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Potential of a rumen bolus containing 1,25-dihydroxyvitamin D_3 glycosides for the prevention of hypocalcaemia in primiparous and multiparous dairy cows

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ABSTRACT

Periparturient hypocalcaemia is a widespread metabolic disorder in dairy cows. Clinical and subclinical cases occur primarily in multiparous (**Multi**) cows, but subclinical cases have also been reported in primiparous (**Primi**) cows. A preventive strategy was investigated by administering the physiologically active vitamin D_3 metabolite, 1,25-dihydroxyvitamin D_3 (1,25-dihydroxycholecalciferol, **1,25(OH)$_2$D$_3$**) as a rumen bolus. The bolus contained tablets of 1,25$(OH)_2D_3$ glycoside extract from *Solanum glaucophyllum* (**SGE**), releasing SGE over several days. The aim was to study the effect of a bolus containing 0 (**C**) or 500 μg (SGE) of 1,25$(OH)_2D_3$ on 1,25$(OH)_2D_3$ and mineral status in periparturient cows up to three weeks into lactation and on colostrum, milk and calves' blood mineral contents. The bolus was administered three to four days prior to expected calving to Primi and Multi cows fed a herbage-based diet (dietary cation-anion difference of +522 mEq/kg DM). One C or SGE bolus was applied to 12 Primi and 12 Multi cows. Blood was regularly sampled (and selected a posteriori for antepartum samples) in regard to the actual calving day (**d0**), immediately prior to bolus application and at day −2, 0.5, 1, 1.5, 2, 4, 8, 11, 15, 18 and 22. Additional samples included urine (at bolus application, d0.5 and d2), colostrum, milk samples (weekly) and calves' blood (d2). Blood serum 1,25$(OH)_2D_3$ increased between d0.5 and d2 in Primi-SGE, but remained unchanged in Primi-C, as did parathyroid hormone (**PTH**) and Ca in all Primi. Urinary Ca of Primi-SGE was increased on d2, indicating regulation of Ca excess. Three Multi-C cows with confirmed clinical hypocalcaemia needed treatment and thus were excluded from the dataset and replaced. Blood serum 1,25$(OH)_2D_3$ and PTH increased while Ca dropped by 40% between d0.5 and d2 in Multi-C, whereas 1,25$(OH)_2D_3$, Ca and PTH remained unchanged in Multi-SGE. Blood serum carboxyterminal telopeptide of type I collagen was higher in Primi than in Multi and increased with time, except in Primi-C. Mineral contents in colostrum, milk and blood serum of calves were not influenced to a relevant degree. In conclusion, Primi-C did not, in contrast to Multi-C, develop subclinical hypocalcaemia (<2.0 mmol Ca/l). Prevention of hypocalcaemia with one SGE bolus applied three to four days prior to expected calving was successful in maintaining blood Ca within normal range in Multi over the critical first two days and up to the first three weeks of lactation, without any observed detrimental effects on cows or calves.

Implications

Hypocalcaemia is a widespread metabolic disorder in dairy cows at onset of lactation affecting mainly multiparous cows, but cases, albeit mostly subclinical, are also reported in primiparous cows. The study demonstrates that multiparous cows were hypocalcaemic when fed a herbage-based diet which is considered risky in this regard. However, the application of a bolus containing a source of the active metabolite of vitamin D_3 extracted from *Solanum glaucophyllum* leaves three to four days prior to expected calving date was successful in preventing hypocalcaemia in multiparous cows. Primiparous cows did not show hypocalcaemia and thus would not require any prevention.

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Introduction

At calving time, the sudden onset of milk production induces a drastic increase in the Ca requirement of dairy cows. The physiological adaptation to maintain blood Ca at constant level during this period consists in the secretion of parathyroid hormone (**PTH**) from the parathyroid glands to stimulate Ca resorption from the bones and to increase renal Ca reabsorption. Under the influence of PTH, the production of the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (**1,25(OH)$_2$D$_3$**), in the kidneys is activated, which in turn increases active intestinal Ca and P absorption (Fukumoto, 2014; Goff, 2018; Oetzel, 2020). Unsuccessful adaptation leads to a decline in blood Ca reaching lowest values around 24 h after calving (Rérat and Schlegel, 2014; Caixeta et al., 2017) and may lead to clinical signs of hypocalcaemia (milk fever). Whereas clinical cases of periparturient hypocalcaemia (serum total Ca <1.4 mmol/l; DeGaris and Lean, 2009) essentially occur in multiparous cows, especially from the third lactation onwards, subclinical cases (serum total Ca 1.4–2.0 mmol/l; DeGaris and Lean, 2009) are reported in multiparous cows with a prevalence of about 50% as well as in primiparous cows with a prevalence between 6 and 25% (Reinhardt et al., 2011; Caixeta et al., 2017; Venjakob et al., 2017). Subclinical hypocalcaemia can predispose cows to other periparturient disorders such as mastitis, retained placenta, metritis, abomasum displacement, leading to reduced milk yield, impaired fertility and finally to increased culling (Chapinal et al., 2011 and 2012; Seifi and Kia, 2017).

To limit the risk of hypocalcaemia occurrence, preventive feeding strategies during the transition period from three to four weeks prepartum until parturition mainly consist in a restrictive use of dietary Ca with sufficient dietary Mg and vitamin D_3 content and/or by favouring a metabolic acidosis (Meschy, 2010; Goff, 2018). This can be achieved by manipulation of the dietary mineral content in order to reach a negative dietary cation–anion difference (**DCAD**) to favour increases in blood Ca concentration of cows immediately after calving (Santos et al., 2019). The basal diet of dairy herds in intensively managed grassland systems contains relatively high amounts of Ca (5–9 g/kg DM) and K (25–32 g/kg DM) originating from herbage (Schlegel et al., 2016 and 2018). The high dietary K leads to a high DCAD, easily exceeding +400 mEq/kg DM (Rérat et al., 2009; Rérat and Schlegel, 2014; Goff, 2018), when using the formula according to Block (1984). Under such conditions, favouring a low dietary Ca and a recommended negative DCAD for dry cows (Santos et al., 2019) is hardly achievable, even with the use of anionic salts (Liesegang et al., 2007). Therefore, other prepartum preventive dietary strategies to limit the risk of hypocalcaemia need to be investigated. One of these strategies could consist in feeding the biologically active metabolite of vitamin D_3 (1,25(OH)$_2$D$_3$) prior to calving, which increased blood Ca by using either a synthetic source (Hove and Kristiansen, 1982; Hove et al., 1983) or a natural source in the form of leaves of *Solanum glaucophyllum,* which are rich in 1,25(OH)$_2$D$_3$ glycosides (Horst et al., 2003; Ishii et al., 2015). However, with an abrupt withdrawal of dietary 1,25(OH)$_2$D$_3$ at the onset of lactation, delayed hypocalcaemia can occur (Horst et al., 2003). These authors reported that whenever the ingested 1,25(OH)$_2$D$_3$, that had maintained the blood Ca level, had cleared from the body, blood Ca rapidly declined, causing delayed start of the endogenous production of 1,25(OH)$_2$D$_3$. Furthermore, the activation of Ca resorption from the intestine under the influence of 1,25(OH)$_2$D$_3$ will normally restore blood Ca back to normal levels within hours (Vieira-Neto et al., 2017; Goff 2018). Thus, a progressive decline in exogenous 1,25(OH)$_2$D$_3$ supply after calving would favour a progressive activation of endogenous 1,25(OH)$_2$D$_3$ production, which may prevent delayed hypocalcaemia.

For this purpose, a rumen bolus was developed to be administered once a few days prior to calving. This bolus consists of tablets based on *S. glaucophyllum* leaf extract (**SGE**), which contain high concentrations of 1,25(OH)$_2$D$_3$ glycosides. These tablets were developed to enable a rapid- and long-acting controlled-release of SGE in the rumen (Herbonis Animal Health GmbH, Augst, Switzerland). The pharmacokinetic response to several boli with different SGE tablet formulations in blood 1,25(OH)$_2$D$_3$, Ca, P and Mg in pregnant dry dairy cows has been described previously (Bachmann et al., 2017; Meyer-Binzegger et al., 2021). According to the most recent bolus formulations, blood Ca increased starting half a day after bolus application, peaked between 2 and 4 days and then constantly decreased to return to basal Ca levels after 11 days (Meyer-Binzegger et al., 2021). The SGE bolus containing 500 μg 1,25(OH)$_2$D$_3$ in rapid and slow release tablets resulted, compared to the other formulations, in the highest and longest elevation in blood 1,25(OH)$_2$D$_3$ and Ca (Meyer-Binzegger et al., 2021). Thus, it is hypothesised that the administration of one SGE bolus within a time window of nine to a half a day prior to actual calving provides the potential to maintain blood Ca. This could prevent periparturient hypocalcaemia up to the critical time of 48 h postpartum, while the slow decrease of 1,25(OH)$_2$D$_3$ supply enables the onset of a progressive endogenous PTH production and endogenous 1,25(OH)$_2$D$_3$ activation, thus reducing the risk of delayed hypocalcaemia during the first few weeks of lactation.

The aim was to study the effect of SGE administered via a rumen bolus prior to calving to primiparous and multiparous cows fed a herbage-based diet with an expected highly positive DCAD on 1,25(OH)$_2$D$_3$, Ca, P and Mg concentrations in blood and urine up to three weeks into lactation. In addition, colostrum, milk and blood from newborn calves were collected to determine if SGE would influence their 1,25(OH)$_2$D$_3$ and mineral contents.

Material and methods

Animals, diet and experimental design

The experiment was set up by blocking cows according to parity at subsequent calving (primiparous, **Primi**; multiparous with $\geq 3^{rd}$ lactation, **Multi**). Within each block, cows were assigned, in sequence to their expected calving date, to either a Control (**C**) or a SGE bolus to obtain four groups (Primi-C, Primi-SGE, Multi-C and Multi-SGE) of cows.

The rumen bolus was composed of a capsule (8 cm × 2.4 cm) filled with dolomite as ballast and it contained either 0 (C) or 500 μg of 1,25(OH)$_2$D$_3$ (SGE, analysed 491 μg, Meyer-Binzegger et al., 2021) bound as glycosides. The SGE was provided as tablets containing *S. glaucophyllum* leaf extracts (Herbonis Animal Health GmbH, Augst, Switzerland). According to Meyer-Binzegger et al. (2021), the response to SGE in increased blood serum Ca started 12 h after administration and lasted for 11 days. Thus, the optimal time window for SGE administration is between nine and a half day before actual calving to obtain an optimal response in blood serum Ca within the first two days postpartum. The mean actual calving date of the dairy herd, used in this experiment (Agroscope, Posieux, Switzerland), is 281 days after successful insemination with a SD of 5 days. Considering these five days of variability, the optimal application of the bolus is three to four days before the expected calving day.

According to a power analysis (parameter: expected blood serum Ca effect of 0.35 with a SD of 0.22 mmol/l), six replicates were required per group. In order to obtain calvings of six Primi and six Multi cows within this time-frame after SGE bolus application, the following procedure was applied: to account for potential drop-outs, a total of 18 pregnant Holstein and Red Holstein heifers

and 18 Multi pregnant, dry Holstein and Red Holstein cows from the Agroscope dairy herd were selected 260 days after successful insemination, corresponding to 21 days prior to expected calving. None of the selected Multi cows had previously shown signs of clinical hypocalcaemia. Three to four days prior to expected calving date, (277–278 days after successful insemination), a rumen bolus (randomly either C or SGE) was administered using a bolus applicator (V-Grip™ Bolus Gun, item 64314, MAI Animal Health, Melksham, Wiltshire, UK). Within three consecutive expected calvings of heifers or cows, respectively, one C-bolus and two SGE boli were provided. Cows were provided more frequently with the SGE bolus in order to take into account cows which would need to be excluded if they calve outside the predefined window of one to nine days after bolus application. In case of observed clinical signs suggesting hypocalcaemia following calving (e.g. tremor, loss of appetite, nervousness, hypersensitivity, weakness, restlessness, shuffling of the back legs, cold extremities), a blood sample was taken to measure Ca concentration. If blood serum Ca was <1.6 mmol/l, milk fever was confirmed and the cow was treated with soluble Ca preparations, excluded from the experiment and replaced with another forthcoming calving cow of the same category (primi- or multiparous).

The cows were housed in a free-stall barn without exterior access until signs of calving were observed or at the latest four days prior to expected calving. From that time until two days after calving, they were all transferred to individual straw-bedded pens (31 m^2) adapted for calvings. Each pen was equipped with an infrared camera to monitor calving whenever personal was not present. Two days following calving, the calf was separated from the cow and the cow returned to the free-stall barn. The free-stall barn was equipped with automatic weighing troughs (RIC System, Insentec B.V., Marknesse, The Netherlands) allowing for individual recordings of the basal diet intake. It was also equipped with an automated concentrate and mineral feeder (Insentec B.V., Marknesse, The Netherlands). The basal diet was fed *ad libitum* throughout the experiment and consisted of hay and maize silage (75/25% of DM). Two pelleted concentrates differing in their protein concentration (formulated at 121 and 587 g CP/kg DM) were provided restrictively during lactation to balance energy and protein contents of the diet. A pelleted prepartum mineral feed (formulated at 13 g Ca, 7 g P and 38 g Mg/kg and 16 000 IU vitamin D_3/kg) was provided at 400 g/d before calving and lactation mineral feed (formulated at 160 g Ca, 25 g P, 70 g Mg/kg and 43 000 IU vitamin D_3/kg) was provided restrictively according to milk yield during lactation.

Sample collection and preparation and data recording

Blood samples were taken from each cow in the morning (0800–0900 h) immediately before bolus application and then every second day until calving. After calving, blood samples were taken either in the morning (0800–0900 h) or evening (1600–1700 h) to obtain samples within 12, 24, 36 and 48 h after calving. When the cows returned to the free-stall barn, blood samples were collected twice a week (on Tuesdays and Fridays for practical reasons, 0800–0900 h). Blood samples were taken from calves within 36–48 h after birth. All blood samples were taken from the jugular vein using serum vacutainers without anticoagulant (Vacuette 20G, Greiner Bio One International GmbH, Solingen, Germany; S-Monovette, Sarstedt AG + Co. KG, Nümbrecht, Germany). The vacutainers were centrifuged, at room temperature, at 1500*g* for 15 min followed by 4000*g* for 2 min within 2 h after collection and the retained serum was stored at −20 °C until analysis. At the time of bolus application and at 12 h and 48 h after calving, urine was spot sampled at midstream urination whenever applicable, otherwise urine was sampled using a catheter. Urine pH was measured immediately after collection (Metrohm 691, Metrohm AG, Herisau, Switzerland), and the samples were stored in 10 ml tubes at −20 °C until analysis. The cows were milked twice daily, and milk yield was recorded. A colostrum sample was taken at first milking (within 6 h after calving) and milk samples were collected on d7, d14 and d21 during the morning and evening milkings. Morning and evening milk samples were pooled per day proportionally to the respective milk yield. The colostrum and pooled milk samples were frozen at −20 °C until analysis. Body condition score of cows was determined and BW was recorded on the last day in the free-stall barn before calving. The date and time of calving and of placenta expulsion were recorded. The calf was weighted within 12 h of birth. The daily basal feed intake in the individual straw-bedded pens was recorded as the difference in weights of distributed feed minus leftovers. Samples of the basal diet were taken once a week and dried for 24 h at 60 °C to determine DM. Dried samples were pooled every second week. Samples of the other feeds were collected once a week and pooled every 4 weeks. Pooled feed samples were milled (1.0 mm, Brabender mill, Duisburg, Germany) and stored in brown glass bottles until analysis.

Chemical analyses

Based on the blood sampling plan for the cows, the day of sampling and the number of samples from actual calving were not identical between cows in the period prior to calving (based on expected calving day) and from two days onwards after calving (twice a week). Hence, the sampling days were standardised and blood samples corresponding best to the standardised days were selected for analysis. Detailed information is given below in chapter Data analysis.

The selected blood serum samples of the cows and the calves were analysed within 12 months. Serum $1,25(OH)_2D_3$ concentration was only measured in samples up to 15 days postpartum using ELISA kits (Kit 2112, Immundiagnostik, Bensheim, Germany) as the values were expected to be back to baseline after this time (Meyer-Binzegger et al., 2021). The serum samples as well as the urine samples were colorimetrically analysed for Ca, P and Mg concentrations using commercial assay kits (Greiner Diagnostik GmbH, Bahlingen, Germany). For creatinine in urine, the analysis was done colorimetrically using a commercial creatinine kit (Creatinine 164L, Biotecnica Instruments SpA, Roma, Italy). All kits were used in combination with a BT1500 autoanalyser (Biotecnica Instruments SpA, Roma, Italy). Parathyroid hormone and the bone resorption biomarker carboxyterminal telopeptide of type I collagen (**CTx**) were determined in blood serum from four and seven selected sampling days, respectively. Commercial ELISA kits were used to determine the concentrations of PTH (63-3100, Quidel Corporation, Athens, USA) and CTx (AC-02F1, Immunodiagnostic Systems Ltd., Boldon, UK) with an Asys UVM 340 microplate reader (Biochrom Ltd., Cambridge, UK) following instructions provided by the manufacturer. Dry matter, ash, CP, crude fibre, NDF and ADF contents of the pooled feed samples were determined according to Oberson et al. (2019). Calcium, P, Mg, K and Na contents in the pooled feed samples were quantified after solubilisation in 65% nitric acid + Millipore water and microwave digestion (Multiwave 7000, Anton Paar Switzerland AG, Buchs, Switzerland) with an inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 7300 DV, Perkin Elmer, Schwerzenbach, Switzerland). Sulphur content was directly determined by infrared absorption after combustion at 1360 °C (TruMac CNS, Leco Instrumente GmBH, Mönchengladbach, Germany). Colostrum and milk samples were analysed for Ca, P and Mg by graphite furnace atomic absorption spectroscopy. All analyses were performed in the accredited Agroscope laboratories in duplicate, except DM and ash as a single analysis.

Data analysis

The nutrient content of the total diet was calculated as the cows' daily intake of each feed component multiplied by its respective measured concentration. The DCAD (mEq/kg of DM) contents of the prepartum and lactation diets were calculated as = $(Na^+ + K^+) - (Cl^- + S^{2-})$ according to Block (1984). The Cl content of hay was defined according to Schlegel et al. (2016 and 2018) and based on the reference values (Agroscope, 2020) for the other feedstuffs. Urinary Ca, P and Mg concentrations were divided by urinary creatinine concentration and are presented on basis of that ratio.

The standardised days from actual calving (**d0**) were defined as the median distribution of actual sampling days from d0: d-4, d-2, d0.5, d1, d1.5, d2, d4, d8, d11, d15, d18 and d22. The blood samples which were closest to the defined standardised days were selected for analysis, except for the standardised d-4 which was defined as the sample taken at bolus application. The SD of the actual days within each standardised day was below 1 day, except on d-4 (bolus application). On d-4, the SD was 3.5 days for C cows (earliest 13 days and latest 1 day prior actual calving) and 2.7 for SGE cows (earliest 8 days and latest 1 day prior actual calving).

The individual cow and the individual calf, respectively, were considered as the experimental unit. After graphical examination of the repeated individual blood concentrations, the following mixed model of SYSTAT 13 (SYSTAT Software Inc.) was defined: $Y_{ijkl} = \mu + Parity_i + Bolus_j + Parity_i \times Bolus_j + Day_k + Parity_i \times Bolus_j \times Day_k + Cow_l + \varepsilon_{ijkl}$ where Y_{ijkl} is the response, μ the least-squares mean, $Parity_i$ the fixed effect of parity (i = Primi, Multi), $Bolus_j$ the fixed effect of bolus (j = C, SGE), Day_k the fixed effect of standardised blood sampling d (k = −4, −2, 0.5, 1, 1.5, 2, 4, 8, 11, 15, 18 and 22), Cow_l the random effect of the individual and ε_{ijkl} the random error. The remaining data were analysed using the general linear model of SYSTAT 13 (SYSTAT Software Inc.) according to the following model: $Y_{ij} = \mu + Parity_i + Bolus_j + Parity_i \times Bolus_j + \varepsilon_{ij}$ where Y_{ij} is the response, μ the least-squares mean, $Parity_i$ the fixed effect of the parity (i = Primi, Multi), $Bolus_j$ the fixed effect of the applied bolus (j = C, SGE), and ε_{ij} the random error. The codes for the statistical models are given in Supplementary Material S1. To calculate the P-values of data which were not normally distributed (blood serum $1,25(OH)_2D_3$, PTH and CTx, and urinary Ca/creatinine), those were log transformed. Comparisons among least square means were calculated using Tukey's contrasts. Differences were considered as significant when $P \leq 0.05$. Unless otherwise stated, mean results are presented as LSM ± SE.

Results

Analysed and calculated nutrients in the basal diet, the concentrates, the mineral feeds and calculated nutrients in the prepartum and lactation diet are presented in Supplementary Table S1. The prepartum diet had a highly positive DCAD content (+522 mEq/kg DM), mainly as the consequence of a high K concentration (29.8 ± 1.0 g/kg DM, mean ± SD) in the herbage-based basal diet.

Three cows (Multi-C) showed clinical signs of hypocalcaemia and had a blood serum Ca level below the defined threshold of 1.6 mmol/l (1.54, 0.73 and 0.72 mmol/l at 32 h, 17 h and 24 h postpartum, respectively). In order to obtain six animals per group, three other Multi cows replaced these animals. Prepartum BW (687 ± 20 and 818 ± 20 kg) and body condition score (3.25 ± 0.11 and 3.15 ± 0.11) of Primi and Multi cows, as well as the number of calvings (incl. the one during the experiment) of Multi cows (4.7 ± 0.4 and 4.2 ± 0.4), were comparable between the groups C and SGE (Bolus, $P > 0.10$). Feed intake prior and after calving was higher (Parity, $P < 0.001$) in Multi (17.4 ± 0.4 and 20.0 ± 0.3 kg DM/d, respectively) than in Primi cows (11.4 ± 0.4 and 14.6 ± 0.3 kg DM/d, respectively), but similar between C and SGE groups (Bolus, $P > 0.10$). Colostrum yield (6.2 ± 2.8 kg) and weekly milk yield (27.9 ± 1.4, 39.2 ± 1.5 and 40.8 ± 1.6 kg/d, respectively in weeks 1 to 3) of Multi cows was also higher (Parity, $P < 0.001$) than the in Primi cows (3.9 ± 2.5 kg colostrum and 18.3 ± 1.4, 24.9 ± 1.5 and 26.6 ± 1.6 kg/d, respectively in weeks 1 to 3). Colostrum and milk yields did not differ between groups C and SGE (Bolus, $P > 0.10$). The 24 calves (40.8 ± 1.6 kg BW) were born 115 ± 35 h after bolus application and cows expulsed their placenta 4.6 ± 0.8 h after calving, all similar among groups ($P > 0.10$).

Cow's blood composition

Cow blood serum concentrations of $1,25(OH)_2D_3$, Ca, P and Mg are presented in Figs. 1–4 and in Supplementary Tables S2–S5. Compared to the values on d-4 (baseline), serum $1,25(OH)_2D_3$ concentration increased from d0.5 to d2 and then dropped again to the baseline (Day, $P < 0.001$). However, this change over time differed between groups (Parity × Bolus × Day, $P < 0.01$) as it remained stable in Primi-C and Multi-SGE cows, whereas it was increased in Primi-SGE prior to calving on d-2 and in Multi-C after calving (d1 to d2) compared to their lowest level reached (d4 to d8 and d8 to d15, respectively).

Compared to baseline serum Ca (2.26 ± 0.08 mmol/l), the concentration decreased from d0.5 to d2 and then raised again to baseline (Day, $P < 0.001$). However, this change over time was only observed in Multi-C and Multi-SGE, while Ca remained stable in Primi-C and Primi-SGE cows (Parity × Bolus × Day, $P < 0.001$). From d0.5 to d2 after calving, the Ca concentration of Multi-C cows dropped by 40% compared to baseline and reached values <2.0 mmol/l. In Multi-SGE cows, Ca level peaked after bolus application on d-2 to a higher concentration than the levels observed in Primi-C and Multi-C cows. After this peak, Ca levels of Multi-SGE returned to values similar to baseline until the end of the experiment and never decreased to values <2.0 mmol/l.

Compared to baseline serum P (1.41 ± 0.12 mmol/l), the concentration peaked on d-2 and then dropped to baseline (Day, $P < 0.001$). However, a change over time was only observed in Primi-SGE and Multi-SGE while it remained stable in Primi-C and Multi-C cows (Parity × Bolus × Day, $P < 0.001$). The P concentrations of Primi-SGE and Multi-SGE peaked on d-2 and remained high until d2 before dropping again to baseline until the end of the experiment. Multi-C cows showed a stable P concentration, but their values reached levels below 0.8 mmol/l between d0.5 and d2, which was lower than those of Primi-SGE cows.

Compared to baseline serum Mg (0.94 ± 0.04 mmol/l), the concentration increased slightly from d0.5 to d2 and then dropped again to baseline (Day, $P < 0.001$). This change over time was observed in Primi-C and Multi-C cows, but not in Primi-SGE and Multi-SGE cows which had stable Mg concentrations over time (Parity × Bolus × Day, $P < 0.001$). Overall, multiparous cows had slightly higher Mg values than primiparous cows (Parity < 0.05).

The PTH and CTx concentrations in cows' blood serum are presented in Figs. 5 and 6 and in Supplementary Tables S6 and S7, respectively. While PTH increased after calving (d1.5) compared to baseline (Day, $P < 0.05$), CTx increased with time (Day, $P < 0.001$). The increase in PTH was however solely observed in Multi-C cows on d0.5 and d1.5 (Parity × Bolus × Day, $P < 0.01$). On the other side, the CTx increase was observed in all groups with the exception of Primi-C (Parity × Bolus × Day, $P < 0.001$). Overall, primiparous cows had higher CTx concentrations than Multi-SGE (Parity, $P < 0.01$).

Urine pH and composition

The urine pH and urine composition are presented in Table 1. Urinary pH and creatinine concentrations were similar among

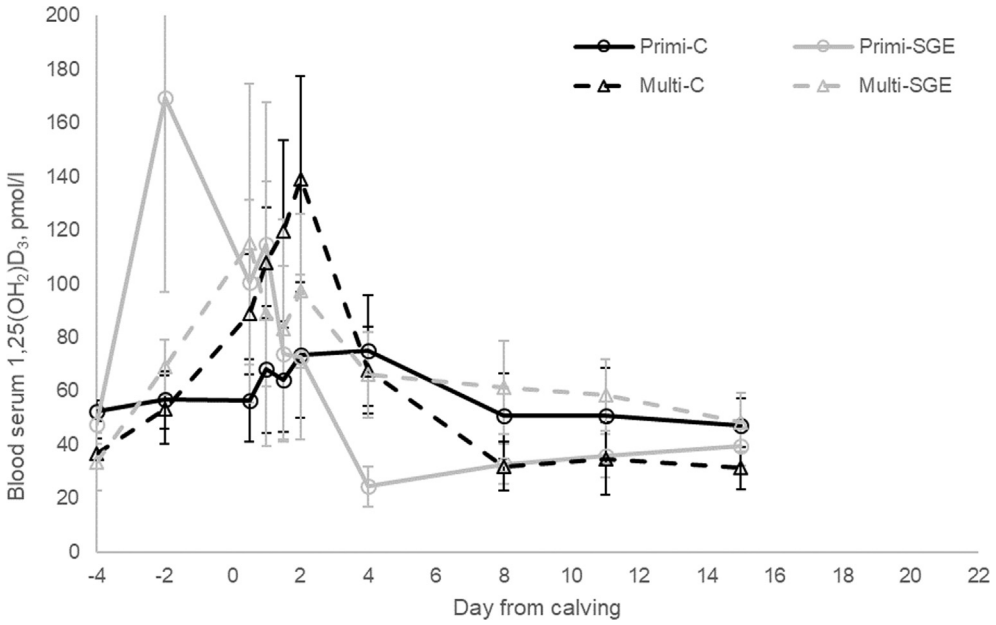

Fig. 1. Blood serum $1,25(OH)_2D_3$ concentration (least square means ± SE) in periparturient primiparous (Primi) and multiparous (Multi) cows given, four days prior to calving, a rumen bolus containing 0 (C) or 500 (SGE) μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *S. glaucophyllum*. Day ($P < 0.01$) and Parity × Bolus × Day ($P < 0.001$).

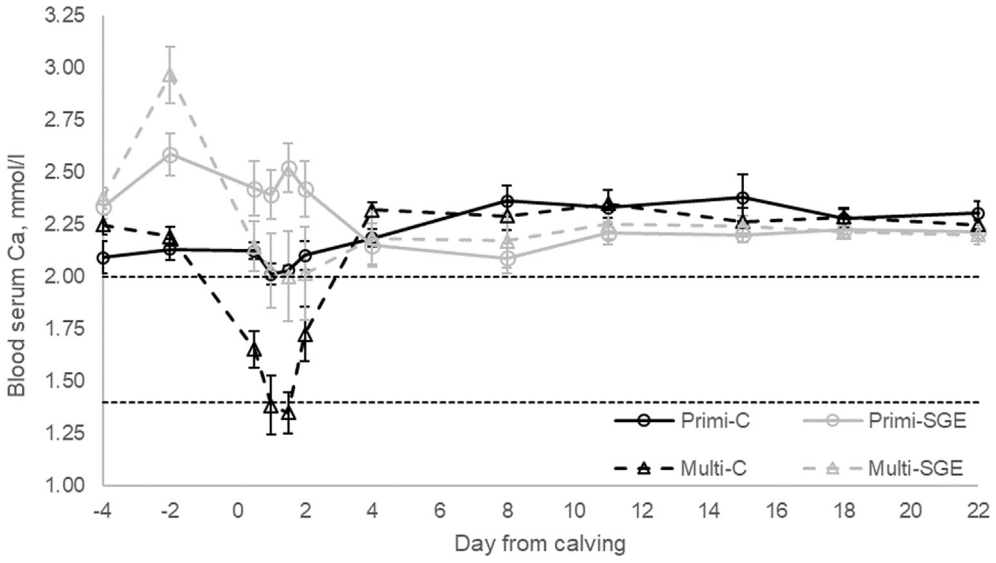

Fig. 2. Blood serum calcium concentration (least square means ± SE) in periparturient primiparous (Primi) and multiparous (Multi) cows given, four days prior to calving, a rumen bolus containing 0 (C) or 500 (SGE) μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *S. glaucophyllum*. Parity ($P < 0.05$), Bolus ($P < 0.01$), Day ($P < 0.001$) and Parity × Bolus × Day ($P < 0.001$). The limits for clinical and subclinical hypocalcaemia (<1.4 and <2.0 mmol/l, respectively) are indicated as dotted lines.

groups, except that creatinine was higher in urine of Primi than Multi cows prior to calving (Parity, $P < 0.05$). Relative to creatinine, urine Ca concentration prior to calving was lower in Primi than in Multi cows (Parity, $P < 0.05$). After calving, it was higher in Primi-SGE compared to the other groups (Parity × Bolus, $P < 0.05$). Relative to creatinine, P and Mg concentrations did not differ among groups, except that prior to calving, urinary P was slightly higher in Multi than in Primi cows (Parity, $P < 0.01$).

Colostrum and calf blood composition

The composition of colostrum, milk and blood serum of calves are presented in Table 2. Calcium in colostrum and milk did not differ among groups, except for the 7% higher value in Multi cows on d7 (Parity, $P < 0.05$). Phosphorus in colostrum did not differ among groups, but milk P was 8% lower in Multi than Primi cows on d14 (Parity, $P < 0.05$) and on d21 (Parity, $P < 0.001$) and was 5% higher in SGE than in C cows on d14 (Bolus, $P < 0.05$). Magnesium in colostrum and milk did not differ among groups. Serum concentrations of $1,25(OH)_2D_3$, P and Mg in two day-old calves did not differ among groups of dams ($P > 0.10$). However, blood serum Ca was 9% higher in calves of Primi-C cows than of Multi-C cows (Parity × Bolus, $P < 0.05$).

Discussion

The risk for periparturient hypocalcaemia is increased with DCAD prior to calving exceeding +200 mEq/kg DM and a negative

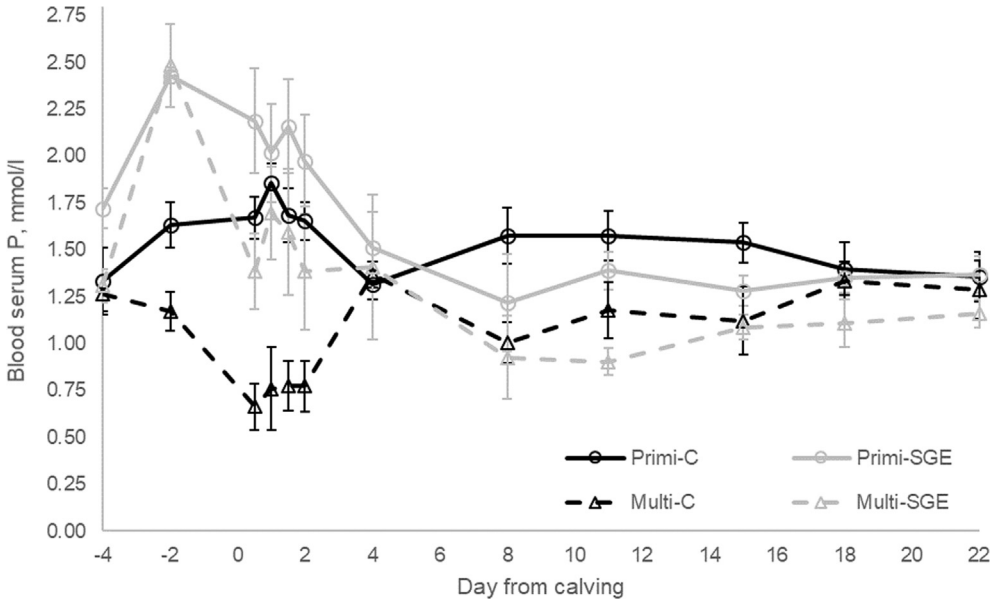

Fig. 3. Blood serum phosphorus concentration (least square means ± SE) in periparturient primiparous (Primi) and multiparous (Multi) cows given, four days prior to calving, a rumen bolus containing 0 (C) or 500 (SGE) μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *S. glaucophyllum*. Parity ($P < 0.001$), Bolus ($P < 0.05$), Day ($P < 0.001$) and Parity × Bolus × Day ($P < 0.001$).

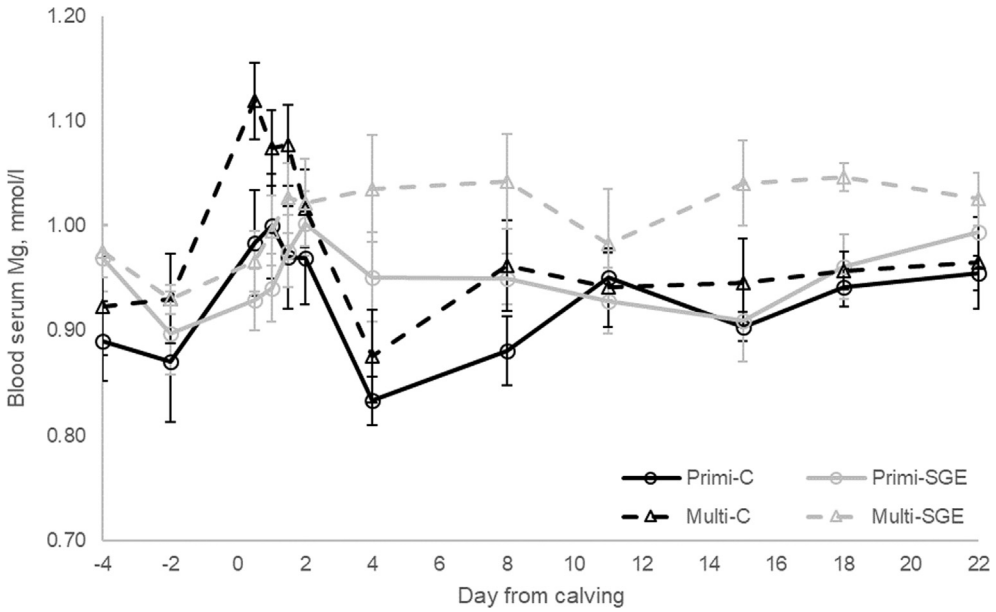

Fig. 4. Blood serum magnesium concentration (least square means ± SE) in periparturient primiparous (Primi) and multiparous (Multi) cows given, four days prior to calving, a rumen bolus containing 0 (C) or 500 (SGE) μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *S. glaucophyllum*. Parity ($P < 0.05$), Day ($P < 0.001$) and Parity × Bolus × Day ($P < 0.001$).

DCAD should be seeked (Santos et al, 2019). However, a negative DCAD is difficult to achieve in intensively managed herbage-based diets as these diets are rich in potassium (Liesegang et al., 2007). This study was conducted under such critical nutritional conditions with an average DCAD of +522 mEq/kg DM. The high urinary pH (>8.0), which is indicative of a state of metabolic alkalosis, confirms that the risk of milk fever occurrence was present as it increases with increasing urinary pH (Santos et al, 2019). Despite these dietary conditions, primiparous cows did not have clinical or subclinical hypocalcaemia (<2.0 mmol Ca/l) at any time following calving. According to Goff et al. (2014), a metabolic alkalosis was induced with a DCAD of +188 mEq/kg DM prior to calving which on one hand reduced the ability of the cow to respond to PTH and on the other hand was detrimental for stimulating 1,25 $(OH)_2D_3$ formation. The stable blood serum PTH in Primi cows and of $1,25(OH)_2D_3$ in Primi-C suggests that primiparous cows did not need to activate physiological mechanisms to enhance intestinal Ca absorption. The more active bone metabolism related to ongoing growth, as illustrated by their higher serum concentration in the bone resorption biomarker CTx in comparison to Multi cows, was probably sufficient to maintain blood Ca. Similar differences in CTx levels according to parity were observed in dairy cows (Gaignon et al., 2018), sows (van Riet et al., 2016) and women (Chubb, 2012). Furthermore, the increased urinary Ca of Primi-SGE compared to Primi-C is indicative of a Ca excess in Primi-SGE which was successfully excreted as no hypercalcaemia

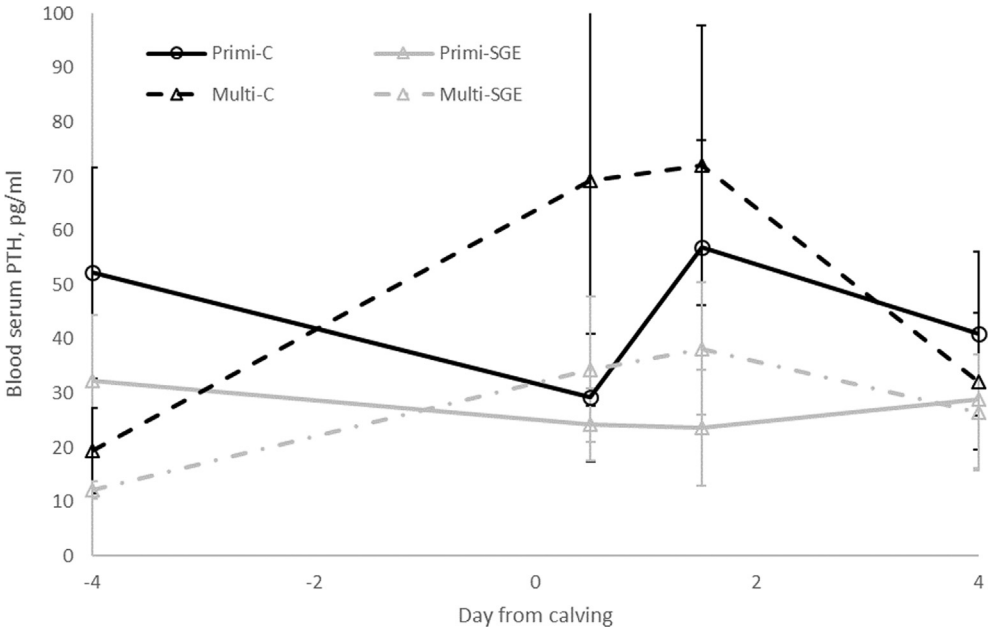

Fig. 5. Blood serum parathyroid hormone concentration (PTH, least square means ± SE) in periparturient primiparous (Primi) and multiparous (Multi) cows given, four days prior to calving, a rumen bolus containing 0 (C) or 500 (SGE) μg $1,25(OH)_2D_3$, provided as 1,25(OH)2D3 glycosides from *S. glaucophyllum*. Day ($P < 0.05$) and Parity × Bolus × Day ($P < 0.01$).

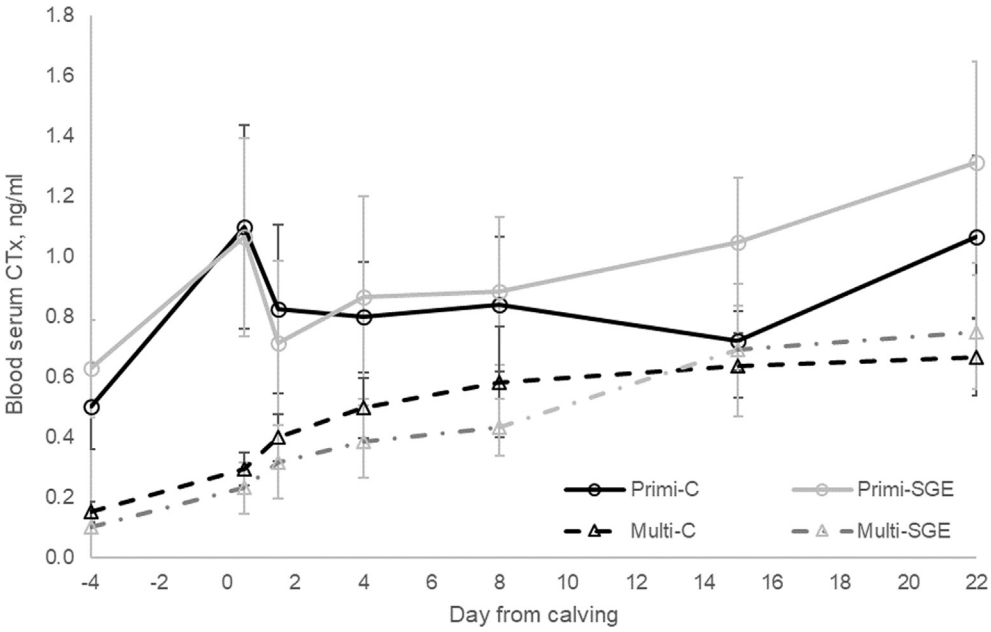

Fig. 6. Blood serum carboxyterminal telopeptide of type I collagen concentration (CTx, least square means ± SE) in periparturient primiparous (Primi) and multiparous (Multi) cows given, four days prior to calving, a rumen bolus containing 0 (C) or 500 (SGE) μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *S. glaucophyllum*. Parity ($P < 0.01$), Day ($P < 0.001$) and Parity × Bolus × Day ($P < 0.001$).

(>2.8 mmol Ca/l, Braun et al., 2009) was observed. This Ca excess in Primi-SGE probably did not originate from bone resorption as CTx profile was comparable between Primi-C and Primi-SGE. A similar observation was reported by Vieira-Neto et al. (2017), where cows having received a subcutaneous injection of 0 vs 300 μg 1,25 $(OH)_2D_3$ following calving, resulted in no different CTx profiles over seven days postpartum. Thus, it is assumed that it could originate from an increased intestinal Ca absorption, independent from PTH, but mediated through the increased blood serum $1,25(OH)_2D_3$ level induced by the provided SGE. Finally, the homeostatic regulation of Ca in Primi-C and Primi-SGE cows was successful in maintaining a constant blood Ca during the first three weeks of lactation.

The situation was quite different in Multi cows. Their higher colostrum and daily milk yield compared to Primi cows induced a higher Ca export of 38% (10.5 vs 7.6 g/d) and 57% (36.8 vs 23.5 g/d), respectively, and Multi cows after a second calving have a lower level of bone remodelling (Gaignon et al., 2018), probably as they achieved full growth. Apart from the three Multi-C cows excluded due to confirmed clinical hypocalcaemia, the Multi-C cows remained clinically healthy throughout the study period. However, their blood Ca profile between d0.5 and d2 classified

Table 1
Urine pH and composition of periparturient primiparous and multiparous dairy cows given, four days prior to calving, a rumen bolus containing 0 or 500 μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *S. glaucophyllum*.

	Group					*P*-value		
Item	Primi-C	Primi-SGE	Multi-C	Multi-SGE	SEM	Parity	Bolus	Parity × Bolus
pH								
Day −4	8.28	8.35	8.33	8.28	0.031	0.73	0.85	0.09
Day 0.5	8.29	8.06	8.36	8.31	0.079	0.06	0.09	0.29
Day 2	8.26	8.17	8.31	8.27	0.040	0.06	0.12	0.55
Creatinine (mmol/l)								
Day −4	9.1	9.9	6.7	6.4	1.15	0.01	0.80	0.62
Day 0.5	12.9	9.8	11.9	10.3	1.82	0.90	0.21	0.70
Day 2	8.4	7.3	7.2	5.8	0.81	0.10	0.14	0.86
Ca (mol/mol creatinine)								
Day −4	0.04	0.07	0.21	0.19	0.061	0.02	0.49	0.43
Day 0.5	0.03	0.24	0.03	0.05	0.062	0.04	0.007	0.16
Day 2	0.04[b]	0.28[a]	0.04[b]	0.05[b]	0.053	0.07	0.02	0.02
P (mol/mol creatinine)								
Day −4	0.05	0.05	0.07	0.06	0.004	0.008	0.31	0.59
Day 0.5	0.07	0.10	0.05	0.04	0.031	0.23	0.66	0.50
Day 2	0.17	0.28	0.07	0.06	0.099	0.14	0.61	0.55
Mg (mol/mol creatinine)								
Day −4	0.99	1.08	1.10	1.04	0.116	0.74	0.90	0.51
Day 0.5	0.69	0.80	0.59	0.55	0.132	0.21	0.79	0.56
Day 2	0.81	1.01	1.08	0.80	0.127	0.85	0.76	0.08

Abbreviations: Primi = primiparous; Multi = multiparous; C = bolus with 0 μg $1,25(OH)_2D_3$; SGE = bolus with 500 μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *Solanum glaucophyllum*.
[a,b] Values within a row with different superscripts differ (Parity × Bolus, $P < 0.05$).

Table 2
Composition of colostrum and of blood serum of two-day-old calves from primiparous and multiparous dairy cows given, four days prior to calving, a rumen bolus containing 0 or 500 μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *S. glaucophyllum*.

	Group					*P*-value		
Item	Primi-C	Primi-SGE	Multi-C	Multi-SGE	SEM	Parity	Bolus	Parity × Bolus
Colostrum/milk Ca (g/kg)								
Day 1	2.06	1.83	1.73	1.65	0.15	0.11	0.32	0.64
Day 7	1.08	1.06	1.13	1.16	0.03	0.03	0.95	0.42
Day 14	1.01	1.01	1.03	1.02	0.02	0.47	0.74	0.77
Day 21	0.98	0.99	0.95	0.97	0.03	0.39	0.67	0.94
Colostrum/milk P (g/kg)								
Day 1	2.07	1.91	1.83	1.69	0.17	0.17	0.39	0.96
Day 7	1.05	1.10	1.07	1.05	0.03	0.64	0.69	0.26
Day 14	0.96	1.03	0.92	0.95	0.02	0.02	0.04	0.43
Day 21	0.96	0.98	0.83	0.90	0.02	<0.001	0.07	0.46
Colostrum/milk Mg (g/kg)								
Day 1	0.338	0.287	0.300	0.242	0.038	0.11	0.06	0.67
Day 7	0.105	0.103	0.108	0.113	0.003	0.06	0.60	0.24
Day 14	0.098	0.099	0.096	0.094	0.003	0.29	0.81	0.73
Day 21	0.098	0.099	0.089	0.093	0.004	0.06	0.57	0.76
Blood serum of calf								
$1,25(OH)_2D_3$ (pmol/l)	218	177	185	147	36.8	0.40	0.30	0.97
Ca (mmol/l)	2.81[a]	2.66[ab]	2.58[b]	2.71[ab]	0.054	0.10	0.89	0.02
P (mmol/l)	2.34	2.47	2.56	2.02	0.185	0.54	0.28	0.09
Mg (mmol/l)	0.97	0.96	0.98	0.91	0.039	0.51	0.36	0.46

Abbreviations: Primi = primiparous; Multi = multiparous; C = bolus with 0 μg $1,25(OH)_2D_3$; SGE = bolus with 500 μg $1,25(OH)_2D_3$, provided as $1,25(OH)_2D_3$ glycosides from *Solanum glaucophyllum*.
[a,b] Values within a row with different superscripts differ (Parity × Bolus, $P < 0.05$).

them as hypocalcaemic (<2.0 mmol Ca/l). This course of serum blood Ca is typical of multiparous cows around calving as previously observed (Rérat et al., 2009; Rérat and Schlegel, 2014). In addition to the reduced blood serum Ca of Multi-C cows during these days, PTH, $1,25(OH)_2D_3$ and CTx levels increased, but urinary Ca remained low. This indicates that Multi-C cows had to activate the homeostatic Ca regulation by enhanced kidney Ca reabsorption (urinary Ca as indicator), as well as intestinal Ca absorption ($1,25(OH)_2D_3$ as indicator) in an attempt, albeit unsuccessful, to maintain their blood Ca level. Potentially, also bone mineral resorption (CTx as indicator) played a role in this homeostatic regulation. Additional analysis of bone accretion biomarkers, such as osteocalcin, would have been supportive to better interpret the skeletal involvement in maintaining serum Ca levels in Primi and especially in Multi cows.

Considering the difficulties in Multi-C cows to sustain serum Ca, the application of SGE to the Multi-SGE cows was successful in preventing hypocalcaemia by maintaining blood Ca concentration within the reference range of 2.0–3.0 mmol/l. As in Primi-SGE cows, it is assumed that intestinal Ca absorption was enhanced in Multi-SGE cows, independently from PTH, through the increased blood serum $1,25(OH)_2D_3$ profile induced directly by the provided

SGE. As in the present study, Vieira-Neto et al. (2017) observed no increased PTH following a subcutaneous injection of $1,25(OH)_2D_3$ after calving. The similar CTx profile in Multi-C and Multi-SGE suggests that their bone resorption remained comparable. Finally, no serum Ca depression was observed between d4 and d22 in the SGE cows. This suggests that the continuous release of 1,25 $(OH)_2D_3$ glycosides from the bolus, which led, according to Meyer-Binzegger et al. (2021), to increased $1,25(OH)_2D_3$ and Ca levels in dry cows from d0.5 to d5 and from d0.5 to d11, respectively, did not cause any delayed occurrence of hypocalcaemia in the present study, as previously reported after abrupt withdrawal of $1,25(OH)_2D_3$ (Horst et al., 2003).

The blood serum P of the control cows was comparable to profiles previously observed in periparturient dairy cows (Rérat et al., 2009; Rérat and Schlegel, 2014). The increased blood P between d-2 and d2 following SGE application is consistent with the increased levels observed for 10 days (peak over four days) in dry cows following SGE application (Meyer-Binzegger et al., 2021). Although the exact mode of action remains unclear, this suggests that the provided source of the physiologically active form of vitamin D_3 not only regulates Ca, but also P absorption in the intestines (Fukumoto, 2014; Goff, 2018; Oetzel, 2020).

Blood serum Mg of the control cows was also comparable to profiles previously observed around calving (Rérat et al., 2009; Rérat and Schlegel, 2014). Blood Mg was within the considered normal values (0.8–1.2 mmol Mg/l; Martens, 2016) for all cows. According to previous studies in dry cows, blood Mg dropped following SGE application (Meyer-Binzegger et al., 2021) or following the feeding of S. *glaucophyllum* leaves (Ishii et al., 2015). In the present study with periparturient cows, blood Mg did not increase with SGE after parturition as it did slightly in control cows, however to a biologically negligible degree. The possibility of an increased renal Mg reabsorption through modification of its excretion threshold under the influence of PTH was suggested previously (Martin-Tereso and Martens, 2014; Martens, 2016). This process may have occurred in Multi-C cows as PTH concentrations increased in these cows after parturition and as their urinary Mg concentration tended to be lower than in Multi-SGE cows. Further research focused on Mg metabolism would be necessary to discuss the observed effects of SGE.

The unchanged mineral contents in colostrum and milk following a single SGE application are in accordance with Bachmann et al. (2017). The unaltered blood $1,25(OH)_2D_3$ and mineral profiles in the two day-old calves are in accordance with values from newborn calves by Ishii et al. (2015) who fed *S. glaucophyllum* leaves to cows from 14 days prior to expected calving. These are however in contrast to the findings by Roux et al. (1979) who reported increased Ca, P and Mg concentrations in colostrum and an improved Ca status of the newborn calves following the feeding of *S. glaucophyllum* leaves for six days. In the end, in all studies, the blood serum Ca of calves were within the reference range. Thus, the biological significance of this slightly, even if statistically significant, higher value in calves of treated cows remains unclear. At any rate, no detrimental effects for the calves were observed after oral $1,25(OH)_2D_3$ administration to cows shortly before calving.

In conclusion, subclinical hypocalcaemia (<2.0 mmol Ca /l) was not observed in primiparous cows fed a high DCAD diet prior to calving, but it did occur in multiparous cows fed the same diet. The application of one SGE bolus three to four days prior to expected calving was successful in the maintenance of blood Ca concentration within the normal range in multiparous cows over the critical first two days of lactation without any detrimental effects for the cows or their calves. In addition, no delayed occurrence of hypocalcaemia was observed within the first three weeks of lactation. The SGE bolus appears to be a promising new alternative oral solution to prevent hypocalcaemia in multiparous cows and testing its efficacy in a field trial would be helpful in confirming the present detailed physiological results.

Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.animal.2021.100414.

Ethics approval

The experimental procedure was approved by the Office for Food Safety and Veterinary Affairs (2018_26_FR), and all procedures were conducted in accordance with the Swiss Ordinance on Animal Protection and Ordinance on Animal Experimentation.

Data and model availability statement

None of the data was deposited in an official repository. Data that support those study findings are available upon request.

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Declaration of interest

The co-author K. Bühler is affiliated with Herbonis Animal Health GmbH, the company that developed and provided SGE. KB was neither involved in data collection nor in data analysis.

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