High dietary calcium supplementation *ante partum* and effects on calcium and bone homeostasis of dairy sheep and goats and growth response of their offspring

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Interpretive Summary

Many studies focused on deficient Ca supply to small dairy ruminants, yet nothing is known about high dietary Ca during the last days *ante partum*. Here we show that multiparous dairy goats and sheep were able to balance their Ca homeostasis despite the high dietary Ca supply with no consequences for their health. However, we observed that animals fed high dietary Ca showed a gradual increase in serum osteocalcin *post partum* and their suckling offspring showed reduced average daily weight gain. The functional background and practical consequences are yet unknown and may direct future research on the matter.

Abstract

This study investigated the effects of high dietary Ca supplementation during 21 days *ante partum* on Ca and bone homeostasis in dairy sheep and goats, and the growth response of their suckling offspring. Multiparous dairy sheep (n=5/group, 10 animals total) and goats (n=6/group, 12 animals total) were randomly assigned to 2 experimental groups. Feeding occurred restrictively (2.3 kg DM/d animal-1 *ante partum* for both species; 2.9 and 3.1 kg DM/d animal-1 for sheep and goats *post partum*, respectively) according to recommendations with the exception of Ca during *ante partum*, where one group received a high Ca diet with CaCO3 (1.3% in DM; 2.49fold recommendations) based on hay and concentrate 3 weeks *ante partum*, and the other group received no extra Ca on top (0.6% in DM; 1.15fold recommendations). Experimental feeding ended with parturition and animals were henceforth fed according to Swiss recommendations for lactating sheep and goats and kept together with their suckling offspring. The observation period spanned from 21 days *ante partum* to 56 days *post partum*. Animals were subject to continuous veterinary surveillance and monitored for signs of milk fever. Data collection comprised quantitative and functional parameters of Ca and bone homeostasis as well as birth weights and daily weight gain of suckling lambs and kids. Data was analyzed by repeated measures and endpoint mixed models. The results of quantitative markers indicated that high dietary Ca *ante partum* significantly increased fecal Ca concentrations until parturition, suggesting efficient physiological mechanisms to manage Ca overload by increasing fecal excretion. No significant differences were observed in serum Ca levels, urinary Ca excretion, or bone mineral density between *ante partum* Ca feeding groups at any point in time of observation, indicating stable Ca homeostasis despite the dietary challenge. Species-specific differences in quantitative markers were noted including serum and colostral Ca levels as well as bone mineral density and mostly reflected results from earlier comparative studies. Serum calcitriol as well as markers of bone formation and resorption were

monitored, revealing significant increases in serum osteocalcin *post partum* in goats and sheep fed high Ca *ante partum*. All other serum markers including calcitriol were not affected by the feeding regime but again by the species of animal, in line with previous works. All repeated measures were significantly affected by time except for urinary Ca and bone specific alkaline phosphatase activity in serum. Offspring of sheep and goats in the high Ca group exhibited significantly reduced average daily weight gain compared to the 0.6 % in DM Ca group, despite similar birth weights. We conclude that while dairy sheep and goats effectively managed high dietary Ca intake without overt signs of hypocalcemia or milk fever under the present experimental conditions, there were notable impacts on offspring growth and potential long-term physiological effects that require further research. These findings contribute to the understanding of mineral nutrition in late gestating dairy goats and sheep and stress the need for further research on balanced dietary strategies to optimize health and productivity in lactating mothers and their offspring.

Key words: Transition phase, Calcium Homeostasis, Small Ruminant, Maternal Nutrition

Nonstandard abbreviations: bAP = bone-specific alkaline phosphatase activity; BMD = bone mineral density; calcitriol = 1,25-$(OH)_2$ vitamin D; CTX = crosslaps; CpG = cytosine linked to guanine in 5'→3' direction of a DNA molecule; ICTP = crosslinked carboxyterminal teleopeptide of type I collagen; qCT = quantitative computer tomography; SAS = statistical analysis software; VDR = vitamin D receptor

Introduction

To avoid a drop in circulatory Ca, the organism has 3 options to stabilize the system, by increasing absorption capacity at the gut barrier, limiting endogenous losses via the gastrointestinal tract and kidneys, and mobilizing Ca from the skeleton (Wilkens and Muscher-Banse, 2020). With respect to the regulation of Ca homeostasis, the animal species under study is an important factor to consider. For example, sheep and goats have been demonstrated to respond quite differently to Ca-restricted feeding with the first tending quicker to bone mobilization while the latter will first attempt to compensate via more efficient absorption from the small intestinal mucosa (Wilkens et al., 2012a). A relevant regulation of renal Ca excretion and reabsorption in response to changes in the endogenous Ca status has been reported for mice (Hoenderop et al., 2002, van Cromphaut et al., 2003, Ko et al., 2009), but was not confirmed in ruminants including sheep, goats (Herm et al., 2015) and cows (Wild et al., 2021). Quantitative adaption to fluctuations in the endogenous Ca status are regulated by a complex system of endocrine factors, that control the regulation of absorption, excretion and bone remodeling, the detailed discussion of which exceeds the capacities of this introduction (see the reviews by Martín-Tereso and Martens (2014) and Wilkens and Muscher-Banse (2020)).

In lactating dairy ruminants, a proper endogenous Ca status is especially important since milk supplies high levels of Ca to the offspring on a daily basis and the high yields and associated Ca losses with milk of modern genotypes disproportionately increase Ca demand compared to the mere needs of the offspring, which do not differ much shortly before and after parturition (Hernández-Castellano et al., 2020). In this context, the last weeks *ante partum* are critical. In high-yielding dairy cows, the sudden onset of lactation after birth in combination with impaired feed intake around parturition puts animals in danger of developing milk fever if the mobilization of bone stores lacks capacity to bridge this gap in dietary intake. This is typically accompanied by hypocalcaemia, mostly also by hypophosphatemia and often hypomagnesemia

(Liesegang et al., 2007, Goff, 2008, Martín-Tereso and Martens, 2014). Symptoms develop within 48 h *postpartum* and may include listlessness, coma and sternal recumbence. The situation for sheep and goats is different. Due to their lower milk yield, meat sheep breeds develop milk fever rarely *post partum* though a higher tendency for carrying twin or even triplet fetuses may increase the risk of developing a hypocalcaemia during the last trimester given an increasing fetal Ca requirement during the last 4 weeks *ante partum* due to the calcification of the fetal skeleton. Sheep typically show preceding anorexia and stiff gait before sternal recumbence. Dairy sheep breeds and most goat breeds combine a high milk yield potential with a high probability for carrying multiple fetuses, consequently clinical events of hypocalcemia *ante* and *postpartum* are likely. High-yielding dairy goats and sheep develop a milk fever comparable to dairy cows but much later at 1-3 weeks postpartum. If prepartum symptoms arise they are less pronounced than the postpartum complex (Oetzel, 1988).

Dietary and metabolic factors that affect the incidence of milk fever are various and the underlying literature is dominated by studies in cows. Amongst relevant factors are the DCAD, Mg, P, and Ca in the *ante partum* diet (Lean et al., 2006). There has been some debate regarding the proper dietary Ca concentration *ante partum*, with some sources suggesting that diet Ca at 1-1.5% in DM or even 2-3% does not affect the incidence of milk fever whereas others recommend going below 1%. An earlier meta-analysis on the matter attempted to bring some order in the chaos. The authors developed two models using data from 51 trials, updating the findings of an earlier meta-analysis by Oetzel (1991). They concluded that dietary calcium exhibits a non-linear relationship with milk fever incidence, with both very low and very high dosages showing protective effects. Model I included *ante partum* DCAD, as well as diet Ca, P, Mg, and diet exposure time as variables while Model II included *ante partum* dietary K and S in replacement of DCAD. Both models confirmed a quadratic effect of dietary Ca on the pathogenesis of milk fever (odds ratios 0.131 and 0.115, respectively). Simulations with Model

I based on dietary Ca varying from 0 to 2.5 % in DM and assuming all other variables remain stable at levels associated with low milk fever risk, yielded a bell curve of predicted milk fever incidence (%) over varying diet Ca with a peak of ~+3.8% incidence increase at 1.35 % in DM dietary Ca. Based on this simulation it has been suggested that dietary Ca concentrations between 1.1-1.5% in DM should be avoided, whereas higher or lower levels gradually reduce the incidence of milk fever (Lean et al., 2006). These findings would explain the aforementioned inconsistency between available studies on the interaction of dietary Ca dosage and milk fever incidence. This raises the interesting question about the situation in other livestock ruminants.

Studies on high dietary calcium (Ca) levels in gestating sheep and goats are limited, and specific guidelines for managing dietary Ca to prevent milk fever in these species do not exist, particularly in Switzerland. Our experimental design was informed by Lean et al. (2006), who found that in cows, dietary Ca levels between 1.1-1.5% in DM (vs. 0.6% in DM) in *ante partum* feed could increase milk fever incidence by up to 3.8%. This study aimed to evaluate the effects of high dietary Ca in dairy sheep and goats during the last 21 days *ante partum* on Ca homeostasis and bone metabolism and to provide initial data on potential risks extending into lactation. Given the naturally high environmental Ca in alpine regions, including Switzerland, where average Ca concentrations in forage can reach 7 g/kg DM (Schlegel et al., 2016), oversupply with dietary Ca is not an unlikely event. Therefore, gestating sheep and goats from our in-house dairy herd were fed diets containing either 0.6% or 1.3% Ca in DM during the final 3 weeks *ante partum*, providing 1.15 and 2.54 times the daily Ca recommendations (Agroscope, 2017). These levels represented a lower Ca intake (<1.1% in DM) to potentially reduce milk fever risk and a higher intake approximating the hypocalcemia risk peak noted by Lean et al. (2006). At the same time, it avoided dietary Ca deficiency in all animals, which would have not benefitted our research. Observations continued until 56 days *post partum*,

during which animals received standard lactation diets with recommended levels of Ca according to current Swiss recommendations (Agroscope, 2017). We hypothesized that goats and sheep fed the higher Ca diet *ante partum* (equivalent to 30.5 g/day) would show signs of hypocalcemia at some point during the observation period, unlike those on the lower Ca diet. This design also allowed for comparing responses in mineral and bone metabolism to dietary Ca overload in 2 species with distinct homeostatic strategies of dealing with variations in Ca supply (Wilkens et al., 2012b).

Material and Methods

Animals and Housing

This study was conducted at the research and laboratory facilities of the Institute of Animal Nutrition and Dietetics, Vetsuisse-Faculty, University of Zurich, Switzerland. Ten and 12 multiparous gestating dairy sheep and goats (East Friesian Dairy Sheep, Saanen goats), respectively, aged between 4 and 6 years were housed in individual pens with concrete floor and wood shavings as bedding material, from 4 weeks before the estimated parturition date. The imbalance in animal numbers between species was due to miscarriages of 2 sheep before the onset of the study. Both species were housed in rooms separate from each other. The goats and sheep were housed individually until their delivery date, afterwards with their respective lambs and kids in lambing/kidding pens. The average body weight just before birth of goats and sheep was 95.2 ± 1.5 kg and 101 ± 2.22 kg, respectively, and 78.3 ± 2.22 kg and 81.0 ± 2.31 kg directly after birth, respectively.

Diets and Experimental design

Animals were randomly divided in 2 experimental groups according to their ear tag numbers by a random sequence generator (https://www.random.org/sequences/). The effective sample size for sheep and goats was 5 and 6 per group, respectively. The feeding trial started 3 weeks before the estimated day of parturition. The diets were adapted to the specific energy and nutrient requirements during gestation and lactation for sheep and goats (Agroscope, 2017). The 0.6 % in DM Ca groups received a balanced diet provided by grass hay (2nd cut) and a commercial high energy concentrate (UFA 765, Herzogenbuchsee, Switzerland). Additionally, for the 1.3 % in DM Ca groups until parturition, the same concentrate was applied but mixed up daily with 2.5 times (16.8 g Ca /day) the recommended daily Ca supply from $CaCO_3$ (Table 1). From parturition onwards, all animals received lactation feeding to meet current feeding recommendations which accounted for 17.6 and 19.0 g Ca per day for sheep and goats,

respectively (Table 1) (Agroscope, 2017). Throughout the study, animals were fed twice per day restrictive amounts of hay (2 kg/animal*d^{-1} ante partum and 2.4 kg/animal*d^{-1} post partum for both species) and concentrate (0.3 kg/animal*d^{-1} ante partum for both species and 0.50 and 0.70 kg/animal*d^{-1} post partum for sheep and goats, respectively) adapted to recommendations for *ante* and *post partum* feeding (with the exception of Ca *ante partum*), and had *ad libitum* access to drinking water (Table 1). The restrictive feeding approach allowed for better feed intake control during single housing of animals. The composition of the commercial concentrate (UFA 765) as communicated by the manufacturer UFA (Union des Fédération Agricoles), is shown in Supplementary Table 1.

Colostrum collection and determination of live weight and growth performance

First milk after parturition was stripped completely from each animal, which ranged between 0.5 – 1.2 L $animal^{-1}$ in both species. Immediately after collection, colostrum was distributed equally over nursing bottles and supplied to the neonates.

Sheep and goats were weighed before morning feeding the day before and directly after parturition. Due to the suckling of the offspring, daily milk yield could not be assessed. The lambs and kids were weighed directly after parturition and once between 33-59 days *post partum*, to determine the average daily weight gain during the suckling phase. The variation in the time frame between first and second weighing was due to the fact that lambs and kids were weaned at different ages to be subject to a different research purpose. To account for this variation in observational periods, we included the time interval between the first weighing at birth and the second weighing (the age of each lamb and kid at second weighing) as a continuous variable in the statistical analysis of weight gain. This approach controls for potential age effects that could bias the estimation of treatment effects (see the subsection on statistical analysis below).

Sample collection and handling

For this experiment, a closed batch of grass hay (2nd cut) and concentrates was ordered and 3 representative samples each were taken according to published standard methods 1.3 ("Regulations of the German Agricultural Society on the Sampling of Feed and Sample Treatment") and 1.5 ("Sampling of Hay") (VDLUFA, 2012) in order to investigate the feed value by chemical analysis. Feed samples were directly subject to chemical analysis after sample collection.

Blood was collected in daily frequency during 28 days before estimated parturition and on days 1, 2, 3, 4, 5, 6, 7, 14, 28, and 56 *post partum*, in any case before morning feeding at 8 a.m. in serum monovettes (9 and 6 mL, Vacuette®, Greiner Bio-One Vacuette Schweiz GmbH) and subject to serum collection (1580 x g, 10 min) after allowing coagulation at room temperature. The serum was stored at -80°C until further usage. After the actual parturition, samples of days -21, -4, -3, -2, -1, 0 (parturition), 1, 2, 3, 4, 5, 6, 7, 14, 28, and 56 were reserved for further analysis.

Urine was collected in a beaker, in parallel to the blood sampling *ante partum* and on days 7 and 14 *post partum*, and homogenized to be separated in two equal subsamples. One of the subsamples was acidified (12 mol/L HCl, pH 1-2) for later Ca determination the other was not for the later determination of creatinine. Both subsamples were stored at -20°C. After parturition, samples of days -21, -14, -7, 0, 7, and 14 were reserved for further usage. Fecal grab samples of each animal were collected in parallel to blood sampling *ante partum* and days -21, -2, -1, and 0 were subject to further analysis. Samples were stored at -20°C until further usage.

All goats were subject to colonic mucosal biopsies by veterinary personal on days -21, -7, 0, and 7. An endoscope (PCF-20, Olympus) equipped with biopsy forceps was inserted 40 cm into the large intestine via the anus to collect 4 samples (1.5-2.0 mm diameter) per animal*time. Two of said samples were immediately put into 4% neutral buffered formalin and the remaining 2 samples were snap frozen in liquid nitrogen and stored at -80°C.

Sample analysis

Feed was analyzed according to VDLUFA (2012) with the following methods. For DM according to Method 3.1 ("Moisture, Water") by oven drying at 105°C for 4 h. Net energy for lactation (Method 25.1 "Determination of Gas Production according to the Hohenheim Gas Test") was assessed by quantification of cumulative gas production (CGP) from rumen fluid over 24h at 39°C and regression estimation of NEL considering CGP and analyzed crude nutrients and fiber fractions (Society of Nutrition Physiology (GfE), 2008a, b). Crude Protein determination (Method 4.1.1 "Determination of Crude Protein") involved the Kjeldahl method of H_2SO_4 extraction in the presence of a catalyst, alkalization with NaOH, titration of NH_3) and multiplying the assessed nitrogen concentration with a factor of 6.25. Starch and ash corrected neutral detergent fiber (aNDFom) (Method 6.5.1 "Determination of Neutral-Detergent-Fiber after amylase digestion (aNDF) as well as after amylase digestion and ashing (aNDFom)") involved treatment with heat-stable amylase for 1h, subsequent cooking in neutral detergent solution, and subsequent ashing of the solid residue). Ash-corrected acid detergent fiber (ADFom) (Method 6.5.2 "Determination of acid-detergent fiber (ADF) and of acid detergent fiber after ashing (ADFom)") was done by 1h cooking in acid detergent solution and ashing of the solid residue. Acid Detergent Lignin (Method 6.5.3 "Determination of Acid Detergent Lignin (ADL)" was quantified by incubating the starch-corrected ADF residue for 3h at RT in 72% H_2SO_4 and subsequent ashing of the residue. The determination of the EE (Method 5.1.2 "Determination of Crude Fat") involved the pre-extraction in hot HCl and subsequent extraction of the dry residue in petro ether. Crude ash (Method 8.1 "Determination of Crude Ash") was measured by dry ashing at 550 °C for 4 h and HCl-insoluble ash (Method 8.2 "Determination of Hydrochloric Acid-insoluble Ash") added a treatment of the crude ash residue with boiling HCl. The dietary mineral profile (Ca, P, Mg) (Method 10 "Determination of selected elements in plant material and feedstuffs with ICP-OES") involved the sample ashing in a furnace,

extraction with HCl with a deviation from the protocol by measuring solution not via ICP-OES but by flame atomic absorption spectrometry (flame-AAS) (ContrAA 700, Analytik Jena).

Blood serum was analyzed for Ca, osteocalcin, 1,25-$(OH)_2$ vitamin D (calcitriol), bone-specific alkaline phosphatase (bAP), crosslinked carboxyterminal teleopeptide of type I collagen (ICTP), and crosslaps (CTX).

In brief, serum Ca was measured via flame-AAS after microwave wet digestion of 2 mL serum per technical replicate in 65% HNO_3 and 30% H_2O_2 (Ethos, MLS GmbH) against a dilution gradient (0.2, 0.4, 0.6, 0.8, 1.0 mg/L) of a commercial AAS-certified standard (ROTI®Star 1 000 mg/l Ca, in 2 % HNO_3; Roth).

Serum osteocalcin was quantified with a commercial EIA test kit (MikroVue Osteocalcin EIA Kit; Quidel Corporation). Intra- and interassay CVs were 5.00% and 5.75% with a sensitivity of 0.45 ng/mL. Serum calcitriol determination was carried out with a RIA kit (1,25VitD RIA, sheep-anti 1,25VitD antibody; Immunodiagnostics Systems GmbH). 9.0, 9.3 <8pmol/L Intra- and interassay CVs were 9.0% and 9.3% with a sensitivity a 8 pmol/L. Serum bAP was analyzed with an immunoassay via a monoclonal anti-bAP and para-nitrophenylphosphate as a substrate (Alkphase-BTM™; Metra Biosystems). Intra- and interassay CVs were 0.58 and 0.52.

Serum ICTP was measured by a RIA immunoassay (UniQ® ICTP RIA, DiaSource) with an intra- and interassay CV of 5.6 and 10.0 and a sensitivity of 0.6 µg/L. Serum CTX was assayed by a commercial ELISA assay (Serum CrossLaps (CTX-I) ELISA; Immunodiagnostics Systems GmbH) with intra- and interassay CVs of 1.7% and 2.5% at a sensitivity of 0.02 ng/mL.

For CTX, ICTP, and calcitriol, data gaps at -4 days and for CTX additionally for -21 days, as depicted in Figures 5 and 6, respectively, were due to compromised sub-samples. All other sampling decisions were intentional, based on a balance between data relevance, workload, and the cost constraints of a study that lacked direct funding.

Dried Feces (105°C, 4 h) was milled (0.5 mm) and 500 mg per technical replicate were subject to Ca analyzes as described for serum above.

Colostrum was defatted by centrifugation (4000 x g, 4°C, 30 min), and subject to the same procedures of sample preparation and Ca analyzes as described for feces.

Urine was analyzed for total Ca with the method described for serum and further analyzed for creatinine by a kinetic color test using a commercial kit (Crea Jaffe DIA00540, Diatools AG) on the Cobas Mira autoanalyzer with an intra- and interassay CV of 0.83% and 0.85% at a sensitivity of 18 µmol/L. The urinary Ca excretion was expressed as ratio to urinary creatinine since the material could not be sampled quantitatively. Creatinine is generally excreted at a relatively constant rate, making it a useful reference compound to normalize mineral concentrations for differences in urine dilution caused by hydration status, collection timing or other individual factors (Sullivan et al., 2012).

Goat colonic mucosal biopsies were subject to semi-quantitative immunohistochemical determination of vitamin D receptor (VDR) abundance as originally described by Liesegang et al. (2008) and Sidler-Lauff et al. (2010). Caprine colonic VDR was labelled after rinsing the tissue with TBS buffer and incubation for 80 min with a biotinylated rat monoclonal antibody (9A7γ, ThermoScientific) at a 1:200 dilution in Tris-buffered saline against unspiked Tris-buffered saline on an adjacent colonic section as negative control and duodenal cross sections of pigs (obtained from the in-house slaughtering facility of the Vetsuisse-Faculty, University of Zurich) (aa 89-105) as positive control based on earlier reported identical cross-reactivity (Milde et al., 1989). For sheep, no suitable antibody was available at the time of study.

Nuclear immunoreactivity was assessed with a binocular microscope (DMLB®, Leica) with a digital camera (DC480, Leica) (magnification 400x, 5-7 microscopic fields). For each sample, 500 cells of the following cell types were evaluated for nuclear staining intensities: surface epithelial cells, intermediate glandular/crypt epithelial cells, superficial glandular/crypt

epithelial cells, basal glandular/crypt epithelial cells. Staining intensities of nuclei were scored 0, 0.5, 1, 2, 3 for negative, very weak, weak, intermediate, and strong, which is correlated to the absence of brown staining (only blue counter staining), light brown, brown, and dark brown, respectively. From this an immune reactivity score was calculated with the following formula:

$$Immune\ Reactivity = 0 \times n(negative) + 0.25 \times n(very\ weak) + 1 \times n(weak) + 4 \times n(intermediate) + 9 \times n(strong)$$

Where n is the number of cells within 500 cells expressing a respective staining intensity, with a range of calculated immune reactivity scores between 0 (no specific staining) to 4500 (all 500 cells with strong staining).

Bone mineral density

Bone mineral density (BMD) was measured with peripheral quantitative computer tomography (Stratec XCT 2000 bone scanner, Stratec Medizinaltechnik GmbH) in the middle of the left metatarsus after length determination with a caliper. Measurements were taken in the mid diaphysis and cortical BMD was automatically calculated (cortical mode 2, >640 mg/cm^3 cortical bone threshold).

Statistical analysis

The individual animal was the smallest experimental unit. All data analysis was performed with SAS 9.4 (SAS Institute Inc.) applying the procedure MIXED. Repeated measures mixed models were applied to analyze the effects of the fixed variables “diet Ca”, “species”, “time”, and interactions considering the individual animal nested under the respective feeding group as a

random factor. Repeated measures mixed modelling involved in any case the mapping of covariance structures appropriate for the respective data structure following guidance by Wang and Goonewardene (2004). Due to the uneven spacing of times of observation for respective datasets, covariance structures like first-order autoregressive (AR(1)) and related structures (e.g. Toeplitz), which require an equal spacing of times, were not considered. The Bayesian Information Criterion (BIC) served as model fit statistic and we chose the candidate yielding the lowest BIC. The respectively mapped covariance structure for different repeated measures datasets were as follows: compound symmetry (serum Ca, urinary Ca), heterogeneous compound symmetry (BMD), first-order ante-dependent (serum bAP, serum ICTP, serum CTX, serum calcitriol, serum osteocalcin, fecal Ca). Due to the limited availability of suitable antibodies, the VDR protein expression over time was only analyzed in colonic biopsies of goats, therefore no species effect could be estimated.

The colostral Ca concentration of sheep and goats was analyzed with an endpoint mixed model with the fixed effect of “diet Ca”, “species”, and interaction (Ca*S), again including the individual animal nested under the respective feeding group as random effect. Birth weights and daily weight gain of the offspring were also analyzed with an endpoint mixed model accounting for the fixed effects of “diet Ca”, “species”, “sex” and interactions (Ca*S, Ca*sex) including the individual animal nested under respective mother as random effect. For the analysis of average daily weight gain, the animals’ age at the second weighing was included as a continuous control variable to account for potential age-related bias in estimating average daily weight gain. In case the residuals of respective mixed models missed the precondition of normality, the analysis was repeated after Log_{10}-transformation of data and displayed as such. This applied to the following datasets: fecal Ca, urinary Ca, serum bAP, serum ICTP, serum CTX. Data was presented in any case as least square mean values with Tukey-adjusted 95% confidence limits. $P \leq 0.05$ was defined as threshold for a TYPE I statistical error.

Earlier datasets were used for prospective power analyses during study planning. Therefore, effect sizes of *ante* and *post partum adaption* of assessed serum parameters were determined with the respective datasets of Liesegang et al. (2007). Effects size of VDR immune reactivity in the goat colon to dietary Ca variation were determined according to the respective dataset of Sidler-Lauff et al. (2010). Bone density effect sizes of their fluctuation from gestation to lactation of milk goats and sheep were estimated with the respective data of Liesegang et al. (2007). Estimation of effects sizes and minimum number of replicates to determine these statistically significant with a minimum statistical power of $1\text{-}\beta = 0.8$ at $\alpha = 0.05$ (McDonald, 2014) occurred with R-4.4.2 (The R Project for Statistical Computing) applying the SIMR package according to Green and MacLeod (2016), with the exception of using the aforementioned earlier published instead of simulated datasets. Based on these estimations, the anticipated minimum power of ≥ 0.8 was reached in any case assuming 6 replicates per feeding group, applying the models described above.

Results

Animal health and development

All sheep and goats remained healthy throughout the entire study period with no signs of pathology and hypocalcemia in particular, based on continuous veterinary surveillance. Due to miscarriage before the study, only 10 instead of anticipated 12 sheep were available. Sheep showed 5 cases of triplets, 4 cases of twins and 1 single birth (n = 24 lambs in total) whereas the majority of goats gave birth to twins with 1 case of triplets and 2 cases of single births (n = 23 kids in total). The sex ratio of sheep and goat offspring was (m/f) 0.71 and 1.30, respectively. All lambs and kids were born with species-dependent birth weights (4.62 Cl[4.22, 5.01] vs. 5.15 Cl[4.80, 5.51] kg for kits vs lambs; P = 0.06) irrespective of sex (4.71 Cl[4.35, 5.07] vs. 5.06 Cl[4.71, 5.41] kg for female vs. male; P = 0.16) and unaffected by the mothers' dietary Ca supply *ante partum* (4.89 Cl[4.53, 5.23] vs. 4.88 Cl[4.54, 5.22] kg at 0.6% vs. 1.3% in DM, respectively; P = 0.94) (Figure 1A).

However, lambs and kids of mothers fed high Ca *ante partum* showed significantly lower daily weight gain from milk intake than animals fed low Ca (0.18 Cl[0.17, 0.20] vs. 0.22 Cl[0.20, 0.24] kg/d at 1.3% vs. 0.6% in DM, respectively; P = 0.02) irrespective of sex and species, which expressed distinct effects on the animals' growth performance (0.19 Cl[0.17, 0.21 vs. 0.21 Cl[0.19, 0.23] kg/d for female vs. male; P = 0.06; 0.25 [0.23, 0.27] vs. 0.15 [0.13, 0.17] kg/d for kids vs. lambs; P < 0.0001) (Figure 1B).

Supplementary Table 2 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of birth weights and daily weight gain of offspring as related to species, sex, and dietary Ca supply of the mothers *ante partum*.

Quantitative markers of calcium metabolism

Analyzed fecal Ca concentrations during the observation period ranged between 1.46 and 5.19 % in DM in sheep as well as 1.17 and 14.9 % in DM in goats whereas the ranges for urinary Ca excretion were 0.005 and 0.41 mmol/mmol creatinine in sheep as well as 0.004 and 1.55 mmol/mmol creatinine in goats. Fecal and urinary data had to be $\log_{10}$-transformed to meet the precondition of normality, accounting for fecal Ca ranges of 0.16 and 0.71 $\log_{10}$(% in DM) for sheep as well as 0.07 and 1.17 $\log_{10}$(% in DM) for goats. The respective log-transformed urinary Ca excretion ranges were -2.31 and -0.38 $\log_{10}$(mmol/mmol creatinine) in sheep as well as -2.42 and -0.19 $\log_{10}$(mmol/mmol creatinine) in goats.

Higher supply with dietary Ca *ante partum* significantly increased fecal $\log_{10}$-Ca concentrations until parturition by 1.43fold vs. 2.87fold in sheep vs. goats and in any case significantly above the control level (0.21 Cl[0.07, 0.34] vs. 0.62 [0.48, 0.75] and 0.35 Cl[0.22, 0.48] vs. 0.66 Cl[0.53, 0.79] $\log_{10}$(% in fecal DM) in 0.6% vs. 1.3% in DM of sheep and goats, respectively; $P < 0.0001$). On average, sheep showed lower fecal Ca than goats (0.41 Cl[0.36, 0.46] vs. 0.47 Cl[0.43, 0.52] log10(% in fecal DM) for sheep vs. goats; $P = 0.05$) but the response patterns over time were different. Sheep in the low Ca group slightly declined fecal Ca until parturition to 0.64fold of the level at -21 days *ante partum*, while sheep in the in the high Ca group responded opposite to this as mentioned before (1.43fold increase until parturition). In contrast, both control and high Ca goats increased the levels until parturition but in different magnitudes (1.75fold vs. 2.87fold for low vs. high Ca). This difference in time patterns between species was reflected by a strong statistical interaction between species and time ($P < 0.0001$) (Figure 2A).

In contrast, the feeding regime had no effect on the urinary Ca excretion (-1.04 Cl[-1.22, -0.86] vs. -0.96 Cl[-1.14, -0.79] log10(mmol/mmol creatinine) at 0.6% vs. 1.3% in DM; $P = 0.56$) (Figure 2B). Values fluctuated over time but a clear trend in adaption of urinary losses was not evident ($P = 0.61$). The only significant effect was in comparison between sheep and goats irrespective of treatment or time, with goats excreting on average 3.26fold the levels of sheep

(-1.26 Cl[-1.44, -1.07]vs. -0.75 Cl[-0.91, -0.58] $\log_{10}$(mmol/mmol creatinine) in sheep vs. goats; P = 0.0003).

Supplementary Table 3 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of fecal Ca concentrations and urinary Ca excretion, expressed in the original measurement scale as related to species, time, and dietary Ca supply *ante partum*.

Figure 3 shows the response of serum Ca and colostral Ca to varying Ca feeding *ante partum*. Serum Ca was not affected by the *ante partum* feeding (2.47 Cl[2.35, 2.59] vs. 2.49 Cl[2.37, 2.61] mmol/L at 0.6% vs. 1.3% in DM; P = 0.80) and gradually and significantly (P < 0.0001) decreased independent of animal species towards the first 4 days *post partum* to gradually increase to previous levels during the further course of the observation period (Figure 3A). In any case, sheep showed on average 1.16fold higher serum Ca levels than goats (2.29 Cl[2.17, 2.40] vs. 2.67 Cl[2.54, 2.79] mmol/L in goats vs. sheep; P = 0.0003).

The colostral Ca concentration (Figure 3B) was 2.35fold higher in sheep than in goats (1.44 Cl[0.69, 2.19] vs. 3.38 Cl[2.56, 4.20] g/L in goats vs. sheep; P = 0.002) but was not affected by the *ante partum* Ca feeding regime (2.36 Cl[1.58, 3.14] vs. 2.46 Cl[1.67, 3.24] g/L at 0.6% vs 1.3% Ca in DM; P = 0.86) (Figure 3B).

Supplementary Table 4 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of serum Ca and colostral Ca as related to species, time, and dietary Ca supply *ante partum*.

Figure 4 highlights the BMD of dairy sheep and dairy goats from -14 day *ante partum* to 56 days *post partum*, as affected by Ca feeding during the last 3 weeks *ante partum*, time and animal species. Higher Ca feeding *ante partum* did not affect bone mineral density (868 Cl[845,

891] vs. 877 Cl[854, 900] mg/cm^3 at 0.6% vs. 1.3% in DM; P = 0.63), which significantly declined towards parturition from 932 Cl[904, 960] to 864 Cl[807, 922] mg/cm^3 in sheep and 845 Cl[817, 873] to 782 Cl[738, 826] mg/cm^3 in goats, to gradually increase *post partum* until day 56 of lactation where it roughly approached in both species the initial level at the beginning of the observation (Sheep: 944 Cl[920, 967] mg/cm^3, Goats: 846 Cl[823, 868] mg/cm^3 P < 0.0001). On average, sheep showed 1.08fold higher bone density than goats (839 Cl[808, 853] vs. 913 Cl[888, 938] mg/cm^3 in goats vs. sheep; P < 0.0001).

Supplementary Table 5 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of BMD as related to species, time, and dietary Ca supply *ante partum*.

<u>Serum calcitriol and markers of bone formation</u>

Serum calcitriol, bAP and osteocalcin of sheep and goats over time in response to varying Ca feeding *ante partum* are shown in Figure 5.

Serum calcitriol was 1.98fold higher in goats than in sheep (70.2 Cl[56.8, 83.6] vs. 139 Cl[128, 149] pmol/L in sheep vs. goats; P < 0.0001) but not different between *ante partum* feeding groups (103 Cl[90.1, 117] vs. 105 Cl[92.3, 119] pmol/L at 0.6% vs. 1.3% in DM; P = 0.75) (Figure 5A). Serum levels significantly changed over time (P < 0.0001) which interacted with species (P < 0.0001) as follows. In sheep, serum levels reduced towards parturition and fluctuated throughout, whereas in goats serum levels increased until 4 days *post partum* to turnaround and approach initial levels until 56 days *post partum*.

Serum activity of bAP ranged between 38 and 251 U/L in sheep as well as 21 and 117 U/L in goats. After Log_{10}-transformation to meet the precondition of normality, these ranges were 1.58 and 2.40 Log_{10}(U/L) in sheep as well as 1.32 and 2.07 Log_{10}(U/L) in goats. Feeding group (1.76 Cl[1.67, 1.85] vs. 1.80 Cl[1.70, 1.90] log_{10}(U/L) at 0.6% vs. 1.3% in DM; P = 0.59) expressed no significant effect on bAP (Figure 5B). This was also true for time, though control goats

showed a temporal numerical drop by ~22% of serum activity around parturition (P = 0.12). Sheep showed on average 1.25fold higher serum activities than goats (1.58 Cl[1.49,, 1.68] vs. 1.97 Cl[1.888, 2.07] $\log_{10}$(U/L) in goats vs. sheep; P < 0.0001).

Serum osteocalcin remained rather stable and comparable between species from -5 days *ante partum* to 5 days and 7 days *post partum*, respectively, after which it gradually increased on average by 2.27fold in sheep and 3.23fold in goats until 56 days *post partum* (P < 0.0001). This difference in magnitude was significant between species (P = 0.007) (Figure 5C). Most notably, this increase in serum osteocalcin was significantly higher in those sheep and especially goats who received higher dietary Ca during the last 3 weeks *ante partum* (29.9 Cl[5.62, 54.1] vs. 39.6 Cl[15.3, 63.8] and 42.2 Cl[17.8, 66.6] vs. 80.5 [58.4, 103] mmol/L at 0.6% vs. 1.3% in DM in sheep and goats, respectively; P = 0.02).

Supplementary Table 6 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of serum calcitriol, bAP, and osteocalcin, expressed in the original measurement scale also in the case of bAP, as related to species, time, and dietary Ca supply *ante partum*.

<u>Serum markers of bone resorption</u>

Serum concentrations of ICTP ranged between 4.87 and 90.2 µg/L in sheep as well as 5.75 and 57.6 µg/L in goats, while CTX ranged 0.50 and 10.3 ng/mL in sheep as well as 0.35 and 8.72 ng/mL in goats. After Log_{10}-transformation to meet the precondition of normality, these ranges were for ICTP 0.69 and 1.95 $\log_{10}$(µg/L) as well as -0.30 and 1.01 $\log_{10}$(µg/L) and for CTX 0.76 and 1.76 $\log_{10}$(ng/mL) as well as -0.46 and 0.94 $\log_{10}$(ng/mL).

Serum ICTP showed no marked changes in both species until parturition, afterwards it significantly increased over the first 2 days post partum (1.49fold vs. 1.60fold in sheep vs. goats) and held a plateau until 7 days postpartum from which on the levels gradually reduced over the following weeks to approach initial values at 56 days *post partum* (P < 0.0001) (Figure

6A). Average serum levels were significantly higher for sheep than goats (1.19 Cl[1.14, 1.23] vs. 1.30 Cl[1.26, 1.35] $\log_{10}$(µg/L) in goats vs. sheep; $P < 0.0001$) with a significant interaction between species and time reflecting fluctuations in differences between species over given times ($P = 0.001$). The feeding *ante partum* did not express any significant effect on serum ICTP levels of dairy sheep and goats (1.24 Cl[1.20, 1.29] vs. 1.25 [1.20, 1.29] $\log_{10}$(µg/L) at 1.3% vs. 0.6% in DM; $P = 0.98$).

Sheep showed markedly higher serum CTX levels during this study than goats ($P < 0.0001$) (Figure 6 B). In sheep serum levels significantly increased from -3 days *ante partum* until 2 days post partum and hold a plateau until day 7 post partum with a subsequent decline during the following weeks approaching initial values at day 57 post partum. In contrast goats showed not much response until parturition after which it peaked at 2 days post partum and fluctuated around that plateau for the next 14 days to decline until day 56 with the high Ca group showing the steepest decline. These responses over time were significant ($P < 0.0001$) but did not statistically interact with other variables like diet Ca or species. Neither on average (0.35 Cl[0.27, 0.43] vs. 0.38 [0.30, 0.46] $\log_{10}$(µg/L) at 1.3% vs. 0.6% in DM; $P = 0.59$) nor over time (Diet Ca*Time: $P = 0.64$) did the feeding *ante partum* affect serum CTX levels in a significant manner.

Supplementary Table 7 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of serum ICTP and CTX, expressed in the original measurement scale, as related to species, time, and dietary Ca supply *ante partum*.

<u>Caprine colonic abundance of mucosal vitamin D receptor</u>

Figure 7 highlights the statistical evaluation of VDR immune reactivity in caprine colonic mucosa in goats fed 0.6% in DM and 1.3% in DM dietary Ca *ante partum*. Figure 8 presents exemplary immunohistochemical images from both groups at parturition.

Vitamin D receptor abundance in caprine colonic mucosa was not significantly affected by diet Ca *ante partum* (2268 Cl[1856, 2680] vs. 2669 Cl[2214, 3125] immune reactivity at 1.3% vs. 0.6% in DM; P = 0.19). In both groups, the scores showed a curvilinear trend over time which could not be statistically confirmed (P = 0.1).

Supplementary Table 8 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of VDR immune reactivity, as related to time and dietary Ca supply *ante partum*.

Discussion

An earlier meta-analysis concluded that the risk for hypocalcaemia in dairy cows significantly increases if dietary Ca during the *ante partum* phase is between 1.1-1.5% in DM (Lean et al., 2006). This hypothesis has yet not been tested in dairy sheep and goats, therefore the present study investigated whether Ca fed at 1.3% in DM (compared to 0.6% in DM) during the last 3 weeks *ante partum* increases the incidence of hypocalcaemia *ante* and/or *post partum* by dysregulating Ca homeostasis.

<u>Effects on quantitative Ca homeostasis and the incidence of hypocalcaemia</u>

Sheep and goats showed in any case significant differences in the expression of observed measures of quantitative Ca homeostasis (fecal Ca, urinary Ca excretion, colostrum Ca, serum Ca, BMD). Differences in magnitude of these parameters between both species have also been observed in several earlier comparative studies. Our observation of higher values in sheep with respect to urinary Ca excretion and colostral Ca and bone mineral density are in line with earlier studies (Liesegang et al., 2006, 2007, Liesegang, 2008). We made the same observation with serum Ca, which agrees to Kohler et al. (2013) and Liesegang (2008), though Wilkens et al. (2014) observed the opposite with goats expressing higher values than sheep. The reason for this discrepancy is unclear at the moment.

The feeding showed mostly no or just numerical effects on quantitative measures. Increasing dietary Ca feeding during the prepartum phase significantly increased fecal Ca concentration by at least 2fold during the last 3 weeks but did not significantly affect serum and colostral Ca concentration and urinary Ca excretion in both species at any given point during the study. Most notably, no signs of hypocalcemia or any pathology were noted *ante* or *post partum*, respectively, although, according to Oetzel (1988) dairy sheep and goats are expected to develop milk fever symptoms comparable to cows but at 1-3 weeks *post partum*. However, milk yields and associated Ca losses have improved since 1988, which would suggest a milk fever-

promoting feeding regime to be more likely today and maybe also earlier than described by Oetzel. Literature on the effects of Ca oversupply in sheep and goats is scarce, particularly with respect to the *ante partum* phase. The only study on sheep by Braithwaite (1979), however, agrees with our observations. The colleague studied gradual differences in dietary Ca intake in adult sheep and observed increasing fecal Ca losses. Fractional Ca absorption (% of intake) was at 31.8 % at the lowest Ca supply (0.22 % in DM) and dropped to a plateau around ~9-10% with gradually increasing diet Ca to 0.6, 1.2, and 2.4% in dietary DM, respectively. In parallel, bone Ca resorption dropped inversely to the increase in Ca intake and inevitable unregulated absorption of surplus Ca was increasingly redirected into the gastrointestinal tract. Given the response of our sheep and goats in the present study, it may be concluded that for our animals and under the present study conditions, we can reject our initial hypothesis of increased milk fever risk when feeding higher Ca in the range proposed by Lean et al. (2006) for dairy cows. It remains to be tested what the outcome would be when feeding Ca sources with a higher absorbability as $CaCO_3$, for example $CaCl_2$ (Goff and Horst, 1993).Neither sheep nor goats showed any relevant response in urinary Ca excretion in response to the feeding regime over time, confirming once more results from earlier studies suggesting livestock ruminants do regulate Ca primarily via the gastrointestinal tract (Herm et al., 2015, Wild et al., 2021). We assume that the animals were able to dampen uptake from the gastrointestinal tract and, in parallel, effectively rerouted unavoidable unregulated Ca uptake, as described by Braithwaite (1979), towards excretory pathways. Future studies must identify the precise physiological mechanisms behind this effective compensation of dietary Ca overload. See in this context also our discussion below, on potentially confounding factors associated with our study.

<u>Effects on serum calcitriol and markers of bone formation and resorption</u>

We observed a core set of serum bone formation and resorption markers, which have been earlier established in ruminants from gestation into lactation (Liesegang et al., 2000, Liesegang et al., 2007). Observed measures of bone formation and resorption showed changes over time, that have earlier been attributed to the species-dependent adaption towards parturition and the onset and further course of lactation (Liesegang et al., 2006, 2007, Liesegang, 2008, Kohler et al., 2013, Wilkens et al., 2014). However, none of the observed parameters responded to the feeding treatment during the last weeks *ante partum* and mostly also *post partum*. Consequently, the BMD was not affected and suggests the feeding intervention with 1.3% in DM Ca in the diet caused no obvious dysregulation of Ca and bone homeostasis in neither species as compared to the lower Ca feeding.

However, serum osteocalcin showed a significant gradual increase in concentration from d7 to d56 of lactation in animals receiving the high dietary Ca *ante partum*. This response was true for both species but most pronounced in the dairy goats. Serum osteocalcin is considered a marker of bone formation, with increasing levels pointing towards rising embedding of Ca in the skeleton (Liesegang et al., 2000). This observation was puzzling given the lack of response of calcitriol as well as markers of bone formation (calcitriol, bAP) and resorption (ICTP, CTX)) and especially since the effect occurred 8d after the suspension of the high Ca feeding. To the best of our knowledge such a response pattern has yet not been reported for serum osteocalcin and it raises questions on its physiological purpose. The rise in serum osteocalcin was not associated with changes in bone mineral density (or concentration; data not shown) nor did other markers of bone formation show an orchestrated response that would suggest any feeding-associated changes in bone metabolism. Therefore, it may be necessary to have look at osteocalcin from a different perspective. Biomedical research efforts in recent years suggest a hormonal role of uncarboxylated osteocalcin beyond bone metabolism, involving among others the regulation of systemic energy metabolism (Lee et al., 2007), muscle energy metabolism

(Mera et al., 2016), brain development (Obri et al., 2018). At this point, the meaning of these results is up to speculation, hence, more targeted research on the matter is urgently needed.

Effects on offspring development

High Ca treatment of mothers *ante partum* was associated with decreased average daily weight gain of suckling lambs and kids. Since the offspring showed no differences in birth weight that were associated to the feeding of the mothers *ante partum*, we rule out quantitative effects on placental nutrient transfer. In our opinion, the most plausible explanation for reduced offspring growth during the suckling phase is an impairment of milk yield and/or quality. Feed intake was not affected in the present study and all animals consumed the necessary dietary dry matter according to recommendations. At the same time, we noted differences in serum osteocalcin in the higher Ca groups, pointing towards a distinct physiological conditioning. This likely has affected the quantity and/or quality of secreted milk and therefore the growth response of their offspring, but this needs yet to be confirmed. To the best of our knowledge there is yet no data available in dairy sheep and goats that allows us a more informed discussion on this matter and future studies must put a focus on the potential effects of our experimental conditions on milk yield and quality as well as mammary physiology.

In addition, a further hypothesis may deserve some attention in future studies. High Ca feeding during the last 3 weeks *ante partum* and the associated physiological adaption of the mothers may have promoted fetal programming in the offspring that impaired their digestive capacity for Ca (and maybe other nutrients) *post partum* , therefore negatively affecting their growth response. The possibility of mineral nutrition during gestation to induce fetal programming in ruminants either for the benefit or disadvantage of the offspring has recently been reviewed by Anas et al. (2023) for cattle, highlighting a multitude of already established interactions as well as future routes for research on the matter. In this context, an earlier study in rats tested the

effects of varying dietary Ca supply during gestation including higher supply at 1.2% in DM (Li et al., 2018). The colleagues observed abnormal expression of hepatic and adipose genes in the offspring as well as dyslipidemia and hepatic lipid accumulation in response. Unfortunately, no data on Ca homeostasis in rat mothers and their offspring was obtained. Nevertheless, it may be argued that high supply with a mineral over several weeks may induce epigenetic changes in the mother, which could be inherited to the offspring. It may therefore be interesting to have a closer look on the epigenetic consequences of high dietary Ca as well as opposite feeding regimes during gestation and their potential to imprint Ca homeostatic regulation and development of the offspring *post partum*.

The geographical region and other potentially confounding factors

Depending on the geographic location, Ca levels in the environment are quite variable. In Switzerland, animals are often confronted with high levels of native Ca in diets, originating from the Ca-rich geological formations, which shape the chemical composition of soil in certain areas. For example an earlier survey of Schlegel et al. (2016) found a mean Ca concentration of 7 g/kg DM in 236 samples of mixed grassland from Posieux, Switzerland, and our own experience with grass hay qualities delivered to our animal facility in the Zurich area are in line with this observation. This leads to quite relevant intake levels of native Ca from feed particularly when grass-based roughage is fed, even when mineral Ca supplements are not applied. Since the organism attempts to maintain Ca homeostasis by fine-tuning absorption, losses, and mobilization of skeletal reserves according to the environmental conditions (Martín-Tereso and Martens, 2014, Wilkens and Muscher-Banse, 2020), it may be argued that sheep and goats raised predominantly on Ca-rich roughage may be better suited to deal with high dietary Ca challenge. However, it may also suggest that during the feeding of high dietary Ca due to active supplementation of inorganic Ca to such raw materials, a metabolic imprinting as

hypothesized earlier may more likely promote the observed adverse effects on daily weight gain of suckling lambs and goat kids. Therefore, the present results must be considered with caution when it comes to the general translation into dairy sheep and goat feeding systems. More data is urgently needed also from regions with less Ca in the soil.

This study is one of the first to compare high Ca responses between sheep and goats. While many biomarkers in our findings align with previous research on bone metabolism and calcium homeostasis principles within and across these species, our study is too preliminary to draw definitive conclusions regarding species-specific effects under the current treatment conditions. Given that "species" is an intrinsic characteristic, it cannot be effectively randomized within a single study limited to one herd per species. However, our findings represent an initial step toward further research in this area, underscoring the need for not only replication of these experimental conditions but also replication across multiple herds to strengthen the reliability of observed responses.

The current Swiss feeding recommendations for sheep and goats suggest comparable dietary calcium levels during the last three weeks *ante partum* (Agroscope, 2017). Based on these guidelines, our study applied the same diet to both species. While these recommendations likely exceed net requirements due to built-in safety margins, it remains an open question whether current genotypes in both species respond similarly to a standardized diet. To enhance future research, it would be beneficial to revisit and, if needed, revise the calcium feeding guidelines for dairy sheep and goats individually, ensuring they reflect species-specific physiological needs more accurately.

Neither sex of offspring nor litter size were balanced in the present study, therefore it may be argued whether this imbalance may have biased the results on daily weight gain during the suckling phase. However, sex was included as a variable in the statistical model and multiple births largely outweighed single births in both species (only 1 single birth in sheep and 2 in

goats). In addition, the random baseline of each animal nested under the respective mother was added as a random factor. We are therefore confident the results truly reflect a physiological response from the varying prepartum Ca feeding of the mothers. Nevertheless, this is the first experiment reporting this phenomenon and further replication of the study ideally in other labs is needed to further confirm our findings.

Conclusion

The present study did not find any signs of hypocalcaemia in sheep and goats challenged with higher Ca during the last 21 days *ante partum*. However, serum osteocalcin showed increasing concentrations in lactating sheep and goats fed higher Ca *ante partum* compared to the respective lower Ca group. Interestingly, although no obvious negative effects of the feeding regime could be observed for the mothers, the offspring of sheep and goats fed higher Ca *ante partum* did grow slower over time compared to the lower Ca groups, despite comparable birth weights. This likely has originated from effects on the physiological status of the mothers, thereby affecting milk yield and quality. Nevertheless, the additional hypothesis of a potential adverse effect of high dietary Ca *ante partum* on the digestive capacity of the offspring via epigenetic imprinting may be an exciting avenue for future research. Our animals were reared in a Ca-rich environment since their birth, therefore, we cannot rule out that the present findings are exclusive for this situation. Follow-up studies are urgently needed and should also comprise observations in production regions with less Ca in the soils.

Notes

Raw datasets can be requested via E-mail to the corresponding author.

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All procedures used in the experiment involving animals were in accordance with the Swiss guidelines for animal welfare at the time of study and were registered and approved by the Cantonal Veterinary Office (ZH 9/2006).

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Chemical composition and daily intake levels of feedstuffs fed during the last 21 days *ante partum* until 56 days of lactation of dairy sheep and goats.

Chemical analysis	Grass hay (2nd cut)	Concentrate	[1]Concentrate + $CaCO_3$
Energy, MJ NEL	5.90	7.00	6.70
Dry matter, g/kg DM	928	884	896
Crude protein, g/kg DM	155	133	115
[1]aNDFom, g/kg DM	654	267	194
[2]ADFom, g/kg DM	357	90.4	70.2
ADL, g/kg DM	38.4	18.9	13.2
Crude fat, g/kg DM	28.0	61.5	49.2
Crude ash, g/kg DM	81.1	54.2	170
HCl-insoluble ash, g/kg DM	13.3	4.30	4.60
Ca, g/kg DM	5.90	6.90	62.3
Mg, g/kg DM	2.00	1.90	1.40
P, g/kg DM	3.30	4.60	4.30

Component, kg DM/d	Sheep		Goats	
	Low Ca	High Ca	Low Ca	High Ca
		ante partum		
Grass hay (2nd cut)	2.00	2.00	2.00	2.0
[4]Concentrate[3]	0.30	0.3	0.30	0.3
$CaCO_3$	-	0.042	-	0.042
Total DM fed	2.30	2.342	2.30	2.342
[5]Nutrient intake, g/d				
Crude protein	350	345	350	345
Energy (MJ NEL)	13.9	13.8	13.9	13.8
Ca	13.9	30.5	13.9	30.5
P	7.98	7.89	7.98	7.89
Mg	4.57	4.42	4.57	4.42
		post partum		
Grass hay (2nd cut), kg/d	2.40	2.40	2.40	2.40
[2]Concentrate, kg/d	0.50	0.50	0.70	0.70
$CaCO_3$, g/d	-	-	-	-
Total DM fed	2.90	2.90	3.10	3.10
[3]Nutrient intake, g/d				
Crude protein	439	439	465	465
Energy (MJ NEL)	17.7	17.7	19.1	19.1
Ca	17.6	17.6	19.0	19.0
P	10.2	10.2	11.1	11.1
Mg	5.75	5.75	6.13	6.13

Commercial concentrate UFA 765 by supplier UFA (Union des Fédérations Agricoles). For the composition of the concentrate based on manufacturer instructions, please see Supplementary Table 1.

[1]aNDFom, starch and ash corrected NDF after treatment with heat-stable amylase and ashing of the solid residue according to Method 6.5.1 (VDLUFA, 2012)

[2]ADFom, ash corrected ADF after ashing of the solid residue according to Method 6.5.2 (VDLUFA, 2012)

[3]860 g of commercial concentrate + 140 g CaCO3.

[4]During ante partum feeding either the basal concentrate from the manufacturer or the artificially Ca-enriched concentrate were fed to ensure comparative feeding of 0.6 % in DM Ca vs. 1.3 % in DM Ca until parturition. During subsequent lactation feeding, no comparative feeding was applied therefore only the basal concentrate was fed to all animals at highlighted daily amounts.

[5]Daily nutrient intake was calculated from daily supplied dry feed (hay, concentrate) and analyzed nutrient concentrations.

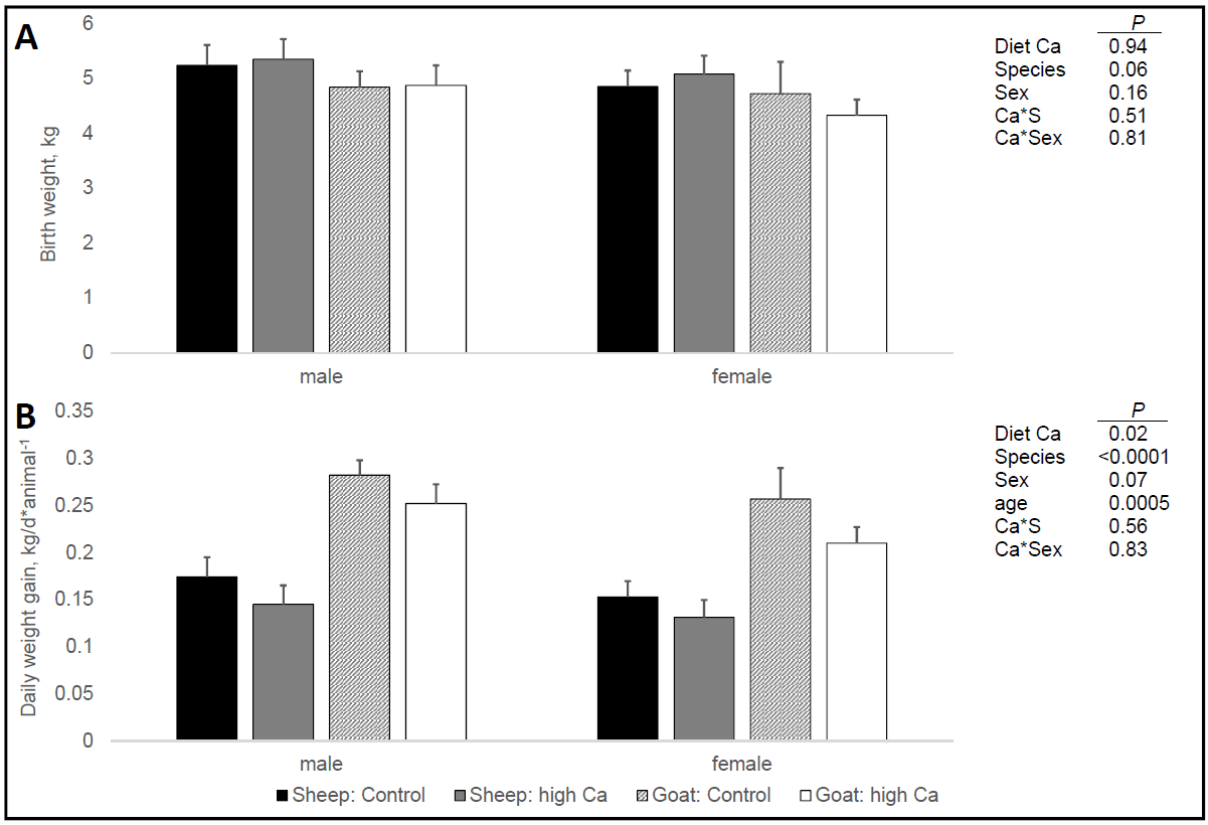

Figure 1. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks *ante partum* as well as sex and species of offspring on the birth weight (A) and daily weight gain during the suckling phase (B). Effective sample size was 5 sheep and 6 goats per group, which gave birth to 24 and 23 lambs and kids in total, respectively. Data is presented as least square mean values with standard errors based on linear mixed model analysis. "Age" refers to the time of second weighing during the suckling phase, which differed between individual animals due to management reasons (range: 33-59 days *post partum*) and was therefore introduced as a continuous control variable to the statistical analysis of daily weight gain data. Ca*S and Ca*Sex refer to statistical interactions between diet Ca and species of animal or sex of offspring, respectively. $P \leq 0.05$ was defined as statistical type I error threshold.

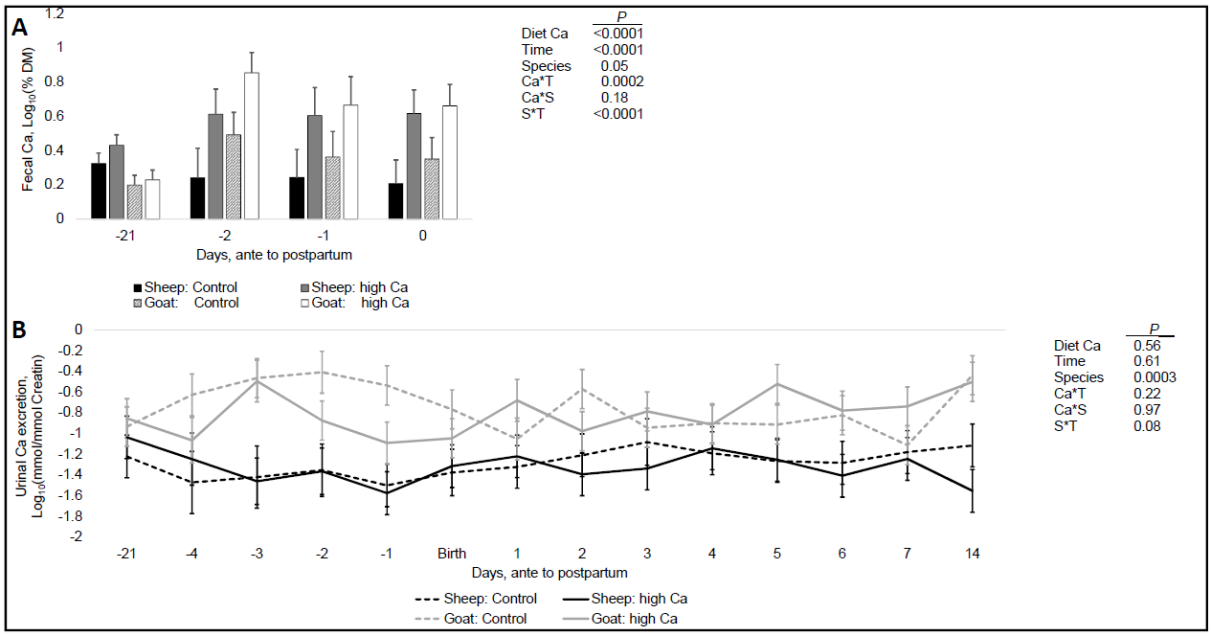

Figure 2. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks *ante partum* as well as time and animal species on the fecal Ca concentration (A) and urinary Ca excretion (B) from 3 weeks *ante partum* to parturition and 14 days *post partum*, respectively. Effective sample size was 5 sheep and 6 goats per group. Data is presented as Log_{10}-transformed least square mean values with standard errors, respectively, based on repeated measures linear mixed model analysis. Ca*T, Ca*S and S*T refer to statistical interactions between diet Ca and animal species or time, respectively, as well as animal species and time. $P \leq 0.05$ was defined as statistical type I error threshold.

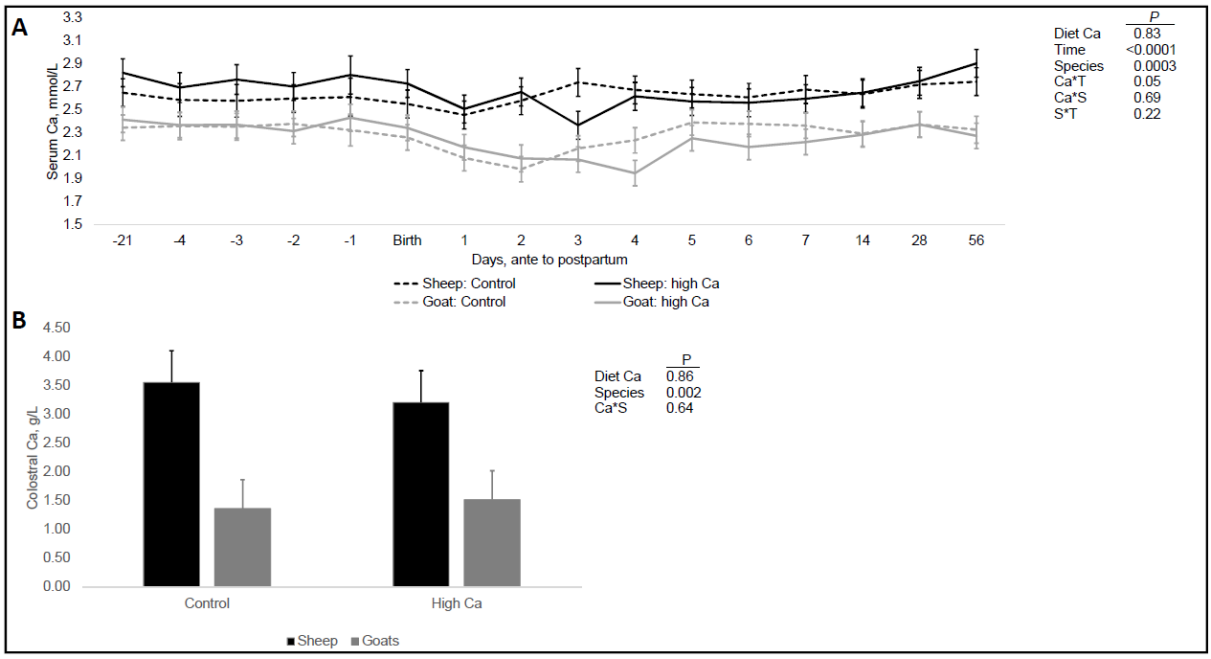

Figure 3. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks *ante partum* as well as time and animal species on serum Ca (A) and colostral Ca (B) from 3 weeks *ante partum* to parturition and 56 days *post partum*, respectively. Effective sample size was 5 sheep and 6 goats per group. Data is presented as least square mean values with standard errors based on repeated measures linear mixed model analysis and regular linear mixed model analysis, respectively. Ca*T, Ca*S and S*T refer to statistical interactions between diet Ca and animal species or time, respectively, as well as animal species and time. P $\leq$ 0.05 was defined as statistical type I error threshold.

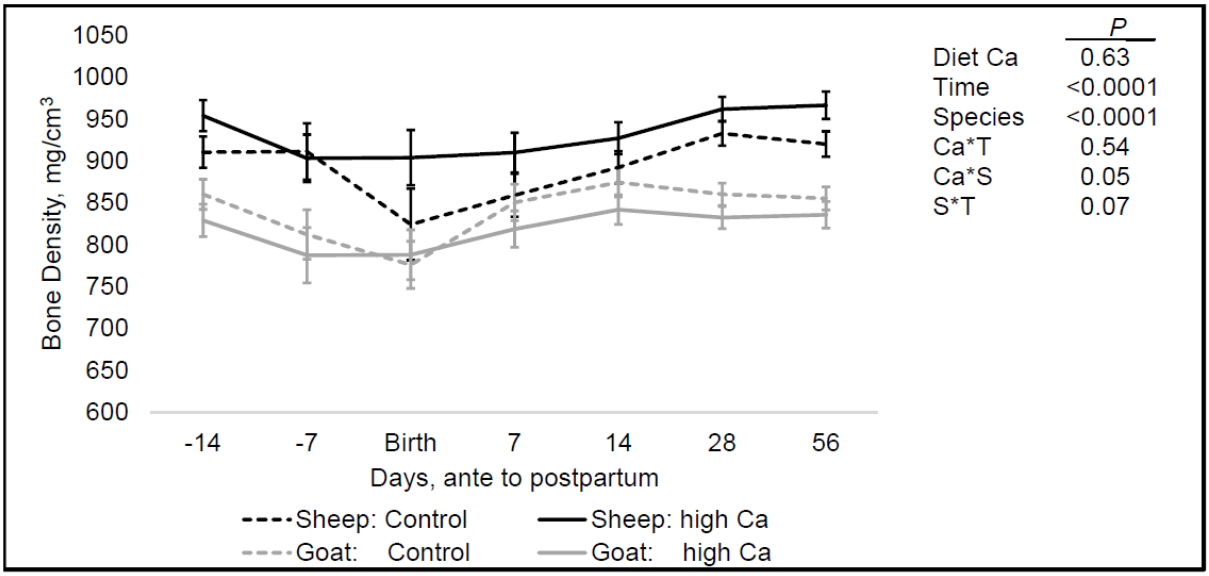

Figure 4. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks *ante partum* as well as time and animal species on bone mineral density from 2 weeks *ante partum* to 56 days *post partum*. Effective sample size was 5 sheep and 6 goats per group. Data is presented as least square mean values with standard errors based on repeated measures linear mixed model analysis. Ca*T, Ca*S and S*T refer to statistical interactions between diet Ca and animal species or time, respectively, as well as animal species and time. P $\leq$ 0.05 was defined as statistical type I error threshold.

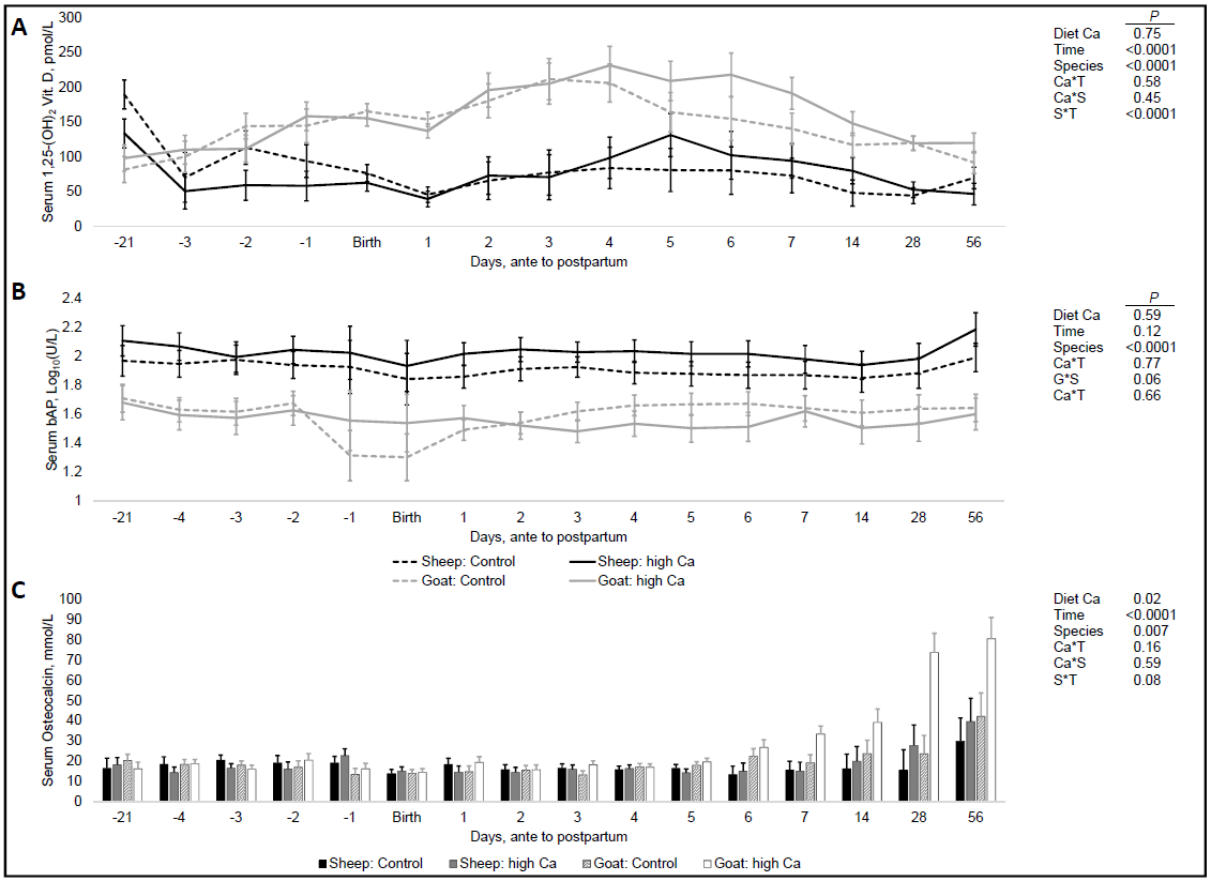

Figure 5. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks *ante partum* as well as time and animal species on serum 1,25(OH)$_2$ Vitamin D (A), serum bone specific alkaline phosphatase activity (B), and serum osteocalcin (C) from 3 weeks and 5 days *ante partum*, respectively, to 56 days *post partum*. Effective sample size was 5 sheep and 6 goats per group. Data is presented as least square mean values (Log$_{10}$-scale in case of bone specific alkaline phosphatase activity) with standard errors based on repeated measures linear mixed model analysis. Ca*T, Ca*S and S*T refer to statistical interactions between diet Ca and animal species or time, respectively, as well as animal species and time. P $\leq$ 0.05 was defined as statistical type I error threshold. Due to compromised sub-samples of analysis of 1,25(OH)2 Vitamin D at -4 days *ante partum*, we cannot provide the respective data.

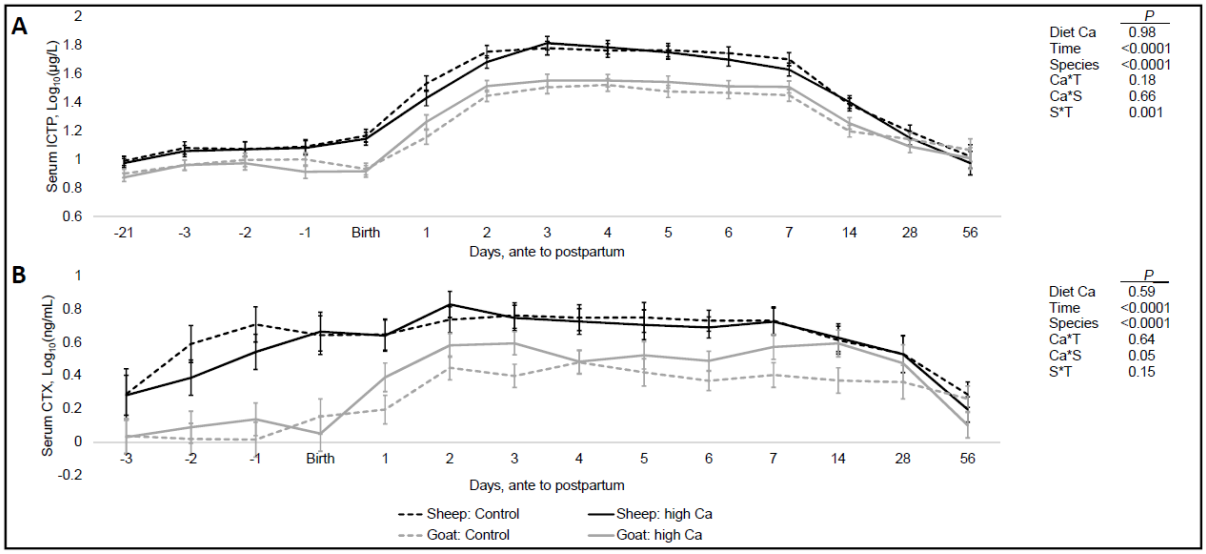

Figure 6. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks *ante partum* as well as time and animal species on serum ICTP (A) and serum CTX (B) from 3 weeks and 3 days *ante partum*, respectively, to 56 days *post partum*. Effective sample size was 5 sheep and 6 goats per group. Data is presented as least square mean values (Log_{10}-scale in case of bone specific alkaline phosphatase activity) with standard errors based on repeated measures linear mixed model analysis. Ca*T, Ca*S and S*T refer to statistical interactions between diet Ca and animal species or time, respectively, as well as animal species and time. ICTP, crosslinked carboxyterminal teleopeptide of type I collagen; CTX, crosslaps. $P \leq 0.05$ was defined as statistical type I error threshold. Due to compromised sub-samples of analysis of CTX at -21 days *ante partum* and for ICTP and CTX at -4 days *ante partum*, we cannot provide the respective data.

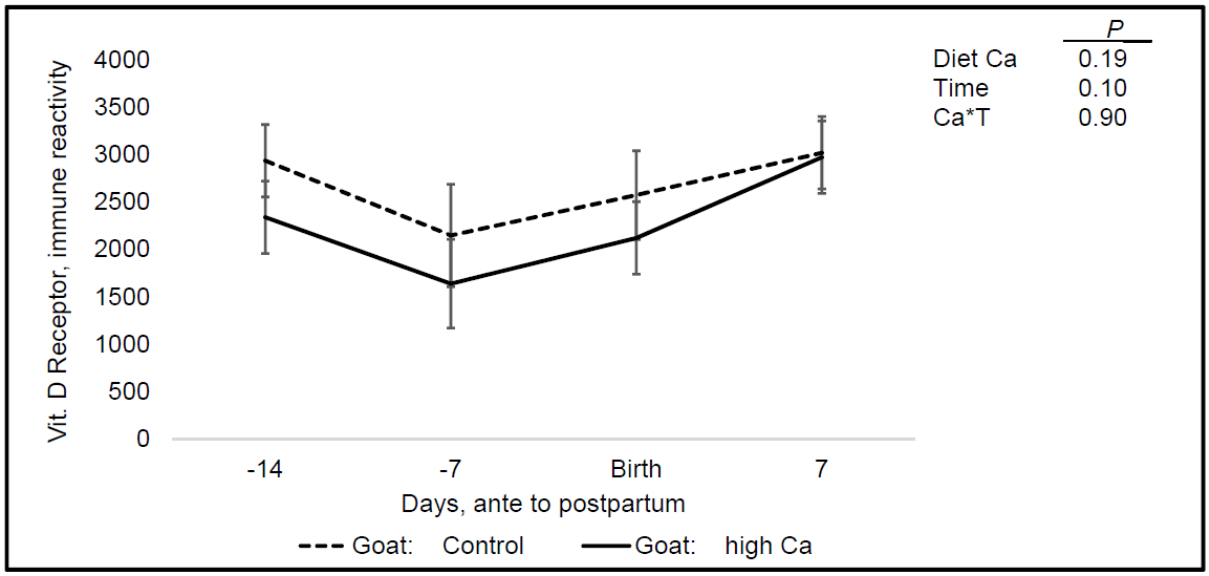

Figure 7. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks *ante partum* as well as time on caprine vitamin D receptor abundance in colonic mucosa from 2 weeks *ante partum* to 7 days *post partum*. Effective sample size was 6 goats per group. Data is presented as least square mean values with standard errors based on repeated measures linear mixed model analysis. Ca*T, refers to statistical interaction between diet Ca and time. $P \leq 0.05$ was defined as statistical type I error threshold. See Figure 8 for exemplary immunohistochemical images.

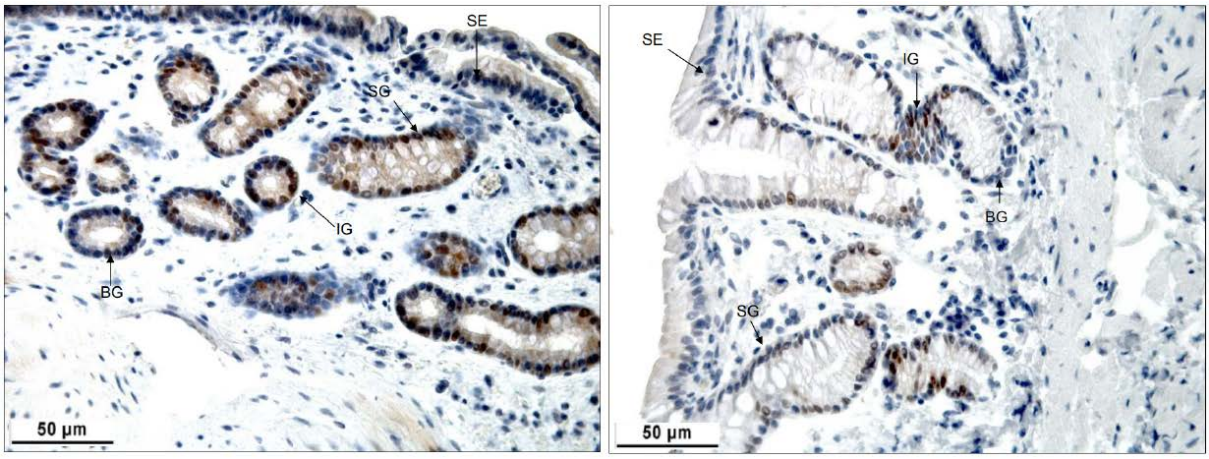

Figure 8. Exemplary immunohistochemical staining of the caprine Vitamin D Receptor in the colon of goats at parturition, fed 0.6 % in DM (left) or 1.3 % in DM (right) dietary Ca during the last 3 weeks prepartum. Arrows mark SE, surface epithelium; SG, surface gland sections; IG, intermediate gland sections; BG, basal gland sections. Samples shown in these photos were taken at parturition with an endoscope equipped with biopsy forceps 40 cm into the large intestine from the anus. Goats fed high Ca prepartum showed reduced immune reactivity to Vitamin D Receptor (VDR) staining, though this could not be statistically confirmed. Figure 7 shows the statistical analysis of VDR immune reactivity over time from -14 days *ante partum* to 7 days *post partum*.