of Rumensin at 250 mg/head per day (monensin; Elanco Animal Health), Flavaco at 10 mg/head per day (flavomycin; International Animal Health Products), vitamins, minerals, and trace elements (Cow-R-Us)).

¹⁰RT premix (Cow-R-Us) containing vitamins, minerals, and trace elements.

¹¹RTT premix (Cow-R-Us) containing vitamins, minerals, biotin, and trace elements.

Table 4. Diet analysis for control rations from each dairy for both experiments calculated by CPM Dairy Ration Analyzer (version 3.10; Cornell-Penn-Miner, Cornell University)¹

Nutrient (% DM unless otherwise specified)	Dairy 1			Dairy 2			Dairy 3			Dairy 4	
	Lactating	Transition: spring 2016	Transition: fall 2017	Lactating	Transition: fall 2018	Transition: spring 2017	Lactating: start	Lactating: winter	Transition: summer 2018	Lactating	Transition: fall 2018
Forage	67.74	53.15	65.03	62.98	53.72	72.7	48.36	67.53	68.08	54.04	56.45
CP	19.34	14.68	17.25	21.72	12.71	15.4	15.72	24.79	15.87	17.87	14.6
RUP (% of CP)	32.32	23.65	23.54	27.72	15.95	24.97	32.07	30.65	26.57	31.69	19.34
RDP (% of CP)	67.68	76.35	76.46	72.28	84.05	75.03	67.93	69.35	73.43	68.31	80.66
RDP	13.09	11.2	13.19	15.7	10.68	11.55	10.68	17.19	11.65	12.21	11.78
Soluble protein (% of CP)	43.1	43.26	49.91	42.77	53.97	48.94	31	47.11	36.46	41.12	44.34
ME (MJ/kg)	10.37	9.28	9.48	10.81	10.17	9.94	11.18	10.4	9.48	10.55	10.09
NE _L (MJ/kg)	6.68	5.98	6.1	6.96	6.55	6.4	7.2	6.7	6.1	6.79	6.5
ADF	22.04	28.42	26.6	20.77	23.26	25.53	21.58	19.68	27.93	21.17	23.17
NDF	41.03	47.28	48.84	33.89	38	42.31	33.47	33.23	46	37.32	42.54
Forage NDF	29.04	31.9	34.8	27.74	29.95	37.32	27.22	29.43	40.92	28.2	32.4
Physically effective NDF	27.49	35.87	35.32	22.85	32.93	33.39	21.56	24.46	39.35	24.74	33.34
Lignin	3.19	4.96	3.58	2.65	3.41	3.28	3.17	3.64	2.61	3.04	3.84
NFC ²	31.12	29.47	24.06	34.84	39.97	33.19	45.67	32.25	30.3	34.16	34.35
Silage acids	1.94	1.8	2.1	0.82	0.53	1.36	0	0	0	0	0.94
Sugar	9.99	12.57	2.72	3.14	10.89	5.56	2.91	2.49	4.86	6.87	5.34
Starch	13.43	11.39	15.5	23.37	21.62	19.17	33.9	21.71	17.77	17.54	22.67
Soluble fiber	5.76	3.71	3.75	7.5	6.92	7.1	8.86	8.05	7.66	9.75	5.39
Ether extract total	4.13	3.55	3.43	4.47	2.61	3.43	3.55	3.97	2.4	5.19	3.35
Ash	8.58	8.44	9.45	8.83	8.07	8	5.35	10.82	8.63	9.55	7.53
Ca	0.6	0.54	0.54	0.48	0.36	0.4	0.16	0.53	0.38	1	0.35
P	0.4	0.36	0.41	0.47	0.27	0.34	0.35	0.46	0.35	0.49	0.47
Mg	0.29	0.45	0.52	0.25	0.49	0.39	0.16	0.37	0.37	0.37	0.5
K	2.39	1.23	1.41	2.28	1.31	1.62	1.94	2.96	2	2.19	1.26
S	0.29	0.47	0.56	0.33	0.46	0.38	0.2	0.35	0.34	0.26	0.38
Na	0.14	0.16	0.12	0.2	0.32	0.24	0.29	1.28	0.23	0.45	0.1
Cl	0.59	1.22	1.12	0.98	1.28	1.15	1.29	1.36	1.76	1.01	0.98
Fe (mg/kg)	153.24	186.57	193.62	826.32	232.89	465.93	1,083.32	473.62	136.06	281.06	178.95
Zn (mg/kg)	82.54	133.65	157.31	44.06	81	62.72	41.14	37.08	56.77	79.51	37.38
Cu (mg/kg)	11.27	21.93	21.33	10.39	22.95	16.82	10.07	8.48	16.19	16.55	8.18
Mn (mg/kg)	27.2	62.3	32.06	59.14	64.04	66.66	93.28	80.97	100.7	79.11	50.18
Se (mg/kg)	0.07	0.05	0.09	0.13	0.4	0.24	0.15	0.1	0.25	0.08	0.07
Co (mg/kg)	0.05	0.12	0.12	0.18	0.57	0.38	0.07	0.04	0.32	0.08	0.09
I (mg/kg)	0.01	0	0.02	0.17	0.64	0.41	0	0.27	0.33	0.01	0.01
DCAD ³ (mEq/100 g)	32.45	-25.7	-24.81	18.5	-17.36	-4.59	13.49	71.02	-9.18	30.61	-14.68
Vitamin D ₃ (IU/kg)	0	0	0	0	0	0	20,000	20,000	0	0	0

¹The transition rations are representative of various seasons, whereas the lactating diets are those at the start of the study, unless otherwise stated. Note for treatment cows 60 g/d of a pellet consisting of 25-(OH)D₃, wheat solubles, lime, and oil was added to transition rations (40 g/d for dairy 1) and 30 g/d was added to the lactation ration (20 g/d for dairy 1), equating to approximately 2 mg/d and 1 mg/d of 25-(OH)D₃, respectively.

²NFC = 100 - (CP + ether extract + ash + NDF-NDICP), where NDICP = neutral detergent insoluble CP (Van Soest, 1994).

³DCAD was calculated by NRC (2001).

the exception that Jersey cows often received lower amounts.

Samples of all new deliveries, harvests, or batches of feeds were taken at each farm, along with periodic TMR samples from dairy 4, stored at -20°C and later analyzed by wet chemistry or near-infrared reflectance spectroscopy at Dairy One Cooperative Inc. Forage Testing Laboratory (Ithaca, NY) according to wet chemistry AOAC International (1999) methods detailed in Golder et al. (2019). The near-infrared reflectance spectroscopy equations were based on methods detailed by Bramley et al. (2012), with the exception of NDF, which was determined as described by Van Soest et al. (1991), using heat-stable amylase without sodium sulfite and the NFC equation that was $\text{NFC} = 100 - (\text{NDF} + \text{CP} + \text{crude fat} + \text{ash})$. When deliveries were frequent and from the same supplier, such as dried distillers grain, samples were pooled for testing.

Urine Testing

To test whether cows were correctly receiving the negative DCAD diet and metabolic acidosis was being achieved, urine was collected from 5 randomly selected cows that had been on a transition diet for more than 5 d. The frequency of testing varied with dairy but was as frequent as every 2 wk for dairy 4, which had a year-round calving pattern. The pH of the urine was tested with a pH 22 LAQUAtwin (Horiba).

Sample Size Determinations

Sample size was estimated using the *rdpower* program in Stata version 14.1 (StataCorp LP), based on 750 cows per group; this would achieve a statistical power of approximately 0.65, for a difference of 20% in hazard of pregnancy, a power of 1.00 for a 1-L difference in milk yield, and a power of 0.90 for 100% difference in risk of a clinical disease incidence of 5% and an effect that increases the probability of pregnancy at an insemination from 35 to 40% with a power $(1 - \beta) = 0.8$ and $\alpha = 0.05$. Therefore, a total of approximately 1,500 to 2,000 cows would be sufficient to achieve a significant difference, based primarily on the difference in hazard of pregnancy in L1 of Exp. 2.

Milk Production Data

All dairies milked twice daily, and all data were recorded on the herd management software program of each dairy. Details of the herd management software, milking equipment, milk meters, milk component meters, and herd recording agencies are described in Table 1.

Weekly Milk Yield. Each dairy had individual inline milk meters that recorded milk yield data from each milking onto the herd management software. For dairies 1 and 2, morning and afternoon milking data were transferred from Microsoft Access 2016 (www.microsoft.com) databases generated by the herd management software to Microsoft Excel 2016 (www.microsoft.com) and summed to give daily total production. If a recording was missed from either milking, the yield from that day was not included. For dairies 3 and 4, daily total milk yields were downloaded from the herd management software into Excel files. Weekly averages were then calculated in RStudio version 1.1.383 (<https://www.rstudio.com>) for each dairy before statistical analysis. In Exp. 1 and L2 of Exp. 2, milk averages were taken from calving date, whereas in L1 of Exp. 2, in which cows commenced the study when already in milk, averages were taken from study d 0.

Milk Components and SCC. Dairies 1 and 2 had inline milk component (fat and protein percent) and SCC meters. The meters at dairy 1 were on each individual milking stall, whereas the meters at dairy 2 were on 25% of the milking stalls. The SCC data from the inline meters were not used from either dairy due to many zero recordings, which are not physiological. Milk fat and protein percentage and SCC were measured at either the morning or afternoon milking on dairies 3 and 4 by a herd recording agency at approximately 60-d intervals. Methods used to ensure equivalency in milk component data among all 4 dairies are detailed in the supplemental material (<https://doi.org/10.6084/m9.figshare.c.5230055.v1>; Golder et al., 2020). For cows in Exp. 1 and those that entered L2 in Exp. 2, herd test results collected between 0 and 59 DIM were used as herd test 1; subsequent test results at 60-d intervals were used for tests 2 to 4. For cows in L1 of Exp. 2, herd test results collected between study d 0 and 59 DIM were used as herd test 1; subsequent test results at 60-d intervals were used for tests 2 to 4. Energy-corrected milk was calculated as $\text{ECM} = [(0.3246 \times \text{milk yield}) + (12.86 \times \text{fat yield}) + (7.04 \times \text{protein yield})]$; NRC, 2001].

Survival and General Censoring

Cows in Exp. 2 were terminated from their treatment 1 group on the date they were dried off, died, were sold, or reached 300 d on study, based on whichever occurred first. Experiment 2 cows were terminated from their second treatment group (during L2) on the date they were dried off, died or were sold, or when they reached 400 d since calving in L2 or the final date of the study, whichever occurred first. The same applied to Exp. 1 cows (those that commenced the study at transition)

except that the maximum length of study was 305 d. Cows that died or were culled were terminated from the weekly milk and herd test data on the date they were removed from the herd. These cows were censored from the survival, reproduction, and health data at that point. Cows that spent more than 3 consecutive weeks on the wrong treatment in lactation ($n = 6$) were terminated from the weekly milk and herd test data at the date of last correct treatment. Survival, health, and reproduction data for these cows were censored at this date.

Health and Reproductive Events

All cows eligible to enter the study contributed data to the health and reproductive records. The diagnosis of disease was primarily by dairy staff, and pregnancy diagnosis was by veterinarians. Health and reproductive data were entered in accordance with standard operating procedures developed with each dairy. These definitions were largely consistent among dairies and are defined in the supplemental material (<https://doi.org/10.6084/m9.figshare.c.5230055.v1>; Golder et al., 2020). Disease and reproductive events, including date of calving, breeding events, pregnancy results, decisions not to breed, clinical disease diagnosis and diagnosis date, disease treatment and dates of treatment, date and reason for death, and data and reason for culling, were recorded daily using the herd management programs and were subsequently exported to Excel for processing before statistical analysis. When a diagnosis was not known, it was recorded as “unknown” and later incorporated into the category “other.” Disorders with a low prevalence, which in most cases did not occur across all 4 dairies, were incorporated into the “other” category to enable analysis. Only the first incidence of each clinical disorder for each cow up to the first 300 d on study was used for analysis. Total clinical disease was calculated as the sum of cows that had at least 1 disorder over the first 300 d of the study.

Reproductive data were gathered on cows from Exp. 1 and those that entered L2 in Exp. 2. Reproductive data were not analyzed from L1 for cows in Exp. 2. Cows were not selectively withheld from breeding based on milk yield, but the split-calving herds (dairies 1 to 3) had a breeding start date. Both the voluntary wait and submission rates can be observed in the Kaplan-Meier survival curves in the results. None of the dairies used synchrony programs. Cows in both dairies 1 and 2 had heat detection collars (SCR Engineers Ltd.). Cows were both right- and left-censored in the study. Cows that died during calving were left-censored because there would have been no intention to breed these. Cows that were designated as do-not-breed were cen-

sored at the date of that decision, and decisions not to breed were made by farm personnel independent of treatment group. Cows that were not pregnant were censored at the last insemination date, resulting in full censoring or pregnancy for cows in the study. For the year-round calving herd (dairy 4), cows that were not pregnant were censored at 300 d. Whereas most cows had a pregnancy diagnosis before removal or movement to the wrong treatment group that allowed determination of pregnancy status at the time of removal, cows that did not have their status confirmed before removal were considered not to be pregnant at the time of removal. Cows that had a confirmed pregnancy diagnosis before 40 d of gestation that then were open at the next pregnancy diagnosis were assigned not pregnant at the earlier diagnosis. Inseminations that were reported ≤ 5 d after parturition were not included in the data set. The pattern of censoring was evaluated to identify any possible anomalies in these data.

Statistical Analysis

All data were analyzed using Stata version 15.1 (StataCorp LLC, <https://www.stata.com>) and the unit of interest is the cow. Data were analyzed as the following 4 data sets: (1) Exp. 1, (2) Exp. 2–L1, (3) Exp. 2–L2, and (4) subgroup.

Milk production data were initially explored for each dairy and evaluated for normality of distribution of milk production responses. We recognized that cows in later lactations were not highly represented in the data, and lactation number was categorized into 4 groups: parity or lactation 1, 2, 3, and ≥ 4 . A nulliparous category was included for Exp. 1. Breed was recorded but was not included in analysis because it is accounted for in the fixed effect of dairy.

A similar linear mixed model was fitted to the weekly milk yield and herd test milk production data for the first 3 data sets and all variables in data set 4. The covariance for each of these mixed models was unstructured. This structure was chosen based on having the lowest Akaike and Bayesian information criteria. Marginal means and contrasts were estimated and used to provide estimates of treatment differences. The SCC data were natural log-transformed for each data set because these were not normally distributed.

Different time-failure models were used to assess survival and reproductive outcomes. A comparison of fit for Cox, exponential, lognormal, and Weibull models indicated that the Weibull model had best fit for most models based on the Akaike and Bayesian information criteria. Contrasts and pairwise comparisons were performed for all models. Kaplan-Meier survival curves were produced for survival, reproduction, and clinical

health disorder outcomes. Only clinical health disorders that had a total of >8 cases were analyzed.

Exp. 1. For the weekly milk and herd test data the model specified was

$$Y_{ijklm} = \mu + \alpha_i + \gamma_j + \theta_k + \delta_{lm} + \beta X_{ijklm} \\ + \omega\alpha\theta\delta\gamma_{ijkl} + \varepsilon_{ijklm},$$

where Y_{ijklm} = dependent variable, μ is the overall mean, α_i is the fixed effect of treatment (i = CON or TRT), γ_j is the fixed effect of dairy (j = dairies 1 to 4), θ_k is the fixed effect of parity (k = primiparous or multiparous), δ_{lm} is the fixed effect of time (l = wk 1 to 15 and herd test 1 to 5) for cow number m (m = 1 to 1,167 for weekly milk and 1,083 for herd test), βX_{ijklm} is the covariable adjustment for time spent on transition, and $\omega\alpha\theta\delta\gamma_{ijkl}$ are the fixed effects of interaction terms including 2-, 3-, and 4-way interactions of treatment, dairy, parity, and time, and ε_{ijklm} is the random error term. The 7-d mean milk volume before the start of the study was considered a covariable but not included in the final model.

A Weibull accelerated time-failure model was used to assess survival (odds of death or being culled per day and censoring pattern) and odds per day of being bred and pregnancy. A random effects logistic regression model (melogit command in Stata) was fitted for the odds of pregnancy at first service. All models accounted for the random effect of dairy and fixed effects of days on transition, treatment group, and parity, and the interaction of group and parity.

Logistic regression mixed models (melogit command in Stata) were used to assess the odds of the clinical health disorders: clinical hypocalcemia, displaced abomasum, dystocia, injury, metritis, pneumonia, retained fetal membranes, and total disease. The model included the fixed effects of treatment group, parity, and days on transition, the interaction between treatment group and parity, and the random effect of dairy. No primiparous cows had clinical hypocalcemia, so parity and the interaction between parity and group were not included in the model for clinical hypocalcemia. A low incidence of displaced abomasum, injury, and pneumonia created nonconvergence when the interaction term between group and parity was included, so it was omitted from these models. Contrasts and pairwise comparisons were performed for all disorders with the interaction term included.

Weibull accelerated time-failure models were used to assess the relative risk of treated or nontreated lameness, mastitis, or “other” disorders as they reflected time-failure events. Models accounted for the random effect of dairy, the fixed effects of treatment group,

parity, and days on transition, and the interaction between group and parity. The interaction between group and parity was omitted for “other” disorders because the model did not converge. Contrasts and pairwise comparisons were performed for all disorders with the interaction term included.

Exp. 2–L1. This data set was analyzed as 2 treatment groups (CON and TRT), as opposed to 4 groups, as the switch in treatment groups had yet to occur. Data from treatment groups A (CON-CON) and C (CON-TRT) were combined as the CON data set, and groups B (TRT-TRT) and D (TRT-CON) were combined as the TRT group.

For the weekly milk and herd test data, the model specified was

$$Y_{ijklm} = \mu + \alpha_i + \gamma_j + \theta_k + \delta_{lm} + \beta X_{ijklm} \\ + \omega\alpha\theta\delta\gamma_{ijkl} + \varepsilon_{ijklm},$$

where Y_{ijklm} = dependent variable, μ is the overall mean, α_i is the fixed effect of treatment (i = CON or TRT), γ_j is the fixed effect of dairy (j = dairies 1 to 4), θ_k is the fixed effect of parity (k = primiparous or multiparous), δ_{lm} is the fixed effect of time (l = wk 1 to 20 and herd test 1 to 4) for cow number m (m = 1 to 2,046 for weekly milk and 2,047 for herd test), βX_{ijklm} is the covariable adjustment for DIM at the start of the study, and $\omega\alpha\theta\delta\gamma_{ijkl}$ are the fixed effects of interaction terms, including 2-, 3-, and 4-way interactions of treatment, dairy, parity, and time, and ε_{ijklm} is the random error term.

A Weibull accelerated time-failure model was used to assess survival (odds of death or being culled per day and censoring pattern). The model accounted for the random effect of dairy and fixed effects of DIM at the start of the study, treatment group and parity, and the interaction of group and parity.

Logistic regression mixed models (melogit command in Stata) were used to assess the odds of the clinical health disorders: clinical hypocalcemia, injury, metritis, pneumonia, and total disease. The model included the fixed effects of treatment group, parity, DIM at the start of the study, and days on trial, the interaction between treatment group and parity, and the random effect of dairy. Parity and the interaction between parity and group was not included in the model for clinical hypocalcemia because no primiparous cows had clinical hypocalcemia. A low incidence of injury and total disease created nonconvergence when the interaction term between treatment group and parity were in the model, so it was omitted. Contrasts and pairwise comparisons were performed for all disorders with the interaction term included.

Weibull accelerated time-failure models were used to assess the relative risk of treated lameness, mastitis, or “other” disorders as they reflected time-failure events. Models accounted for the random effect of dairy, the fixed effects of treatment group, parity, DIM at the start of the study, and the interaction between group and parity. Contrasts and pairwise comparisons were performed for all disorders.

Exp. 2–L2. For the weekly milk and herd test data, the model specified was

$$Y_{ijklm} = \mu + \alpha_i + \gamma_j + \theta_k + \delta_{lm} + \beta X_{ijklm} + \omega\alpha\theta\delta\gamma_{ijkl} + \varepsilon_{ijklm},$$

where Y_{ijklm} = dependent variable, μ is the overall mean, α_i is the fixed effect of treatment (i = CON-CON, TRT-TRT, CON-TRT, or TRT-CON), γ_j is the fixed effect of dairy (j = dairies 1 to 4), θ_k is the fixed effect of parity (k = primiparous or multiparous), δ_{lm} is the fixed effect of time (l = wk 1 to 20 and herd test 1 to 4) for cow number m (m = 1 to 1,530 for weekly milk and 1,523 for herd test), βX_{ijklm} is the covariable adjustment for days on transition, and $\omega\alpha\theta\delta\gamma_{ijkl}$ are the fixed effects of interaction terms, including 2-, 3-, and 4-way interactions of treatment, dairy, parity, and time, and ε_{ijklm} is the random error term. The weekly milk data also had an additional covariable for the time spent on treatment 1 during L1 of the study.

A Weibull accelerated time-failure model was used to assess survival (odds of death or being culled per day and censoring pattern) and odds per day of being bred and pregnancy. A random effects logistic regression model (melogit command in Stata) was fitted for the odds of pregnancy at first service. All models accounted for the random effect of dairy and fixed effects of days on transition and days on trial in L1, treatment group and parity, and the interaction of group and parity.

Logistic regression mixed models (melogit command in Stata) were used to assess the odds of the clinical health disorders: clinical hypocalcemia, dystocia, metritis, pneumonia, retained fetal membranes, other, and total disease. The model included the fixed effects of treatment group, parity, days on transition, and days on trial, the interaction between treatment group and parity, and the random effect of dairy. Parity and the interaction between parity and group was not included in the model for clinical hypocalcemia because no primiparous cows had clinical hypocalcemia. A low incidence of dystocia and pneumonia created nonconvergence when the interaction term between treatment group and parity was in the model, so it was omitted. Contrasts and pairwise comparisons were performed for all disorders with the interaction term included.

Weibull accelerated time-failure models were used to assess the relative risk of injury, treated and not-treated lameness, and mastitis, disorders as they reflected time-failure events. Models accounted for the random effect of dairy, the fixed effects of treatment group, parity, days on transition and days on trial, and the interaction between group and parity. The interaction between group and parity was omitted for injury and not-treated lameness because the model did not converge. Contrasts and pairwise comparisons were performed for all disorders.

Subgroup. For all variables, the model specified was

$$Y_{ijklm} = \mu + \alpha_i + \gamma_j + \theta_k + \delta_{lm} + \beta X_{ijklm} + \omega\alpha\theta\delta\gamma_{ijkl} + \varepsilon_{ijklm},$$

where Y_{ijklm} = dependent variable, μ is the overall mean, α_i is the fixed effect of treatment (i = CON-CON, TRT-TRT, CON-TRT, or TRT-CON), γ_j is the fixed effect of dairy (j = dairies 1 to 4), θ_k is the fixed effect of parity (k = primiparous or multiparous), δ_{lm} is the fixed effect of time (l = sample 1 to 4) for cow number m (m = 1 to 361), βX_{ijklm} is the covariable adjustment for DIM at time of sampling, and $\omega\alpha\theta\delta\gamma_{ijkl}$ are the fixed effects of interaction terms, including 2-, 3-, and 4-way interactions of treatment, dairy, parity, and time, and ε_{ijklm} is the random error term. Inclusion of the baseline sample was examined as a covariable in the subgroup data set but did not improve the model, and not all cows had a baseline measure.

RESULTS

Visual review of the video footage only rarely showed evidence of orts containing treatment pellets in feed bins in the milking parlor.

Survival

Exp. 1. A total of 3 and 23.3% of cows were removed from the study due to death or culling up to d 305 on the study, respectively. A total of 35.8% of the cows were still in milk on d 305 of lactation. The main reasons cows were censored before 305 d on study were that they were dried off or the final date of treatment application was reached. Treatment group did not influence the probability of death or being culled per day over the 305 DIM period [hazard ratio (**HR**) = 0.98 ± 0.13 ; 95% CI = 0.76 to 1.28; $P = 0.764$], or censoring pattern ($HR = 0.96 \pm 0.08$; 95% CI = 0.81 to 1.14; $P = 0.889$). The interaction between treatment group and parity did not influence the probability of survival or the censoring pattern ($P = 0.859$ or 0.689 , respec-

tively). Multiparous cows had a reduced probability of surviving per day (HR = 0.77 ± 0.14 ; 95% CI = 0.54 to 1.10; $P = 0.044$) but lower probability of censoring than primiparous cows (HR = 0.65 ± 0.07 ; 95% CI = 0.53 to 0.80; $P < 0.001$). Days on transition had a significant effect on survival (HR = 1.01 ± 0.004 ; 95% CI = 1.00 to 1.02; $P = 0.017$) but not censoring pattern (HR = 1.00 ± 0.003 ; 95% CI = 1.00 to 1.01; $P = 0.121$).

Exp. 2–L1. Approximately 1.0% of cows were removed from the study due to death, and 12.5% were removed due to culling up to d 300 on the study. A total of 15% of cows were still in milk on d 300 of the study. Most cows were censored before d 300 because they were dried off and subsequently entered L2 of the study. These cows had started the study at a median of 147 DIM. Survival was very similar between treatment groups up to 100 d on study. Treatment group did not affect the likelihood of survival (not dying or being culled; $P = 0.496$); however, treated cows had lower probability of being censored from the study per day (HR = 1.12 ± 0.23 ; 95% CI = 0.75 to 1.67; $P = 0.053$). Parity tended to affect the probability of death or being culled per day, with multiparous cows having increased probability of survival per day than primiparous cows (HR = 1.12 ± 0.23 ; 95% CI = 0.75 to 1.67; $P = 0.053$); however, the opposite pattern was observed for censoring (HR = 0.93 ± 0.07 ; 95% CI = 0.81 to 1.08; $P = 0.001$). The interaction between treatment group and parity was not significant for survival ($P = 0.247$) but tended to influence the censoring pattern ($P = 0.056$). The DIM at the start of the study significantly increased both survival and censoring (HR = 1.003 ± 0.001 , $P < 0.001$ and HR = 1.005 ± 0.0002 , $P < 0.001$). For example, a 100-d increase in DIM at the start would increase survival by 1.3 times and censoring by 1.5 times.

Exp. 2–L2. Approximately 24.2% of cows were censored between the end of L1 and the beginning of the prepartum transition period leading into L2. Overall, treatment group did not influence survival ($P = 0.721$). Multiparous cows had 1.54 ± 0.38 times the probability to be removed per day by death or being culled than primiparous cows (95% CI = 0.96 to 2.49; $P < 0.001$). The overall interaction between treatment group and parity was not significant ($P = 0.155$). Days on trial in L1 decreased survival in L2, but days on transition did not (HR = 1.00 ± 0.001 ; 95% CI = 1.00 to 1.00; $P = 0.020$ and HR = 1.01 ± 0.01 ; 95% CI = 0.99 to 1.02; $P = 0.275$, respectively). Group, parity, the overall interaction of group and parity, and the number of days on transition did not affect the pattern of censoring ($P = 0.194, 0.792, 0.092$, and 0.231 , respectively). The number of days on trial in the previous lactation of the

study did affect the censoring pattern (HR = 1.01 ± 0.00 ; 95% CI = 1.01 to 1.01; $P < 0.001$).

Production

Exp. 1. There were 14,782 weekly milk yield measures from 1,167 cows over 15 wk and 4,195 herd test datapoints from 1,083 cows, averaging 3.9 tests/cow. For LnSCC, there were 2,514 measures from 727 cows, averaging 3.3 tests/cow (dairies 3 and 4 only). Treatment did not affect weekly milk yield (CON = 27.2 ± 2.54 ; TRT = 26.9 ± 2.54 L/d; $P = 0.384$). Parity, week, and their interaction were significant ($P < 0.001$). Group $\times$ parity ($P = 0.982$), group $\times$ week ($P = 0.357$), and their 3-way interaction were not significant ($P = 0.873$). The number of days on transition increased weekly milk yield by 0.03 L/d ($P = 0.007$).

The LnSCC was significantly reduced by 0.2 for the TRT cows, compared with CON cows ($P = 0.002$; Figure 2), whereas treatment did not affect any of the other herd test production measures (Table 5). Parity was significant for all measures except fat and protein percent. All parity by herd test interactions were significant except for fat percent. Group $\times$ parity and the 3-way group $\times$ parity $\times$ herd test interactions were only significant for LnSCC (Table 5). No group $\times$ herd test interactions were significant. Days on transition significantly increased ECM (0.04 L/d), fat percent

Figure 2. Mean $\pm$ SE of LnSCC at 5 herd tests over a 10-mo period at approximately 60-d intervals postpartum for control (CON) and treatment (TRT) cows in experiment 1 (commenced in transition). CON = control cows that were given no 25-(OH)D₃ throughout the study, and TRT = treatment cows that were given 2 mg/d of 25-(OH)D₃ during transition followed by 1 mg/d of 25-(OH)D₃ in lactation.

Table 5. Estimated marginal means $\pm$ SE for the effects of treatment¹ and parity at herd recording over a 10-mo period at 60-d intervals in Exp. 1²

Variable	Group			Parity			P-value					
	CON	TRT		Primiparous	Multiparous	Group (G)	Parity (P)	Test (T)	G $\times$ P	G $\times$ T	P $\times$ T	G $\times$ P $\times$ T
Milk yield (L/d)	25.9 $\pm$ 2.20	25.7 $\pm$ 2.20		23.2 $\pm$ 2.20	29.24 $\pm$ 2.20	0.577	<0.001	<0.001	0.691	0.207	<0.001	0.908
ECM ³ (kg/d)	28.4 $\pm$ 1.71	28.2 $\pm$ 1.71		25.5 $\pm$ 1.72	31.9 $\pm$ 1.72	0.559	<0.001	<0.001	0.523	0.140	<0.001	0.526
Fat (%)	4.22 $\pm$ 0.21	4.22 $\pm$ 0.21		4.20 $\pm$ 0.21	4.23 $\pm$ 0.21	0.898	0.595	<0.001	0.522	0.451	0.106	0.580
Fat yield (kg/d)	1.06 $\pm$ 0.055	1.06 $\pm$ 0.055		0.96 $\pm$ 0.056	1.20 $\pm$ 0.056	0.631	<0.001	<0.001	0.419	0.170	<0.001	0.219
Protein (%)	3.48 $\pm$ 0.15	3.49 $\pm$ 0.15		3.50 $\pm$ 0.15	3.47 $\pm$ 0.15	0.379	0.061	<0.001	0.926	0.702	<0.001	0.530
Protein yield (kg/d)	0.90 $\pm$ 0.059	0.89 $\pm$ 0.059		0.81 $\pm$ 0.060	1.00 $\pm$ 0.060	0.583	<0.001	<0.001	0.760	0.533	<0.001	0.697
TS (kg/d)	1.96 $\pm$ 0.10	1.95 $\pm$ 0.10		1.76 $\pm$ 0.10	2.20 $\pm$ 0.10	0.579	<0.001	<0.001	0.541	0.187	<0.001	0.524
LnSCC ⁴	4.21 $\pm$ 0.045	4.01 $\pm$ 0.050		4.05 $\pm$ 0.037	4.31 $\pm$ 0.75	0.003	0.002	<0.001	0.042	0.680	0.014	0.001

¹CON = control cows that were given no 25-(OH)D₃ throughout the study; TRT = treatment cows given 2 mg/d of 25-(OH)D₃ during transition followed by 1 mg/d of 25-(OH)D₃ in lactation.

²Models include the fixed effects days on transition; and treatment group, parity, dairy, and herd test and their interactions and the random effects of identity within dairy. The effect of dairy and its interactions are not reported.

³ECM = [(0.3246 $\times$ milk yield) + (12.86 $\times$ fat yield) + (7.04 $\times$ protein yield)]; NRC, 2001].

⁴Only contains data from dairies 3 and 4.

(0.008%/d) and yield (0.002 kg/d), total solids (0.003 kg/d), and LnSCC (0.007/d).

Exp. 2–L1. There were 34,402 measures of weekly mean milk yield from 2,046 cows over 20 wk and 6,845 herd test datapoints from 2,047 cows, averaging 3.3 tests/cow. For LnSCC, there were 4,013 observations from 1,304 cows, averaging 3.1 tests/cow (dairies 3 and 4 only). Treatment did not affect weekly milk yield ($P = 0.258$) with mean yields of 25.0 ± 1.76 and 24.8 ± 1.76 L/d for CON and TRT groups, respectively; however, parity, week, and their interaction were highly significant ($P < 0.001$). The DIM at study commencement decreased both weekly milk yield and all herd test measures, except fat and protein percent and LnSCC, which increased ($P < 0.001$) but did not differ between treatment groups.

There was no effect of treatment on any herd test measure for this group of cows or the interactions of group $\times$ parity, group $\times$ herd test, or the 3-way interaction ($P > 0.050$; Supplemental Table; <https://doi.org/10.6084/m9.figshare.c.5230055.v1>; Golder et al., 2020). Parity and herd test and their interaction were all significant at $P < 0.001$ for all measures, except protein percent for parity ($P = 0.034$).

Exp. 2–L2. There were 26,377 measures of weekly milk yield from 1,530 cows over 20 wk and 5,261 herd test datapoints from 1,523 cows, averaging 3.5 tests/cow. For LnSCC, there were 3,174 observations from 964 cows, averaging 3.3 tests/cow from dairies 3 and 4. Overall, group did not affect weekly milk yield ($P = 0.313$), but there was a group $\times$ week interaction ($P = 0.013$). Figure 3 shows that milk yield was more persistent for the CON-TRT group. Means $\pm$ SE were 31.6 ± 2.05 , 31.7 ± 2.05 , 32.3 ± 2.06 , and 31.5 ± 2.07 L/d for groups A to D, respectively. Parity, week, and parity $\times$ week were also highly significant ($P < 0.001$). Group $\times$ parity was not significant ($P = 0.476$), nor was the 3-way interaction between group, parity, and week ($P = 1.000$). The number of days on trial during L1 ($P = 0.005$) and the number of days on transition ($P < 0.001$) both increased weekly milk yield.

The TRT-TRT group had the highest milk yield at herd test (29.7 ± 3.41 vs. 29.4 ± 3.41 L/d for CON-CON; $P = 0.188$). This was reflected in a tendency for lower protein percent ($P = 0.061$) and numerically lowest fat percent ($P = 0.358$), compared with other groups, resulting in equivalent fat and protein yield and ECM (Table 6). The LnSCC was numerically lowest for TRT-TRT (4.04 ± 0.08 vs. 4.19 ± 0.08 for CON-CON; Table 6), whereas other milk measures were not affected. Parity affected all milk measures ($P < 0.001$), except protein percent ($P = 0.473$). Interactions were significant between group and herd test for fat and protein percent, protein yield, and LnSCC ($P < 0.020$;

Supplemental Figure S1; <https://doi.org/10.6084/m9.figshare.c.5230055.v1>; Golder et al., 2020). Parity $\times$ herd test was highly significant for all measures ($P < 0.001$) except fat and protein percent (Table 6). The 3-way interaction was not significant for any measure except protein percent ($P = 0.039$). Days on transition increased fat yield ($P = 0.022$) and decreased protein percent ($P = 0.044$).

Reproduction

Exp. 1. There were 1,036 cows in this data set from dairies 2 to 4 (CON = 573 and TRT = 463). Of these cows, 817 were bred (78.9%), and 71% of the bred cows were pregnant by 300 d. The main reason for a cow not being bred was culling. A total of 55.5% of the CON and 45.2% of TRT cows were pregnant. Of the 580 total cows that were pregnant, 264 (45.5%) were pregnant at first service.

Group did not influence the probability of being bred per day (HR = 0.87 ± 0.07 , 95% CI = 0.74 to 1.02; $P = 0.483$; Supplemental Figure S2; <https://doi.org/10.6084/m9.figshare.c.5230055.v1>; Golder et al., 2020). Primiparous cows tended to have a higher probability of being bred per day than parous cows (HR = 0.78 ± 0.09 , 95% CI = 0.63 to 0.97; $P = 0.060$). Overall,

there was not a significant group $\times$ parity interaction ($P = 0.263$). The days spent on transition increased the probability of being bred per day (HR = 1.01 ± 0.003 , 95% CI = 1.00 to 1.01; $P = 0.010$). For example, cows that had 40 days on transition had a ~30% increase in probability of being bred per day.

Treatment had no effect on days to pregnancy (HR = 0.88 ± 0.09 , 95% CI = 0.73 to 1.06; $P = 0.266$). Primiparous cows had a higher rate of pregnancy per day than multiparous cattle (HR = 0.42 ± 0.06 , 95% CI = 0.32 to 0.56; $P < 0.001$; reference group is primiparous). There was a significant interaction between treatment and parity ($P = 0.013$; Figure 4). Multiparous TRT cows had higher probability of pregnancy per day than multiparous CON cows (HR = 1.41 ± 0.23 , 95% CI = 1.02 to 1.95), and, therefore, a 22-d median decrease in time to pregnancy (Figure 4). However, primiparous CON cows had a higher probability of pregnancy than primiparous TRT cows (HR = 0.88 ± 0.09 , 95% CI = 0.73 to 1.06; Figure 4). The days on transition did not affect days to pregnancy (HR = 1.00 ± 0.004 , 95% CI = 0.99 to 1.01; $P = 0.587$).

Treatment did not influence the odds of pregnancy at first service [odds ratio (OR) = 0.96 ± 0.16 , 95% CI = 0.69 to 1.35; $P = 0.820$]. Of the pregnant CON cows, 46.9% (149/318) were pregnant at first service,

Figure 3. Mean $\pm$ 95% CI of milk volume over a 20-wk period postpartum for treatment groups of cows in their second lactation (L2) of experiment 2 [commenced the study mid-previous lactation (L1)]. Treatment groups were as follows, with the mg/d of 25-(OH)D₃ fed indicated in parentheses for L1, ~21 d in prepartum transition, and L2, respectively: CON-CON = control-control (0–0–0); TRT-TRT = treatment-treatment (1–2–1); CON-TRT = control-treatment (0–2–1), and TRT-CON = treatment-control (1–0–0).

Table 6. Estimated marginal means $\pm$ SE for the effects of treatment¹ and parity at herd recording over an 8-mo period at 60-d intervals in Exp. 2-L2²

Variable	Group				Parity		P-value			
	CON-CON	TRT-TRT	CON-TRT	TRT-CON	Primiparous	Multiparous	Group (G)	Parity (P)	Test (T)	G $\times$ P $\times$ T
Milk yield (L/d)	29.4 $\pm$ 3.41	29.7 $\pm$ 3.41	29.6 $\pm$ 3.42	28.8 $\pm$ 3.42	27.9 $\pm$ 3.41	30.1 $\pm$ 3.41	0.188	<0.001	<0.001	0.338
ECM ³ (kg/d)	32.3 $\pm$ 2.99	32.3 $\pm$ 2.99	32.2 $\pm$ 3.00	31.6 $\pm$ 3.00	30.3 $\pm$ 2.99	33.0 $\pm$ 2.99	0.400	<0.001	<0.001	0.923
Fat (%)	4.36 $\pm$ 0.24	4.29 $\pm$ 0.24	4.31 $\pm$ 0.24	4.34 $\pm$ 0.24	4.23 $\pm$ 0.24	4.37 $\pm$ 0.24	0.358	<0.001	<0.001	0.124
Fat yield (kg/d)	1.23 $\pm$ 0.10	1.22 $\pm$ 0.10	1.22 $\pm$ 0.10	1.20 $\pm$ 0.10	1.14 $\pm$ 0.10	1.26 $\pm$ 0.10	0.421	<0.001	<0.001	0.997
Protein (%)	3.44 $\pm$ 0.17	3.41 $\pm$ 0.17	3.43 $\pm$ 0.17	3.45 $\pm$ 0.17	3.44 $\pm$ 0.17	3.43 $\pm$ 0.17	0.061	0.473	<0.001	0.078
Protein yield (kg/d)	0.98 $\pm$ 0.086	0.98 $\pm$ 0.086	0.98 $\pm$ 0.086	0.97 $\pm$ 0.086	0.94 $\pm$ 0.086	1.00 $\pm$ 0.086	0.746	<0.001	<0.001	0.716
TS (kg/d)	2.21 $\pm$ 0.18	2.20 $\pm$ 0.18	2.21 $\pm$ 0.18	2.17 $\pm$ 0.18	2.08 $\pm$ 0.18	2.26 $\pm$ 0.18	0.516	<0.001	<0.001	0.977
LnSCC ⁴	4.19 $\pm$ 0.08	4.04 $\pm$ 0.08	4.10 $\pm$ 0.11	4.27 $\pm$ 0.12	3.76 $\pm$ 0.09	4.26 $\pm$ 0.07	0.101	<0.001	<0.001	0.248

¹Treatment groups were as follows, with the amount (mg/d) of 25-(OH)D₃ fed indicated in parentheses for lactation 1 of the study (L1), ~21 d in prepartum transition, and lactation 2 of the study (L2), respectively: CON-CON = control-control (0-0-0); TRT-TRT = treatment-treatment (1-2-1); CON-TRT = control-treatment (0-2-1), and TRT-CON = treatment-control (1-0-0).

²Models include the fixed effects of days on transition; and treatment group, parity, dairy, and herd test and their interactions and the random effects of identity within dairy. The effect of dairy and its interactions are not reported.

³ECM = [(0.3246 $\times$ milk yield) + (12.86 $\times$ fat yield) + (7.04 $\times$ protein yield); NRC, 2001].

⁴Only contains data from dairies 3 and 4.

whereas of the pregnant TRT cows, 43.9% (115/262) were pregnant at first service. The odds of pregnancy at first service was lower for multiparous cows than for primiparous cows (OR = 0.54 $\pm$ 0.09, 95% CI = 0.39 to 0.76; P < 0.001). Of the pregnant primiparous cows, 47.2% (205/434) were pregnant at first service, whereas of pregnant multiparous cows, 40.4% (59/146) were pregnant at first service. Overall, treatment $\times$ parity was not significant (P = 0.472). Days on transition did not affect the odds of pregnancy at first service (OR = 1.0 $\pm$ 0.01, 95% CI = 0.99 to 1.01, P = 0.865).

Exp. 2-L2. Of the 1,565 cows in this data set, 1,330 (85.0%) were bred. Of those bred, 72.1% were pregnant. From the CON-CON, TRT-TRT, CON-TRT, and TRT-CON groups, 62.6, 58.3, 61.5, and 64.9%, respectively, of cows were pregnant by 300 DIM.

Group influenced the probability of being bred per day (P = 0.016), with TRT-TRT cows having from 16 to 29% lower probability to be bred per day than all other groups (Figure 5A; CON-TRT vs. TRT-TRT HR = 1.29 $\pm$ 0.11; 95% CI = 1.09 to 1.53; TRT-CON vs. TRT-TRT HR = 1.22 $\pm$ 0.11; 95% CI = 1.02 to 1.47; and TRT vs. CON-CON HR = 0.84 $\pm$ 0.06 95% CI = 0.73 to 0.98). Parity did not influence time to first breeding (HR = 0.94 $\pm$ 0.10, 95% CI = 0.76 to 1.16; P = 0.141). The overall interaction between group and parity was not significant (P = 0.451), but primiparous CON-TRT cows had greater probability of being bred per day than primiparous TRT-TRT cows (HR = 1.48 $\pm$ 0.21, 95% CI = 1.12 to 1.95). The days in L1 of the study and days in transition both affected time to first breeding (HR = 1.00 $\pm$ 0.00, 95% CI = 1.00 to 1.00; P = 0.029 and HR = 1.02 $\pm$ 0.003, 95% CI 1.01 to 1.03; P < 0.001, respectively). For example, cows that spent 20 d on transition would have had ~40% higher probability of being bred per day compared with a cow that had 1 d of transition feeding. If a cow spent 100 d on study in the first lactation of the study, she would have had ~80% higher probability of being bred per day compared with a cow that was on trial for a single day.

Overall, group only tended to affect the probability of pregnancy per day (P = 0.067; Figure 5B); although CON-CON cows had 17 $\pm$ 7.0% and TRT-CON cows 27 $\pm$ 13% higher probability to be pregnant per day than TRT-TRT cows (TRT-TRT vs. CON-CON HR = 0.83 $\pm$ 0.07; 95% CI = 0.70 to 0.98 and TRT-CON vs. TRT-TRT HR = 1.27 $\pm$ 0.13; 95% CI = 1.03 to 1.56). Primiparous cows had a greater probability of pregnancy per day than multiparous cattle (HR = 0.73 $\pm$ 0.09, 95% CI = 0.57 to 0.92; P < 0.001). The overall interaction between group and parity was not significant (P = 0.812), but 16 pairwise comparisons were significant. Of these comparisons, all but 2 are comparisons within the same parity, reflecting that parity had a greater effect

Figure 4. Kaplan-Meier survival curves for days to pregnancy for primiparous and multiparous cows by treatment group in experiment 1 (commenced in transition). CON = control cows that were given no 25-(OH)D₃ throughout the study, and TRT = treatment cows that were given 2 mg/d of 25-(OH)D₃ during transition followed by 1 mg/d of 25-(OH)D₃ in lactation.

fect than treatment. Multiparous TRT-TRT had lower probability of pregnancy than multiparous CON-CON cows (HR = 0.80 ± 0.08 , 95% CI = 0.66 to 0.97), and multiparous TRT-CON cows had higher probability of pregnancy per day than multiparous TRT-TRT cows (HR = 1.30 ± 0.17 , 95% CI = 1.02 to 1.67). Neither days in L1 or on transition affected days to pregnancy ($P = 0.620$ and 0.064 , respectively).

In total, 1,330 cows had been bred and were included in the odds of pregnancy at first service data set. A total of 41.4, 41.3, 48.2, and 46.6% of the pregnant cows from the CON-CON, TRT-TRT, CON-TRT, and TRT-CON groups were pregnant at first service, respectively. Group ($P = 0.813$), parity (OR = 0.84 ± 0.11 , 95% CI = 0.64 to 1.09; $P = 0.179$), or their interaction ($P = 0.934$) did not influence the odds of pregnancy at first service. Days on study in the first lactation increased the odds of being pregnant at first service (OR = 1.00 ± 0.00 , 95% CI = 1.00 to 1.00; $P = 0.011$), whereas there was a tendency for days on transition to increase the odds (OR = 0.99 ± 0.007 , 95% CI = 0.97 to 1.00; $P = 0.050$).

Health

Exp. 1. There were 1,182 cows in this data set. The clinical health disorder with highest incidence was mas-

titis, with an average of 13.6% of cows having at least 1 case during the first 300 d on trial, followed by metritis, which occurred in an average of 11.2% cows. Treatment did not influence odds or probability per day of any health disorder (Table 7). Multiparous cows had 2.87 ± 1.43 times higher odds of retained fetal membranes than primiparous cows ($P = 0.034$). Multiparous cows had 1.88 ± 0.28 times higher odds of at least one clinical disease over the 300 d of the study than primiparous cows ($P < 0.001$; Table 7). There was a significant interaction between group and parity for probability of mastitis per day ($P = 0.006$), with multiparous CON cows having 2.74 ± 0.69 times (95% CI of 1.67 to 4.48) greater probability per day of mastitis than primiparous CON cows (Figure 6). Primiparous TRT cows had 1.67 ± 0.40 times (95% CI of 1.04 to 2.66) higher probability than primiparous CON cows. The primiparous CON had the lowest probability of mastitis per day (Figure 6). Days on transition increased the probability of mastitis by 3%/d ($P < 0.001$; Table 7).

Exp. 2-L1. There were 2,064 cows in this data set. Mastitis was the clinical health disorder with highest incidence (11.4% of cows; Table 8). Treatment did not influence the odds or probability per day of any of the health disorders. Multiparous cows had 2.07 ± 0.30 ($P < 0.001$) times greater odds of having at least 1 clinical disease than primiparous cows (Table 8). The prob-

Figure 5. Kaplan-Meier survival curves for (A) days to first breeding and (B) for days to pregnancy for cows in the second lactation (L2) of experiment 2 [commenced the study mid-previous lactation (L1)]. Treatment groups were as follows, with the mg/d of 25-(OH)D₃ fed indicated in parentheses for L1, ~ 21 d in prepartum transition, and L2, respectively: CON-CON = control-control (0–0–0); TRT-TRT = treatment-treatment (1–2–1); CON-TRT = control-treatment (0– 2–1), and TRT-CON = treatment-control (1–0–0).

Table 7. Percentage, odds ratios (OR) or relative risks, and significance of clinical health disorders for control and treatment¹ cows in Exp. 1 (study commenced during transition)²

Disorder	Group (%)		OR (<i>P</i> -value) ³			
	CON	TRT	Group (G)	Parity (P)	G × P	Days on transition
Clinical hypocalcemia ⁴	1.4	1.9	1.37 (0.502)			1.02 (0.416)
Displaced abomasum ⁵	0.16	0.37	2.32 (0.493)	3.54 (0.305)		0.966 (0.648)
Dystocia	0.62	0.74	1.18 (0.841)	1.85 (0.455)	(0.214)	1.01 (0.532)
Injury ⁵	0.47	0.74	1.47 (0.615)	5.54 (0.052)		1.04 (0.053)
Lame not treated ⁶	5.6	6.0	0.263 ⁷ (0.266)	1.36 ⁷ (0.118)	(0.224)	0.995 ⁷ (0.797)
Lame treated ⁶	3.1	4.1	1.12 ⁷ (0.307)	0.581 ⁷ (0.358)	(0.502)	1.00 ⁷ (0.994)
Mastitis ⁶	13.2	13.8	1.66 ⁷ (0.720)	2.74 ⁷ (0.004)	(0.006)	1.03 ⁷ (<0.001)
Metritis	11.5	10.8	1.07 (0.749)	1.20 (0.461)	(0.475)	0.993 (0.296)
Pneumonia ⁵	1.4	2.6	1.98 (0.120)	0.428 (0.115)		0.966 (0.164)
Retained fetal membranes	2.6	1.7	0.612 (0.264)	2.87 (0.034)	(0.689)	0.956 (0.110)
Other ^{5,6}	4.7	5.6	1.17 ⁷ (0.543)	1.57 ⁷ (0.130)		1.01 ⁷ (0.338)
Total disease	33.2	36.7	1.12 (0.367)	1.88 (<0.001)	(0.746)	1.01 (0.163)

¹Control cows (CON) were given no 25-(OH)D₃, and treatment cows (TRT) were given 2 mg/d of 25-OHD₃ during transition and 1 mg/d of 25-OHD₃ during lactation.

²The logistic regression models include the random effect of dairy and the fixed effects of days on transition, treatment group, and parity and the interaction between treatment group and parity. Time-failure models include the random effect of dairy, the fixed effects of treatment group, parity, and days on transition, and the interaction between group and parity.

³Group (G) = control; Parity (P) = primiparous; *P*-value is an overall *P*-value.

⁴Parity and parity and group interaction not included in the model.

⁵Parity and group interaction not included in the model.

⁶Time-failure models.

⁷Hazard ratio.

ability of mastitis per day was increased by 3.61 ± 1.10 times for multiparous cows (vs. primiparous cows; $P < 0.001$) and was also decreased by DIM at start and days on trial ($P = 0.018$ and < 0.001 , respectively; Table 8). There was a significant interaction between group and parity for the probability of having had a disorder categorized as “other” per day, with multiparous TRT cows having 78.3% lower probability per day of having an “other” disease compared with primiparous TRT cows, and 75.3% lower probability than multiparous CON cows. The DIM at start of the study decreased the odds of injury and total clinical disease. Days on trial decreased the odds of injury and clinical hypocalcemia but increased the odds of metritis.

Exp. 2–L2. There were 1,565 cows in this data set, but the distribution per group for cows that swapped treatment groups was approximately half that of cows that remained in the same group. Mastitis was the clinical disorder with the highest incidence (Table 9). On average, 41.1% of the cows had at least 1 clinical health disorder over the 300-d trial period. Days on transition increased the odds of clinical hypocalcemia and other disease but reduced the odds of retained fetal membranes and metritis (Table 9). Multiparous cows had 2.13 ± 0.27 times greater odds of total clinical disease than primiparous cows ($P < 0.001$). Group or its interaction with parity did not influence the odds or probability per day of health disorders (Tables 9).

Multiparous cows had 3.03 ± 1.19 times ($P = 0.005$) higher probability to have an untreated lameness per day and had 1.57 ± 0.35 times higher probability of mastitis per day than primiparous cows ($P < 0.001$; Table 9). Days on trial in L1 increased the odds of metritis ($P = 0.013$).

Subgroup

Sample day was significant for all measures, whereas the 3-way interaction between group, parity, and sample day was not significant for any of the measures.

Plasma 25-(OH)D₃ Concentration. All treatment groups had very similar baseline concentrations of 25-(OH)D₃ (Figure 7) with a mean $\pm$ SD of 41.9 ± 15.6 ng/mL. There were some differences in baseline means between dairies (Table 1). The baseline concentrations for all groups were lower than the concentrations at subsequent samplings. Figure 7 shows the mean 25-(OH)D₃ concentration for the CON-CON group remained very stable across the 4 postbaseline samplings at approximately 75 ng/mL and that the swap from treatment 1 to treatment 2 was successful, a large contributor to the highly significant group-by-sample interaction. Treatment group was highly significant with 25-(OH)D₃ concentrations highest in the TRT-TRT group overall (239.4 ± 13.3 ng/mL) with a concentration 156.4 ng/mL higher than the CON-CON. The CON-TRT and

Figure 6. Kaplan-Meier survival curves for days to mastitis for primiparous and multiparous cows by treatment group in experiment 1 (commenced in transition). CON = control cows that were given no 25-(OH)D₃ throughout the study, and TRT = treatment cows that were given 2 mg/d of 25-(OH)D₃ during transition (~ 21 d prepartum to parturition) followed by 1 mg/d of 25-(OH)D₃ in lactation.

TRT-CON groups had similar overall concentrations that were both higher than those of the CON-CON and lower than the TRT-TRT group. There was at least a 155 ng/mL difference in 25-(OH)D₃ concentration between treatment and control groups at each sampling point. The 25-(OH)D₃ concentration for the TRT-TRT group did not accumulate over each sampling with a numerically lower concentration observed at sampling d 2 (200 d posttreatment), compared with 1 (100 d posttreatment) and a significantly lower concentration at sampling d 4 (200 d postcalving in L2), compared with 3 (100 d postcalving in L2; Figure 7). Both parity ($P < 0.001$) and DIM ($P = 0.025$) were significant, with primiparous cows having 23.4 ng/mL higher 25-(OH)D₃ concentrations than multiparous cows, but the group $\times$ parity interaction was not significant ($P = 0.908$; Table 10).

Serum Ca and P Concentrations. Group and the interaction of group and parity had no effect on Ca concentration, but there was a tendency for Ca to be higher in primiparous cows ($P = 0.070$; Table 10). Group $\times$ sample day interaction was significant for Ca, with concentrations decreased at the second sampling (200 d posttreatment) compared with the first for all groups ($P = 0.016$; Figure 8C). This decrease was the

most pronounced for the TRT-TRT group. The TRT-TRT and CON-TRT groups had greater variability than the other groups.

Serum P concentration was higher in the TRT-TRT than the CON-CON and CON-TRT cows ($P = 0.003$; Figure 8D). All groups other than the TRT-TRT were similar. Despite the TRT-TRT cows appearing to have a higher P concentration than the TRT-CON cows, due to the group $\times$ parity interaction, pairwise comparisons were not significant. Serum P concentration was the only variable within the subgroup with a significant group $\times$ parity interaction ($P = 0.029$). Primiparous CON-CON cows (2.00 ± 0.072 mM) had lower P concentrations than primiparous TRT-TRT cows (2.18 ± 0.069 mM) and TRT-CON cows (2.18 ± 0.073 mM), whereas multiparous TRT-TRT cows had higher P concentrations (2.02 ± 0.058 mM) than TRT-CON cows (1.89 ± 0.060 mM). Parity, DIM, and parity $\times$ sample day were significant for P concentrations (Table 10). The P concentrations were 0.17 mM higher in primiparous cows than in multiparous cows ($P < 0.001$).

Milk Yield and Components. Treatment only tended to affect milk yield ($P = 0.074$) but altered protein percent ($P = 0.034$; Table 10). There was a 1.5-L difference in milk yield between the TRT-TRT

Table 8. Percentage, odds ratios (OR) or relative risk, and significance of clinical health disorders for control and treatment¹ cows in Exp. 2–L1 (study commenced during lactation)²

Disorder	Group (%)		OR (P -value) ³				
	CON	TRT	Group (G)	Parity (P)	G $\times$ P	DIM start	Days on trial
Clinical hypocalcemia ⁴	0.19	0.78	3.82 (0.093)			0.998 (0.674)	0.990 (0.026)
Injury ⁵	1.1	0.78	0.517 (0.211)	3.60 (0.104)		0.982 (<0.001)	0.979 (<0.001)
Lame not-treated	5.7	6.4	1.24 (0.428)	3.44 (<0.001)	(0.787)	0.999 (0.458)	1.00 (0.603)
Lame treated ⁶	4.9	3.8	0.840 ⁷ (0.378)	1.32 ⁷ (0.383)	(0.840)	1.00 ⁷ (0.735)	1.00 ⁷ (0.218)
Mastitis ⁶	11.1	11.6	0.999 ⁷ (0.933)	3.61 ⁷ (<0.001)	(0.928)	0.997 ⁷ (0.018)	0.995 ⁷ (<0.001)
Metritis	1.2	0.48	0.337 (0.093)	0.649 (0.507)	(0.238)	0.992 (0.205)	1.01 (0.030)
Pneumonia	1.1	0.78	0.451 (0.200)	0.865 (0.817)	(0.074)	1.000 (0.907)	0.995 (0.145)
Other ⁶	1.8	1.1	2.52 ⁷ (0.573)	2.17 ⁷ (0.397)	(0.010)	0.999 ⁷ (0.586)	0.997 ⁷ (0.586)
Total disease ⁵	19.0	18.2	0.944 (0.626)	2.07 (<0.001)		0.996 (<0.001)	1.00 (0.935)

¹Control cows (CON) were given no 25-(OH)D₃ and treatment cows (TRT) were given 1 mg/d of 25-(OH)D₃.

²The logistic regression models include the random effect of dairy and the fixed effects of DIM at the start of the study, days on trial, treatment group, parity, and the interaction between treatment group and parity. The time-failure models include the random effect of dairy, the fixed effects of treatment group, parity, DIM at the start of the study, days on trial, and the interaction between group and parity.

³Group (G) = control; Parity (P) = primiparous; P -value is an overall P -value.

⁴Parity and parity by group interaction not included in the model.

⁵Parity and group interaction not included in the model.

⁶Time-failure models.

⁷Hazard ratios.

Table 9. Percentage, odds ratios (OR) or relative risks, and significance of clinical health disorders for treatment group¹ cows in Exp. 2-L2 (second lactation of the study after commencing mid-previous lactation)²

Disorder	Group (%)				Group OR ³ (<i>P</i> -value)			OR (<i>P</i> -value)	
	CON-CON	TRT-TRT	CON-TRT	TRT-CON	TRT-TRT	CON-TRT	TRT-CON	Parity ⁴	Days on transition
Clinical hypocalcemia ⁵	1.2	1.5	1.8	1.2	0.138 (0.556)	1.21 (0.758)	0.717 (0.649)		1.04 (0.030)
Dystocia ⁶	1.3	1.9	1.8	1.2	1.58 (0.362)	1.80 (0.354)	1.15 (0.851)	2.57 (0.087)	1.01 (0.768)
Injury ^{6,7}	1.2	0.57	0.73	0.40	0.500 ⁸ (0.328)	0.620 ⁸ (0.562)	0.365 ⁸ (0.356)	3.98 ⁸ (0.189)	1.02 ⁸ (0.536)
Lame not treated ^{6,7}	1.7	2.1	5.5	3.6	1.73 ⁸ (0.226)	2.01 ⁸ (0.098)	1.21 ⁸ (0.683)	3.03 ⁸ (0.005)	1.00 ⁸ (0.871)
Lame treated ⁷	3.1	5.0	4.8	4.8	1.52 ⁷ (0.714)	1.43 ⁷ (0.940)	1.53 ⁷ (0.576)	1.08 ⁸ (0.793)	1.00 ⁸ (0.747)
Mastitis ⁷	25.0	22.8	20.9	21.8	0.687 ⁸ (0.205)	0.602 ⁸ (0.150)	0.524 ⁸ (0.098)	1.57 ⁸ (0.001)	1.01 ⁷ (0.381)
Metritis	7.5	7.8	3.3	7.3	0.885 (0.269)	0.587 (0.276)	1.71 (0.456)	1.28 (0.397)	0.976 (0.054)
Pneumonia ⁶	0.77	0.19	0.73	0.40	0.252 (0.219)	1.53 (0.660)	0.827 (0.875)	3.22 (0.278)	0.952 (0.939)
Retained fetal membranes	2.3	2.1	2.2	2.4	1.14 (0.463)	1.15 (0.943)	1.14 (0.978)	1.93 (0.165)	0.924 (0.087)
Other	3.1	2.9	2.9	2.8	0.941 (0.920)	1.30 (0.478)	1.09 (0.880)	0.979 (0.953)	1.03 (0.011)
Total disease	42.6	43.0	37.4	41.5	0.963 (0.392)	0.854 (0.230)	0.996 (0.639)	2.13 (0.001)	0.998 (0.660)

¹Treatment groups were as follows, with the amount (mg/d) of 25-(OH)D₃ fed indicated in parentheses for lactation 1 of the study (L1), ~21 d in prepartum transition, and lactation 2 of the study (L2), respectively: CON-CON = control-control (0-0-0); TRT-TRT = treatment-treatment (1-2-1); CON-TRT = control-treatment (0-2-1), and TRT-CON = treatment-control (1-0-0).

²The logistic regression models include the random effect of dairy and fixed effects of days on transition diet, days on trial in lactation 1 of the study, treatment group, parity, and the interaction between treatment group and parity. The time-failure models include the random effect of dairy, the fixed effects of treatment group, parity, days on transition, days on trial and the interaction between group and parity. The interaction between group and parity was $P > 0.005$ for all disorders and is not reported.

³Referent is CON-CON.

⁴Referent is primiparous.

⁵Parity and group interaction not included in the model.

⁶Parity and group interaction not included in the model.

⁷Time-failure models.

⁸Hazard ratios.

Figure 7. Mean $\pm$ SE for plasma 25-(OH)D₃ concentration from a subgroup of cows from experiment 2 at 5 sampling points. Sampling days correspond to approximately (0) a baseline sample taken prior to study commencement, (1) 100 d after study commencement, (2) 200 d after study commencement, (3) 100 d postpartum in the second lactation of the study (L2), and (3) 200 d postpartum in L2. A change between treatment group 1 [administered in lactation 1 (L1) of the study] and treatment group 2 (administered in prepartum transition and L2) occurred upon cows entering the transition period. This occurred between sample d 2 and 3 as indicated by the dashed line. Treatment groups were as follows, with the mg/d of 25-(OH)D₃ fed indicated in parentheses for L1, ~21 d in prepartum transition, and L2, respectively: CON-CON = control-control (0-0-0); TRT-TRT = treatment-treatment (1-2-1); CON-TRT = control-treatment (0-2-1), and TRT-CON = treatment-control (1-0-0).

(29.7 L) and TRT-CON (28.8 L) cows, but this was not significant (Table 10; Figure 8A). Protein percent was highest for the TRT-CON cows, which corresponds with the lower milk yield, whereas CON-CON cows had the lowest protein percent (Table 10; Figure 8B). Parity was significant for all measures except for protein percent ($P = 0.757$) and BCS ($P = 0.090$). Parity $\times$ sample day was significant for ECM, fat and protein yield, total solids, and BW. The DIM decreased all measures except fat and protein percents and LnSCC (Table 10).

DISCUSSION

The objective of this study was to evaluate milk performance, reproduction, and health of dairy cows after feeding 25-(OH)D₃ in 2 experiments with differing durations of supplementation of 25-(OH)D₃. The first experiment supplemented 25-(OH)D₃ daily from ~21 d prepartum, in conjunction with negative DCAD diets, through to the end of the subsequent lactation. The second began 25-(OH)D₃ supplementation mid-lactation and, upon transition, some of the enrolled

cows swapped treatment groups until the end of the subsequent lactation. This is the largest study to investigate 25-(OH)D₃ use in dairy cattle and one of very few to evaluate the efficacy of its supplementation during lactation. Target sample sizes at enrollment were met; however, with higher censoring (24.2%) between the switch from treatment 1 to treatment 2 in Exp. 2, the power was slightly lower than 0.8.

A negative DCAD diet was effectively delivered to most cows in the current study. Negative DCAD diets increase parathyroid hormone sensitivity, resulting in increased Ca concentrations in blood (Goff et al., 2014), and amplify the effects of 25-(OH)D₃ on Ca metabolism (Wilkens et al., 2012; Lean et al., 2014). Cows that did not receive a sufficient transition period could have been negatively affected by intake of 25-(OH)D₃, particularly if they were older (at higher risk of hypocalcemia). In particular, we suspect these cows would have increased odds and risk of clinical hypocalcemia and removal, based on findings by Martinez et al. (2018a); however, this was not formally evaluated in the study because of the low incidence of clinical hypocalcemia. Martinez et al. (2018a) demonstrated

Table 10. Estimated marginal means $\pm$ SE in Exp. 2 for the effects of treatment¹ and parity on milk yield, BW, BCS, serum Ca, serum P, and plasma 25-(OH)D₃ concentrations at 100 and 200 d poststudy commencement and 100 and 200 d postpartum in the second lactation on the study²

Variable	n	Group				Parity			P-value			
		CON-CON	TRT-TRT	CON-TRT	TRT-CON	Primiparous	Multiparous	Group (G)	Parity (P)	Sample (S)	G $\times$ P	
											G $\times$ S	P $\times$ S
Milk yield (kg/d)	1,050	26.8 $\pm$ 1.77	27.1 $\pm$ 1.77	27.8 $\pm$ 1.77	26.3 $\pm$ 1.78	26.1 $\pm$ 1.77	27.3 $\pm$ 1.74	0.074	0.015	<0.001	0.321	0.526
ECM ^{3,4} (kg/d)	526	28.4 $\pm$ 2.32	28.3 $\pm$ 2.31	28.3 $\pm$ 2.33	28.3 $\pm$ 2.32	26.7 $\pm$ 2.31	29.1 $\pm$ 2.27	0.997	<0.001	<0.001	0.955	0.927
Fat ³ (%)	526	4.38 $\pm$ 0.46	4.27 $\pm$ 0.46	4.33 $\pm$ 0.46	4.45 $\pm$ 0.46	4.14 $\pm$ 0.46	4.46 $\pm$ 0.46	0.400	<0.001	<0.001	0.866	0.796
Fat yield ³ (kg/d)	526	1.07 $\pm$ 0.039	1.05 $\pm$ 0.038	1.06 $\pm$ 0.039	1.06 $\pm$ 0.038	0.97 $\pm$ 0.037	1.10 $\pm$ 0.033	0.975	<0.001	<0.001	0.997	0.860
Protein ³ (%)	526	3.65 $\pm$ 0.22 ^a	3.72 $\pm$ 0.22 ^{bd}	3.67 $\pm$ 0.22 ^{ad}	3.75 $\pm$ 0.22 ^{bc}	3.69 $\pm$ 0.22	3.70 $\pm$ 0.22	0.034	0.757	<0.001	0.367	0.526
Protein yield ³ (kg/d)	526	0.91 $\pm$ 0.083	0.93 $\pm$ 0.083	0.91 $\pm$ 0.083	0.92 $\pm$ 0.08	0.89 $\pm$ 0.083	0.93 $\pm$ 0.081	0.919	0.043	<0.001	0.736	0.972
TS ³ (kg/d)	526	1.98 $\pm$ 0.13	1.98 $\pm$ 0.13	1.97 $\pm$ 0.13	1.98 $\pm$ 0.13	1.86 $\pm$ 0.13	2.03 $\pm$ 0.12	0.999	<0.001	<0.001	0.948	0.916
LnSCC ³	522	4.40 $\pm$ 0.16	4.23 $\pm$ 0.16	4.36 $\pm$ 0.17	4.16 $\pm$ 0.16	4.10 $\pm$ 0.16	4.37 $\pm$ 0.14	0.394	0.030	0.002	0.907	0.075
BW (kg)	1,035	608 $\pm$ 31.9	607 $\pm$ 31.9	612 $\pm$ 32.0	601 $\pm$ 32.0	568 $\pm$ 32.0	620 $\pm$ 31.6	0.630	<0.001	<0.001	0.396	0.367
BCS	1,042	2.84 $\pm$ 0.055	2.81 $\pm$ 0.054	2.89 $\pm$ 0.056	2.86 $\pm$ 0.057	2.90 $\pm$ 0.055	2.83 $\pm$ 0.048	0.364	0.090	0.005	0.344	0.853
Serum Ca (mM)	1,047	2.26 $\pm$ 0.028	2.27 $\pm$ 0.028	2.26 $\pm$ 0.028	2.27 $\pm$ 0.028	2.29 $\pm$ 0.028	2.26 $\pm$ 0.025	0.988	0.070	<0.001	0.362	0.016
Serum P (mM)	1,047	1.96 $\pm$ 0.057 ^a	2.06 $\pm$ 0.057 ^{bc}	2.00 $\pm$ 0.058 ^a	1.96 $\pm$ 0.058 ^{ac}	2.12 $\pm$ 0.058	1.95 $\pm$ 0.054	0.003	<0.001	<0.001	0.029	0.574
Plasma 25-(OH)D ₃ (ng/mL)	1,049	83.0 $\pm$ 13.4 ^a	239.4 $\pm$ 13.3 ^b	169.7 $\pm$ 13.5 ^c	163.4 $\pm$ 13.6 ^c	181.5 $\pm$ 13.5	158.1 $\pm$ 12.7	<0.001	<0.001	<0.001	0.908	<0.001
												0.776
												0.519
												0.025

^{a-d}Means within a row not sharing a common superscript differ significantly ($P < 0.05$).¹Treatment groups were as follows, with the amount (mg/d) of 25-(OH)D₃ fed indicated in parentheses for lactation 1 of the study (L1), ~21 d in prepartum transition, and lactation 2 of the study (L2), respectively: CON-CON = control-control (0-0-0); TRT-TRT = treatment-treatment (1-2-1); CON-TRT = control-treatment (0-2-1), and TRT-CON = treatment-control (1-0-0).²Models include the random effects of identity within dairy and sample and the fixed effects DIM; and treatment group, parity, and sample and their interactions. The effect of dairy and its interactions are not reported.³Only contains data from dairies 1 and 2.⁴ECM = [(0.3246 $\times$ milk yield) + (12.86 $\times$ fat yield) + (7.04 $\times$ protein yield)]; NRC, 2001].

that cows fed a positive DCAD diet supplemented with 3 mg/d of 25-(OH)D₃ had a 30% incidence of clinical hypocalcemia compared with 0% for those on either a negative DCAD diet supplemented with 3 mg/d of 25-(OH)D₃ or cholecalciferol and 15.8% for those on a positive DCAD diet with 3 mg/d of vitamin D₃.

Calciol Concentrations

The vitamin D status of animals is reliably indicated by the concentration of 25-hydroxyvitamin D [25(OH)D]; refers to combined concentrations of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ in serum or plasma

(Nelson et al., 2016; Wilkens et al., 2020). Concentrations of >20 ng/mL of 25-(OH)D₃ are recommended for maintenance of Ca homeostasis (NRC, 2001) and those <5 ng/mL are suggested to indicate deficiency in cattle (Horst et al., 1994). The similar baseline 25-(OH)D₃ concentrations (41.9 ± 15.6 ng/mL) between all 4 treatment groups in Exp. 2 indicates that all groups started with comparable vitamin D status and status was sufficient. Differences in baseline means between dairies may reflect differences in diet and season at sampling. Interestingly, Nelson et al. (2016) found that season within herds did not affect 25(OH)D concentrations; however, of the 4 herds sampled, none spent

Figure 8. Mean $\pm$ SE for (A) milk yield, (B) milk protein percent, (C) serum Ca concentrations, and (D) serum P concentrations from a subgroup of cows from experiment 2 at 4 sampling points. Sampling days correspond to approximately (0) a baseline sample taken prior to study commencement, (1) 100 d after study commencement, (2) 200 d after study commencement, (3) 100 d postpartum in the second lactation of the study (L2), and (4) 200 d postpartum in L2. A change between treatment group 1 [administered in lactation 1 (L1) of the study] and treatment group 2 (administered in prepartum transition and L2) occurred upon cows entering the transition period. This occurred between sample d 2 and 3 as indicated by the dashed line. Treatment groups were as follows, with the mg/d of 25-(OH)D₃ fed indicated in parentheses for L1, ~21 d in prepartum transition, and L2, respectively: CON-CON = control-control (0–0–0); TRT-TRT = treatment-treatment (1–2–1); CON-TRT = control-treatment (0–2–1), and TRT-CON = treatment-control (1–0–0).

>4 daylight hours outside, unlike the current study, in which cows were housed outside. The mean baseline 25-(OH)D₃ and CON-CON group concentrations across the 4 samplings (~41.9 and 75 ng/mL, respectively) were consistent with those reported by Nelson et al. (2016) from 702 cows from 12 dairy herds supplemented with vitamin D₃ across the United States. Nelson et al. (2016) reported a mean serum 25-(OH)D concentration of 68 ± 22 ng/mL, ranging from 40 to 100 ng/mL regardless of lactation stage or housing system.

It appears there was minimal contamination of control cows with 25-(OH)D₃ in the current study because there was a difference of at least 155 ng/mL in 25-(OH)D₃ concentration between the CON and TRT groups at each sampling, and concentrations in the CON-CON cows were very stable. Further, video review indicated that treatment pellets in residual orts were only rarely observed. Intake of 25-(OH)D₃ by controls would have pushed the hypotheses to the null.

The similar overall mean concentrations of 25-(OH)D₃ in the CON-TRT and TRT-CON groups indicate that there was no accumulation of 25-(OH)D₃ from the previous lactation. This is not surprising because the mean half-life of 25-(OH)D₃ in blood circulation in cattle ranges from approximately 14 to 34 d (Wilkens et al., 2013). The highly significant interaction between group and sample day for plasma 25-(OH)D₃ concentration was expected because 25-(OH)D₃ accumulates in the blood over time with daily 25-(OH)D₃ supplementation (Weiss et al., 2015; Rodney et al., 2018a; Poindexter et al., 2020) and may reach a plateau. It is plausible that a plateau in 25-(OH)D₃ concentration had been reached within the first 100 d of supplementation in both lactations of Exp. 2. Poindexter et al. (2020) found that 25-(OH)D concentrations in mid-lactation dairy cows stabilized between d 28 and 56 at 272 to 278 ng/mL, respectively, with 3 mg/d of 25-(OH)D₃ supplementation, but when 1 mg/d of 25-(OH)D₃ was supplemented (consistent with our study), concentrations only reached 180 ng/mL by d 56 and had not stabilized. The highest group mean for 25-(OH)D₃ concentration in our study was 266.2 ± 14.69 ng/mL at 100 d postpartum in L2 (sampling 3) for the CON-TRT cows. Rodney et al. (2018a) found that mid-lactation cows supplemented with 4 mg/d of 25-(OH)D₃ had plasma concentrations approaching 250 ng/mL after 30 d of supplementation, with no signs of a plateau between this and a sampling at 20 d. More studies are required to determine the concentrations at which 25-(OH)D₃ stabilizes in the blood, the dose rates, and timeframes to achieve stabilization.

Variation exists in the metabolism of individual cows, and not all cows respond to 25-(OH)D₃ supplementation (Rodney et al., 2018a). Nelson et al. (2016) found relatively large standard deviations for serum 25(OH)

D concentrations, unlike the current study. Unlike Ca, calcidiol is not under tight homeostatic control.

Toxicity Thresholds

Some animals in the current study were supplemented with 25-(OH)D₃ for up to 700 d over 2 lactations. Consequently, the potential for vitamin D toxicity should be considered. Cows were supplemented with 1 mg/d of 25-(OH)D₃, which is equivalent to 40,000 IU or 66 IU/kg of liveweight (LW) of vitamin D₃ during the lactation periods and 1 mg/d of 25-(OH)D₃ or 80,000 IU or 132 IU/kg of LW of vitamin D₃ during transition. Thresholds for vitamin D toxicity are thought to be between 200 and 300 ng/mL of 25-(OH)D₃ in the blood (Horst et al., 1994). This concentration range was derived from several studies reporting hypervitaminosis D when high doses of vitamin D₃ were administered to cattle (Swan, 1952; Capen et al., 1966; Littledike and Horst, 1979, 1982), often via a single intramuscular injection. The NRC (2001) maximum tolerable concentration of vitamin D₃ is 2,200 IU/kg of LW when fed for >60 d, and the growth rate can be impaired when doses are in the range of 200 to 400 IU/kg. Tomkins et al. (2020) suggested that the risk of vitamin D toxicity is considerably lower from 25-(OH)D than from vitamin D₃. Those authors administered ~240,000 IU of vitamin D₃ equivalent for ~120 d and the equivalent of 1,300 IU/kg of vitamin D₃ [6 mg/d of 25-(OH)D₃] in slow-release boluses containing 25-(OH)D₃ to beef heifers and did not observe signs of toxicity or differences in animal health or performance over their lifetime. In dairy cattle supplemented with up to 4 mg/d of 25-(OH)D₃ with blood 25-(OH)D₃ concentrations approaching or exceeding 250 ng/mL, clinical signs of hypercalcemia were not reported (Martinez et al., 2018a; Rodney et al., 2018a,b; Poindexter et al., 2020), except for a numerically highest incidence of clinical hypocalcemia in Weiss et al. (2015). Despite blood concentrations >200 ng/mL of 25-(OH)D₃ in Exp. 2, no signs of toxicity or hypercalcemia were observed in the current study; serum Ca concentrations remained unchanged and were below 2.7 mM, the threshold considered to define hypercalcemia (Littledike and Horst, 1982). Survival and censoring patterns were also not influenced by treatment. The only negative response observed for treated cattle was for reproduction; however, other observations demonstrated positive effects on reproduction. These series of recent findings in lactating dairy cattle are consistent with a safety evaluation study of 40 weaned Holstein calves in which 10 calves/group were supplemented with either 30 IU of vitamin D₃/kg of feed or 1.7, 5.1, or 8.5 µg/kg 25-(OH)D₃ over a 90-d period (Celi et al., 2018). No growth de-

pression or adverse effects of 25-(OH)D₃ were observed for any hematology, serum chemistry, gross pathology, or histology measures, or during clinical examinations (Celi et al., 2018).

Ca and P Concentrations

Increased blood 25-(OH)D₃ concentrations in response to 25-(OH)D₃ supplementation can increase Ca absorption and therefore blood Ca concentrations (Carnagey et al., 2008; McGrath et al., 2012; Wilkens et al., 2012). This is not always consistent because blood Ca concentrations are under tight homeostatic control and Ca requirements, and therefore, Ca metabolism, differ with physiological stage. Absorption, accretion in bone or other Ca pools, and excretion of Ca differ between the prepartum transition and postpartum periods and between nonpregnant lactating and nonlactating cattle (Ramberg et al., 1970; Horst et al., 2005).

Regardless of a minimum of 155 ng/mL difference in 25-(OH)D₃ concentration between CON and TRT groups at all times, the mean serum Ca concentrations were similar among treatment groups, probably reflecting the tight homeostatic control of blood Ca concentrations. Similarly, ionized and total Ca blood concentrations were not affected by 25-(OH)D₃ supplementation compared with vitamin D₃ supplementation in positive and negative DCAD diets in a study by Rodney et al. (2018b).

Serum P concentrations, however, were increased in TRT-TRT cows compared with all other groups. Phosphorus is important for the repair of all body tissues, and its metabolism is interconnected with that of Ca for body growth, bone mineralization, and muscle development. Phosphorus metabolism differs from Ca metabolism because, as long as it is in an absorbable form, P is readily absorbed, regardless of whether it is in excess (Challa and Braithwaite, 1989). Ruminants can tolerate a large range of circulating P (Underwood and Suttle, 1999), which likely explains part of the increase in concentrations in the TRT-TRT cows. Rodney et al. (2018a) reported a curvilinear increase in serum P concentration in response to increasing supplementary 25-(OH)D₃ in mid-lactation cattle over time. Vitamin D increases both Ca and P retention (McGrath et al., 2012) and, in combination with calcitriol and parathyroid hormone, triggers osteoclasts to resorb bone, releasing Ca and P, which may also have contributed to some of the increase in serum P in the TRT-TRT cows.

Health

Overall, there was one positive significant response of 25-(OH)D₃ supplementation on the incidence of

postcalving diseases; a lower probability/day of the incidence of “other” disease in multiparous TRT cows compared with their CON counterparts, suggesting that our hypothesis was not supported. A lack of response could reflect no true effect of 25-(OH)D₃ on the incidence of clinical postcalving health disorders, use of a dose rate or length of treatment that was not optimal, or insufficient study power to detect differences in all disorders. We suspect underreporting of some clinical health disorders and some misdiagnosis of more rare disorders due to the use of dairy producer diagnosis. These factors would have driven the hypothesis toward the null. It should be noted that the reporting of both clinical and subclinical health disorders is likely to be greater at research farms where animals are monitored more closely and often by veterinarians. As 25-(OH)D₃ enhances immune function (Nelson et al., 2018), this may explain the benefit observed for “other” diseases. Potential health benefits should not be dismissed because Martinez et al. (2018a) found a reduced risk of retained fetal membranes and metritis for cows supplemented with 25-(OH)D₃ during the last 21 d of gestation only compared with those supplemented with vitamin D₃.

Mastitis and LnSCC responses to treatment were not always consistent among the 3 data sets. In Exp. 1, LnSCC was reduced by 0.2 with 25-(OH)D₃ supplementation for all cows, and primiparous TRT cows had a higher probability/day of mastitis than primiparous CON. In general, SCC is regarded as a measure for udder health and immune response. Subclinical SCC does not always reflect clinical mastitis, which may account for part of the inconsistencies. Merriman et al. (2018) found that intramammary treatment with 25-(OH)D₃ had little effect on acute response to endotoxin-induced mastitis and did not affect milk SCC. Mastitis incidence or SCC did not differ over the first 49 d postpartum for cows supplemented with 3 mg/d of 25-(OH)D₃ and fed a negative DCAD diet during the 21 d precalving (Martinez et al., 2018a,b). However, inclusion of 3 mg/d of 25-(OH)D₃ for 56 d decreased the severity of mastitis in lactating cows challenged with *Streptococcus uberis* (Poindexter et al., 2020). Mammary epithelial cells use 25-(OH)D₃ (Nelson et al., 2018). Further, Lippolis et al. (2011) showed reduced signs of mastitis including significantly lower bacteria counts in milk and lower SCC in cows with induced *Strep. uberis* infections that were treated with intramammary 25-(OH)D₃ compared with control cows.

Age

A significant effect of parity was evident throughout the experimental outcomes and was more influential

than treatment in most cases, particularly in reproductive and health outcomes. This was to be expected because the risk of disease increases with age.

A higher Ca demand is associated with increased milk production with age (Horst et al., 2005). The capacity to mobilize Ca from bone and active transport of Ca from the intestine also decreases with age (Van Mosel et al., 1993; Horst et al., 2005). These factors contribute to an increased incidence of milk fever with age. Wilkens et al. (2013) showed that the half-life of 25-(OH)D₃ in the blood of cattle fed 4 or 6 mg of 25-(OH)D₃ for 10 d leading up to parturition was longer in second-lactation cows than in older cows (third and greater lactations). Therefore, it is not surprising that older cows appear to respond differently to 25-(OH)D₃ supplementation and that both plasma 25-(OH)D₃ and serum P concentrations were higher and serum Ca tended to be higher in primiparous cattle in the current study. In contrast, Rodney et al. (2018b) found that serum total Ca concentration was higher and P was not higher, whereas 25-(OH)D₃ concentrations were numerically lower in nulliparous versus parous cattle. The common practice of managing nulliparous cows differently from parous cows both in the prepartum period and in their first year of lactation facilitates supplementation of different supplementation rates and lengths of 25-(OH)D₃, which we hypothesize could optimize responses to 25-(OH)D₃ supplementation across herds.

Milk Responses

This is the first study in which 25-(OH)D₃ was supplemented in both the transition period and the subsequent lactation. Weiss et al. (2015), similar to our Exp. 1, found a lack of milk response up to 28 DIM for cows fed only during the transition period with 5.4 mg/d of 25-(OH)D₃ in a negative DCAD diet, compared with those fed vitamin D₃ plus negative DCAD. Our finding of improved milk persistency for CON-TRT cows in L2 of Exp. 2 is more consistent with results by Martinez et al. (2018b), who found that supplementation of 25-(OH)D₃ during the last 21 d of gestation increased milk yield in the next 49 DIM over cows fed vitamin D₃; however, 25-(OH)D₃ was not supplemented in the lactation ration.

The lack of production response to 25-(OH)D₃ supplementation mid-lactation is consistent with that observed over 21 d by Poindexter et al. (2020), who supplemented mid-lactation cows with 3 mg/d of 25-(OH)D₃, and Rodney et al. (2018a), who supplemented mid-lactation cows with 0, 0.5, 1, 2, or 4 mg/d of 25-(OH)D₃ for 30 d.

The inconsistent results between our experiments and among other experiments suggest that more studies are

needed to determine optimal supplementation management of 25-(OH)D₃ for milk benefits. Possible reasons for the minimal overall benefits of 25-(OH)D₃ supplementation on milk production or component measures across our 2 experiments may be that supplementing 25-(OH)D₃ in prepartum transition only is optimal, the dose rate or supplementation lengths were not optimal, or that all cows may not have received a negative DCAD diet prepartum or for a long enough period.

Reproduction

There is ample evidence that calcium status may influence reproductive performance (Borsberry and Dobson, 1989; Martinez et al., 2018a). Martinez et al. (2018a) found a 55% increased rate of pregnancy and a 19-d reduction in median time to pregnancy for 25-(OH)D₃-treated cows on a negative DCAD diet compared with cows treated with vitamin D₃. This supports our observations in Exp. 1 that 25-(OH)D₃ feeding resulted in a 22-d median decrease in time to pregnancy in multiparous cows and 41% increased odds of pregnancy per day compared with CON multiparous cows.

Both the inconsistency in reproductive responses between the 2 experiments and the negative effects in Exp. 2 (of both the lower probability to be bred per day for TRT-TRT cows and lower probability of pregnancy per day for the multiparous TRT-TRT cows) emphasize the complexity and importance of this field. The concentrations of 25-(OH)D₃ in blood were >250 ng/mL for the TRT-TRT cows in L2, and we hypothesize that the treatment dose may have been too high over an extended period to have beneficial effects on reproduction. The lower-than-targeted mean days on transition may have contributed to the negative effects. Because this study was conducted on commercial dairies, transition feeding, breeding, and management were not as tightly controlled as in a research facility, which may have further contributed to the inconsistent reproductive response.

CONCLUSIONS

Our hypothesis that feeding 25-(OH)D₃ during lactation and in prepartum in conjunction with negative DCAD diets would improve milk production, increase the probability of pregnancy, and reduce the incidence of postcalving diseases, was not supported overall. Concentrations of 25-(OH)D₃ in blood of CON cows were consistent with those of cows in the United States, and treatment resulted in concentrations more than 155 ng/mL higher with little evidence of adverse effects. There were benefits from reduced LnSCC in treatment cows and for reproduction, with treated multiparous cows

having a 22-d median decrease in the time to pregnancy. Use of a negative DCAD transition cow diet, in general, is supported, with findings of increased milk production and improved health and reproduction with increased length of transition feeding. Further research is required on dose rate during lactation and length of supplementation with 25-(OH)D₃.

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