Effects of excess dietary calcium supplementation during the transition phase on calcium and bone metabolism of dairy sheep and goats and growth response of their suckling offspring

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Abstract

This study investigated the effects of excess dietary Ca supplementation during the transition phase on Ca and bone metabolism in dairy sheep and goats, as well as the growth response of their suckling offspring. Multiparous dairy sheep (n=6/group) and goats (n=5/group) were randomly assigned to a control and treatment group. The experimental group received a high Ca diet (1.3% DM) based on hay and concentrate three weeks *ante partum*, compared to the control group (0.6% DM). Experimental feeding ended with parturition and animals were henceforth fed according to recommendations for lactating sheep and goats and kept together with their suckling offspring. The observation period spanned from 21 d *ante partum* to 56 d *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 metabolism 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 excess dietary Ca *ante partum* significantly increased fecal Ca concentrations until parturition by 2 $\log_{10}$-orders of magnitude, 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 (BMD) between control and experimental groups at any point in time of observation, indicating stable Ca homeostasis despite the dietary excess. However, species-specific differences were noted, with sheep generally showing significantly higher serum and colostral Ca levels, as well as BMD, compared to goats. 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 species of animal. Vitamin D receptor abundance in goats' colonic mucosa were numerically lower during parturition when high Ca was fed. 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 control, despite similar species-specific birth weights. We conclude that while dairy sheep and goats can effectively manage high dietary Ca intake without overt signs of hypocalcemia or milk fever, there are 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 periparturient dairy ruminants and highlight the need for balanced dietary strategies to optimize health and productivity in both dams and their offspring.

Key words: Mineral, Homeostasis, Hypocalcemia, Milk Fever

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

Calcium is essential for structure and function of the animal organism and already in 1905 an experimental model of dietary Ca deficiency was successfully applied in dairy cows (Babcock, 1905).

To avoid a drop in circulatory Ca, the organism has three options to stabilize the system, by mobilizing Ca from the skeleton, increasing absorption capacity at the gut barrier, and limiting endogenous losses via the kidneys and gastrointestinal tract (Wilkens and Muscher-Banse, 2020). With respect to the regulation of Ca metabolism, 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., 2012). 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 review by Martín-Tereso and Martens (2014) and Wilkens and Muscher-Banse (2020)).

In lactating animals, proper Ca supply is especially important since milk supplies high levels of Ca to the offspring on a daily basis (Hernández-Castellano et al., 2020). In this context, the transition phase during the last weeks *prepartum* is 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 ewes and nannies 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 *pre*- 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% 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. Authors developed a model with data from 51 trials that estimates the incidence of milk fever as a function of breed of cow as well as *prepartum* dietary DCAD, Ca, P, Mg, and diet exposure time. They concluded that the response of milk fever incidence to dietary Ca *ante partum* follows a bell curve, which suggests that concentrations between 1.1-1.5% DM should be avoided, whereas higher or lower levels gradually reduce the incidence of milk fever (Lean et al., 2006).

Studies applying high dietary Ca levels in the feeding of periparturient animals are rare, especially with sheep and goats. Therefore, the present study was conducted, which aimed to collect such data and attempted to test the conclusions of Lean et al. (2006) under controlled conditions. Therefore, periparturient sheep and goats of our in-house herd of dairy sheep and dairy goats were either given diets with 0.6% DM or 1.3% DM of Ca during the last three weeks *ante partum* and observed until 56 d of lactation. Our working hypothesis was that goats and sheep supplied higher diet Ca (equivalent to 30-36 g/d) will show signs of hypocalcemia whereas the lower supplied animals do not. It further allowed us to observe the response of mineral and bone metabolism to dietary Ca overload in two species with distinct strategies to maintain Ca homeostasis.

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 twelve 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 four weeks before the estimated parturition date. 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 two groups according to their ear tag numbers by a random sequence generator (https://www.random.org/sequences/): one control and one experimental group per species. The effective sample size for sheep and goats was five and six per group, respectively. The feeding trial started three 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 control groups received a balanced diet provided by hay and a commercial high energy concentrate (UFA 766, Herzogenbuchsee, Switzerland). Additionally, for the experimental groups until parturition, the same concentrate was applied but mixed up daily with 2.5 times (16.8 g Ca /day) the recommended daily calcium requirement as $CaCO_3$ (Table 1). From parturition onwards, all animals received lactation feeding according to good practice. Animals were fed twice per day restrictive amounts of hay and concentrate adapted to their requirements *ante* and *post*

partum, and had *ad libitum* access to drinking water. The composition of the commercial concentrate (UFA 766) as communicated by the manufacturer, is shown in Supplementary Table 1.

Zootechnical performance

Sheep and Goats were weighed closely 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 at 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 management issues. This variation in observational periods was considered as a control variable in the analysis of the growth performance data (see subsection on statistical analysis below).

Sample collection and handling

For this experiment, a closed batch of hay and concentrates was ordered and three representative samples each were taken according to good practice (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 before morning feeding on days -21, -7, -6, -5, -4, -3, -2, -1, 0 (parturition), 1, 2, 3, 4, 5, 6, 7, 14, 28, and 56 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. Urine was collected in a beaker on days -21, -14, -7, 0, 7, and 14 before morning feeding and stored at -20°C until further usage. Fecal grab samples of each animal were collected at days -21, -2, -1, and 0 before morning feeding and stored at -20°C until further usage. 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 two samples were snap frozen in liquid nitrogen and stored at -80°C.

Sample analysis

Feed was analyzed for dry matter, crude nutrients, fiber fractions, HCl-insoluble ash, and mineral profile (Ca, P, Mg) according to standard procedures (VDLUFA, 2012).

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) as described by Liesegang et al. (2007).

Feces and colostrum were analyzed for total Ca, and urine for total Ca and creatinine. The urinary Ca excretion was expressed as ratio to urinary creatinine since the material could not be sampled quantitatively. These analyzes and calculation have been described in detail by Liesegang et al. (2007).

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) in sufficient detail. For sheep, no suitable antibody was available at the time of study.

Bone mineral density

Bone mineral density (BMD) was determined by quantitative computer tomography (qCT); the respective equipment and approach has been described earlier in detail (Liesegang et al., 2007).

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 “group”, “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 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 “group”, “species”, and interaction, again including the individual animal nested under the respective feeding group as random effect. 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. 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.

The sample size of the study was designed to meet a minimum statistical power of $1-\beta = 0.8$ at $\alpha = 0.05$ (McDonald, 2014) for the primary outcome variables (quantitative and functional measures of Ca metabolism). Earlier datasets on small ruminant Ca metabolism from our institute served as datasets for prospective power analysis (Liesegang et al., 2007, Liesegang, 2008).

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. Sheep showed five cases of triplets, four cases of twins and one single birth (n = 22 lambs in total) whereas the majority of goats gave birth to twins with one case of triplets and two 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 (5.12 vs. 4.69 kg for lambs and kits, respectively; P = 0.06) irrespective of sex (P = 0.16) and unaffected by the mothers' dietary Ca supply *ante partum* (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 control animals (0.16 vs. 0.14 kg/d and 0.27 vs. 0.23 kg/d for lambs and kids, respectively, P = 0.02) irrespective of sex and species, which in both cases had distinct effects on the animals' growth performance (males grew faster than females; kids grew faster than lambs) (P = 0.07 and P < 0.0001, respectively) (Figure 1B).

Quantitative markers of calcium metabolism

Analyzed fecal Ca concentration during the observation period ranged between 1.46 and 5.19 % DM in sheep as well as 1.17 and 14.9 % 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-transformed to meet the precondition of normality, accounting for fecal Ca ranges of 0.16 and 0.71 $\log_{10}$(% DM) for sheep as well as 0.07 and 1.17 $\log_{10}$(% 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.

Excess supply with dietary Ca *ante partum* significantly increased fecal Ca concentration until parturition by at least two Log_{10}-orders of magnitude ($P < 0.0001$) irrespective of animal species, which expressed a distinct effect on total analyzed fecal Ca ($P = 0.05$) (Figure 2A). In contrast, the feeding regime had no effect on the urinary calcium excretion ($P = 0.56$), which was only different with respect to the animal species (goats expressing higher values than sheep) ($P = 0.0003$) and remained rather stable from 3 weeks *ante partum* to 14 d *post partum*.

Serum Ca was not affected by the *ante partum* feeding group ($P = 0.83$) and gradually and significantly ($P < 0.0001$) decreased independent of animal species towards parturition to gradually increase again during lactation (Figure 3A). In any case, sheep showed higher serum Ca levels than goats ($P = 0.0003$). The colostral Ca concentration was significantly lower in goats compared to sheep ($P = 0.002$) but was not affected by the *ante partum* Ca feeding regime ($P = 0.86$) (Figure 3B).

Figure 4 highlights the bone mineral density of dairy sheep and dairy goats from transition to 56 d in milk, as affected by Ca feeding during the last three weeks *ante partum*, time and animal species. Excess Ca feeding *ante partum* did not affect bone mineral density ($P = 0.63$), which significantly declined towards parturition to gradually increase *post partum* until d 56 of lactation ($P < 0.0001$). On average, sheep showed higher bone density than goats ($P < 0.0001$).

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

Serum calcitriol was significantly changed with respect to time ($P < 0.0001$) and animal species ($P < 0.0001$) but not by the *ante partum* feeding regime ($P = 0.75$), although numerical differences between species-dependent treatment groups were evident (Figure 5A). Serum

levels in sheep gradually reduced towards parturition and fluctuated throughout whereas in goats serum levels gradually increased until 4 d *post partum* to turnaround and reduce until 56 d *post partum*. Sheep showed higher serum levels in the first week of observation after which the ratio changed for the benefit of the goats throughout the rest of the study.

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 and time expressed no significant effect on bAP throughout the whole experiment ($P = 0.59$ and 0.12, respectively) (Figure 5B). Sheep showed in any case significantly higher serum activities than goats ($P < 0.0001$).

Serum osteocalcin remained rather stable and comparable between species von 5 d *ante partum* to 7 d *post partum*, after which it significantly and gradually increased in both species ($P < 0.0001$), with goats showing highest values and steepest increase over time until d 56 *post partum* ($P = 0.007$) (Figure 5C). Most notably, this increase in serum osteocalcin was significantly higher in those sheep and especially goats who received excess dietary Ca during the last three weeks *ante partum* ($P = 0.02$).

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

Serum ICTP showed no marked changes in both species until parturition, afterwards it significantly increased over the first week post partum to gradually reduce until 56 d *post partum* ($P < 0.0001$) (Figure 6A). Average serum levels over time were significantly higher for sheep than goats ($P < 0.0001$). At no time did the feeding *ante partum* express any significant effect on serum ICTP levels of dairy sheep and goats ($P = 0.98$).

Sheep showed markedly higher serum CTX levels during this study than goats ($P < 0.0001$) (Figure 6 B). In both species serum levels significantly increased until the mid of week 1 *post*

partum. Afterwards, levels gradually declined until 56 d *post partum* ($P < 0.0001$). No significant effect of the *ante partum* Ca feeding was observed ($P = 0.59$).

Caprine colonic abundance of mucosal vitamin D receptor

Figure 7 highlights the statistical evaluation of VDR immune reactivity in caprine colonic mucosa until parturition, in goats fed 0.6% DM and 1.3% DM dietary Ca during transition. Figure 8 presents exemplary immunohistochemical images in addition. Vitamin D receptor abundance in caprine colonic mucosa was not significantly affected by *ante partum* feeding ($P = 0.19$) or time ($P = 0.1$), respectively, though showed numerically reduced abundance from 14 d *ante partum* to parturition in goats fed the high Ca diet.

Discussion

An earlier meta-analysis concluded that the risk for hypocalcaemia in dairy cows significantly increases if dietary Ca during the peripartal phase is between 1.1-1.5% 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% DM (compared to 0.6% DM) during the last three weeks *ante partum* increases the incidence of hypocalcaemia *ante* and/or *post partum* by dysregulating Ca homeostasis.

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

Sheep and goats showed in any case significant differences in the expression of observed measures of quantitative Ca metabolism (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 on the matter. 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 peripartal phase significantly increased fecal Ca concentration by at least 2fold during the last three 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. This suggests that the organism was able to dampen Ca absorption and/or recirculate passively absorbed surplus Ca back into the gastrointestinal tract. Furthermore, the generous peripartal Ca supply did not impair the animals' ability to

compensate the sudden increase in Ca losses due to the onset of lactation, though the opposite is expected for dairy cows based on the meta-analysis by Lean et al. (2006). Calcitriol, the active form of vitamin D in the mammal organism, improves jejunal Ca absorption when dietary Ca supply is deficient. Conversely, the efficiency of jejunal Ca absorption is markedly lower when the dietary Ca supply is sufficient (Abdel-Hafeez et al., 1982). It may be speculated that a dietary excess supply with Ca may further impair active calcitriol-dependent Ca absorption. The numerically reduced abundance of colonic mucosal VDR in our treatment goats *ante partum* is in support of this notion and highlights the adjustment of the homeostatic regulation towards a dampening of Ca absorption capacity in response to high Ca during the peripartal phase. Consequently, bone mineral density was not affected by the dietary treatment at any time of observation. Literature on the effects of Ca oversupply in sheep and goats is scarce, particularly with respect to the peripartal phase. The only study on sheep by Braithwaite (1979), however, agrees with our observations. The colleague studied gradual differences in dietary Ca intake in wethers and observed increasing fecal Ca losses while fractional Ca absorption remained stable at ~10% when feeding 0.6, 1.2, and 2.4% Ca in dietary DM, respectively. In parallel, bone Ca resorption dropped inversely to the increase in Ca intake and passively absorbed surplus was increasingly redirected into the gastrointestinal tract. Given the comparable response of our goats in the present study, we hypothesize that the counter-regulation of quantitative Ca homeostasis to dietary Ca excess is comparable between both species, apart from the above discussed differences in baseline values of observed parameters. Neither sheep nor goats showed any relevant response in urinary calcium excretion in response to the feeding regime, confirming once more results from earlier studies suggesting livestock ruminants do not clear calcium surplus via the kidneys (Herm et al., 2015, Wild et al., 2021).

Taken together, sheep and goats were able to stabilize quantitative Ca homeostasis and did not express signs of hypocalcemia or milk fever neither *ante* nor *post partum*, despite a drastic increase in dietary Ca during the last weeks *ante partum* from 0.6 to 1.3% DM.

Effects on serum markers of bone formation and resorption

We observed a core set of serum bone formation and resorption markers, which have been earlier established in ruminants transitioning from gestation to 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 of gestation and mostly also *post partum*. Consequently, the BMD was not affected and suggests the feeding intervention with 1.3% DM Ca in the diet caused no obvious dysregulation of Ca and bone homeostasis in neither species.

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 calcium in the skeleton (Liesegang et al., 2000). This observation was puzzling given the lack of response of other 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. If the rise in serum osteocalcin indeed suggests increasing bone formation activity from d 8 to d 56 of lactation, this did not affect

the bone mineral density (or concentration; data not shown). However, the necessity for increased bone formation in the high Ca group during lactation appears questionable, especially since the challenge feeding was stopped 8d before the rise in serum levels. Biomedical research efforts in recent years suggest a hormonal role of uncarboxylated osteocalcin, involving the regulation of systemic energy metabolism (Lee et al., 2007), muscle energy metabolism (Mera et al., 2016), brain development (Obri et al., 2018), and others. 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*, it can be concluded that this response was due to effects on either the physiological status of the mothers or their offspring or both. The aforementioned differences in serum osteocalcin are pointing towards a distinct physiological conditioning of mothers in the treatment group. This may have affected the quantity and quality of secreted milk and therefore the growth response of their offspring, but this needs yet to be confirmed. Furthermore, high Ca feeding during the last three weeks *post partum* and the associated physiological adaption of the mothers may have promoted fetal programming in the offspring that impaired their digestive capacity for calcium and maybe other nutrients 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 exiting future routes for research on the matter. Furthermore, an earlier study in rats tested the effects of

varying dietary Ca supply during gestation including excess supply at 1.2% DM (Li et al., 2018). They observed abnormal expression of hepatic and adipose genes in the offspring in response thereby promoting dyslipidemia and hepatic lipid accumulation. Unfortunately, no data on calcium homeostasis in rat mothers and their offspring was obtained. Nevertheless, it may be argued that excess supply with a mineral over several weeks may induce epigenetic changes in the mother, which have the potential to be inherited to the offspring. For example, piglets exposed to excess zinc supply over 14 days post-weaning show not only downregulation of active zinc transport at the gut mucosa (Martin et al., 2013) but in fact a methylation of CpG islands of the respective transporter gene, which further impairs its transcription (Karweina et al., 2015). This has yet not been shown for calcium but is has been demonstrated that Ca^{2+} signaling pathways are involved in epigenetic modifications (Sharma et al., 2014). It may therefore be interesting to have a closer look on the epigenetic consequences of dietary Ca-excess as well as opposite feeding regimes during gestation and their potential to epigenetically imprint Ca homeostatic regulation and development of the offspring.

The geographical region as a potential confounding factor

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 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 dietary Ca overload than such in other areas. However, it may also suggest that during Ca excess due to active supplementation of inorganic Ca to such raw materials, a metabolic imprinting as hypothesized for the offspring from the present study 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.

Conclusion

The present study did not find any signs of hypocalcaemia in sheep and goats challenged with excessive Ca during the transition phase. However, serum osteocalcin showed increasing concentrations in lactating sheep and goats fed high Ca *ante partum* compared to the respective control. Interestingly, though no obvious negative effects of the feeding regime could be observed for the mothers, the offspring of sheep and goats fed high Ca during the transition phase did grow slower over time compared to control, despite comparable birth weights. This may have originated from either effects on the physiological status of the mothers, thereby affecting milk yield and quality, or the offspring or both. The 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.

This study received no external funding.

We thank Dr. med. vet. Sybil Lüthi for excellent technical assistance in sample and data collection. We further thank the technical staff in the animal and laboratory facilities of the Institute of Animal Nutrition and Dietetics, Vetsuisse-Faculty, University of Zurich, Switzerland.

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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Wilkens, M. R. and A. S. Muscher-Banse. 2020. Review: Regulation of gastrointestinal and renal transport of calcium and phosphorus in ruminants. Animal 14: s29-s43.

569 Table 1. Chemical composition and daily intake levels of feedstuffs fed during transition and lactation
570 of dairy sheep and goats.

Chemical analysis, per kg DM	Hay (2nd cut)	Concentrate	Concentrate + $CaCO_3$
Energy, MJ NEL	5.90	7.00	6.70
Dry matter, g	928	884	896
Crude protein, g	155	133	115
Crude fiber, g	263	69.3	64.2
NDF, g	654	267	194
ADF, g	357	90.4	70.2
ADL, g	38.4	18.9	13.2
Crude fat, g	28.0	61.5	49.2
Crude ash, g	81.1	54.2	170
HCl-insoluble ash, g	13.3	4.30	4.60
Ca, g	5.90	6.90	62.3
Mg, g	2.00	1.90	1.40
P, g	3.30	4.60	4.30

Component, kg DM/d	Sheep		Goats	
	Control	Treatment	Control	Treatment
		ante partum		
Hay (2nd cut)	2.0	2.0	2.0	2.0
Concentrate[1]	0.3	0.3	0.3	0.3
$CaCO_3$	-	0.042	-	0.042
Total DM fed	2.3	2.342	2.3	2.342
Nutrient intake, g/d				
Crude protein	350	344	350	344
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		
Hay (2nd cut), kg/d	2.4	2.4	2.4	2.4
Concentrate, kg/d	0.5	0.5	0.7	0.7
$CaCO_3$, g/d	-	-	-	-
Total DM fed	2.9	2.9	3.1	3.1
Nutrient intake, g/d				
Crude protein	438	429	465	452
Energy (MJ NEL)	17.7	17.5	19.1	18.8
Ca	17.6	45.3	19.0	57.8
P	10.2	10.1	11.1	11.0
Mg	5.75	5.5	6.13	5.78

571 Commercial concentrate UFA 765. For the composition of the concentrate based on manufacturer
572 instructions, please see Supplementary Table 1.

573

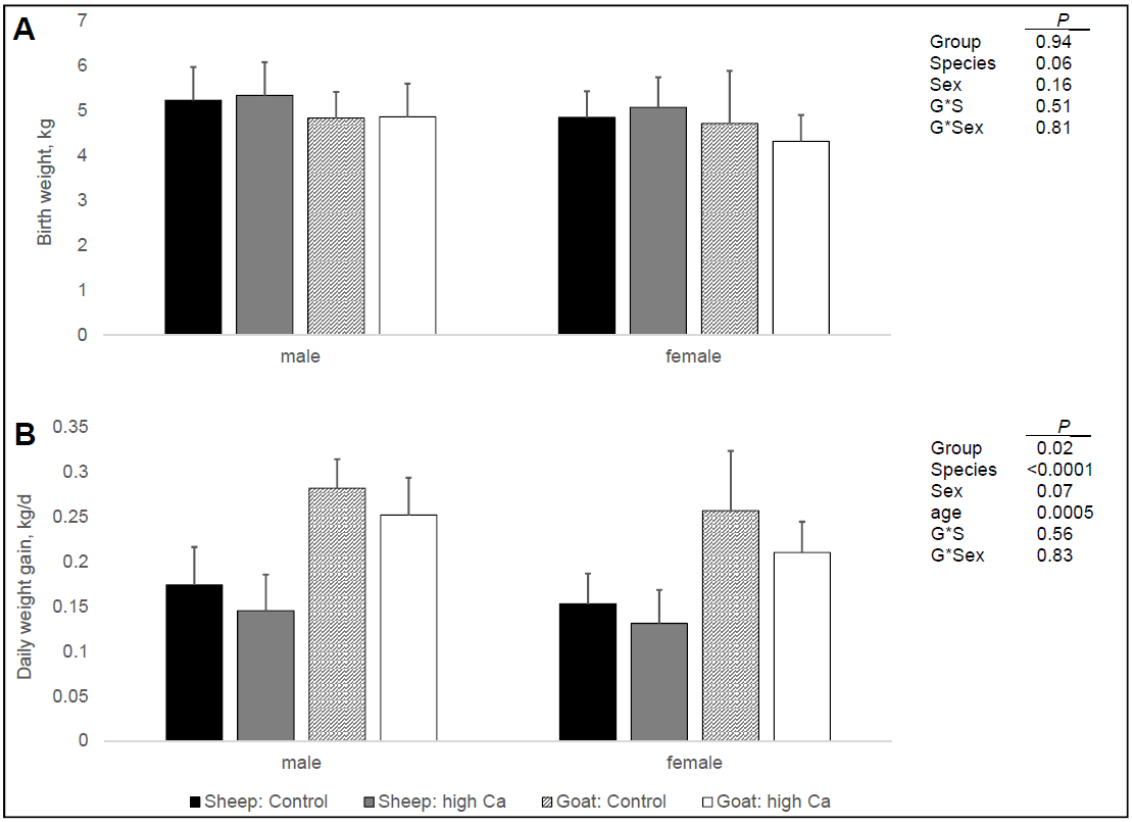

Figure 1. Effects of varying dietary Ca (0.6 vs. 1.3% DM) of dairy sheep and goats during the last three weeks *ante partum* as well as sex and species of offspring on the birth weight (A) and daily weight gain during the suckling phase (B). Effective sample size was five sheep and six goats per group, which gave birth to 22 and 23 lambs and kids in total, respectively. Data is presented as least square mean values with Tukey-adjusted upper 95% confidence limits 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 d *post partum*) and was therefore introduced as a continuous control variable to the statistical analysis of daily weight gain data. G*S and G*Sex refer to statistical interactions between feeding group and species of animal or sex of offspring, respectively. $P \leq 0.05$ was defined as statistical type I error threshold.

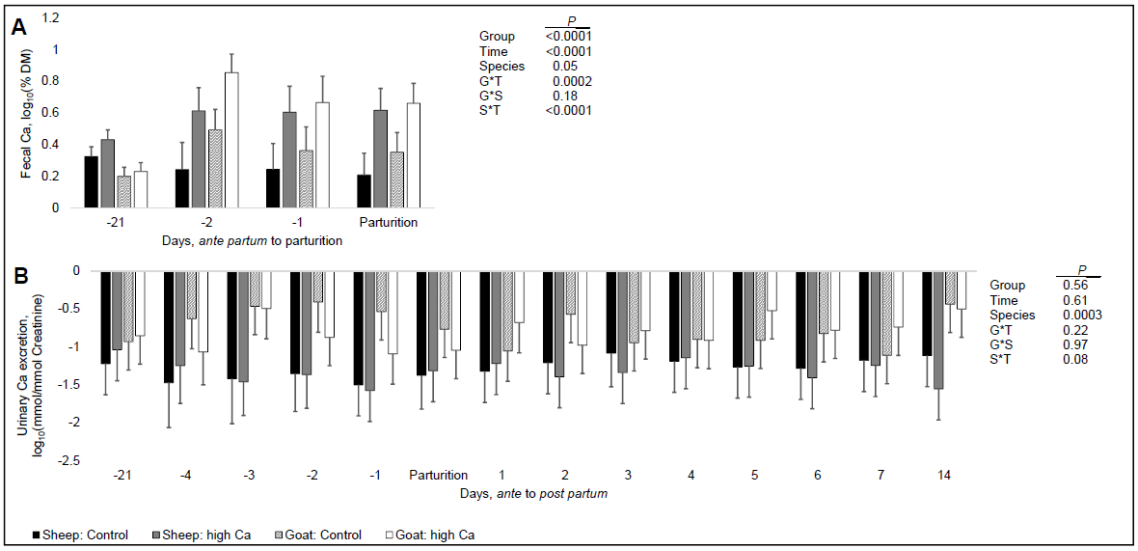

Figure 2. Effects of varying dietary Ca (0.6 vs. 1.3% DM) of dairy sheep and goats during the last three weeks *ante partum* as well as time and animal species on the fecal calcium concentration (A) and urinary calcium excretion (B) from three weeks *ante partum* to parturition and 14 d *post partum*, respectively. Effective sample size was five sheep and six goats per group. Data is presented as Log_{10}-transformed least square mean values with Tukey-adjusted upper or lower 95% confidence limits, respectively, based on repeated measures linear mixed model analysis. G*T, G*S and S*T refer to statistical interactions between feeding group 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.

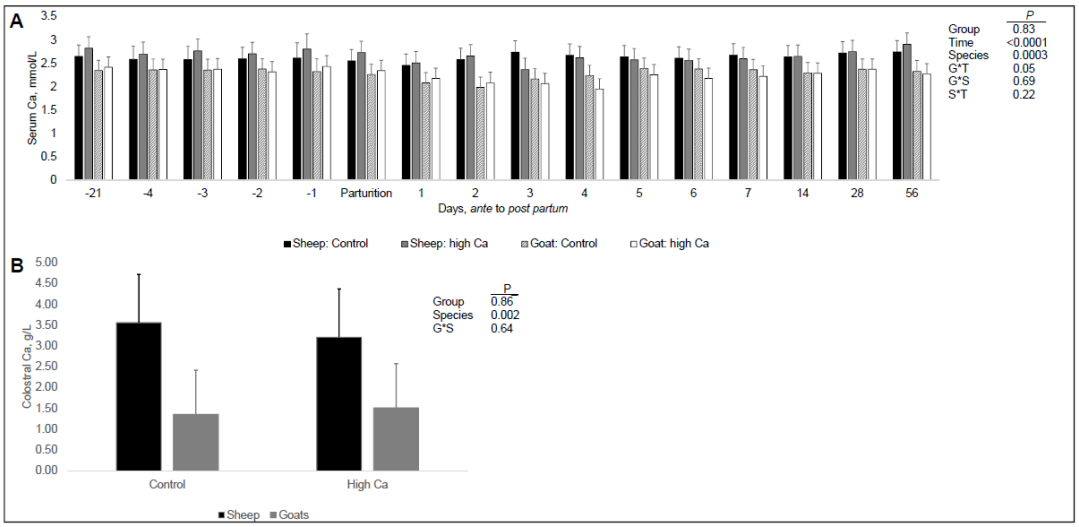

Figure 3. Effects of varying dietary Ca (0.6 vs. 1.3% DM) of dairy sheep and goats during the last three weeks *ante partum* as well as time and animal species on serum calcium (A) and colostral calcium (B) from three weeks *ante partum* to parturition and 56 d *post partum*, respectively. Effective sample size was five sheep and six goats per group. Data is presented as least square mean values with Tukey-adjusted upper 95% confidence limits based on repeated measures linear mixed model analysis and regular linear mixed model analysis, respectively. G*T, G*S and S*T refer to statistical interactions between feeding group 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.

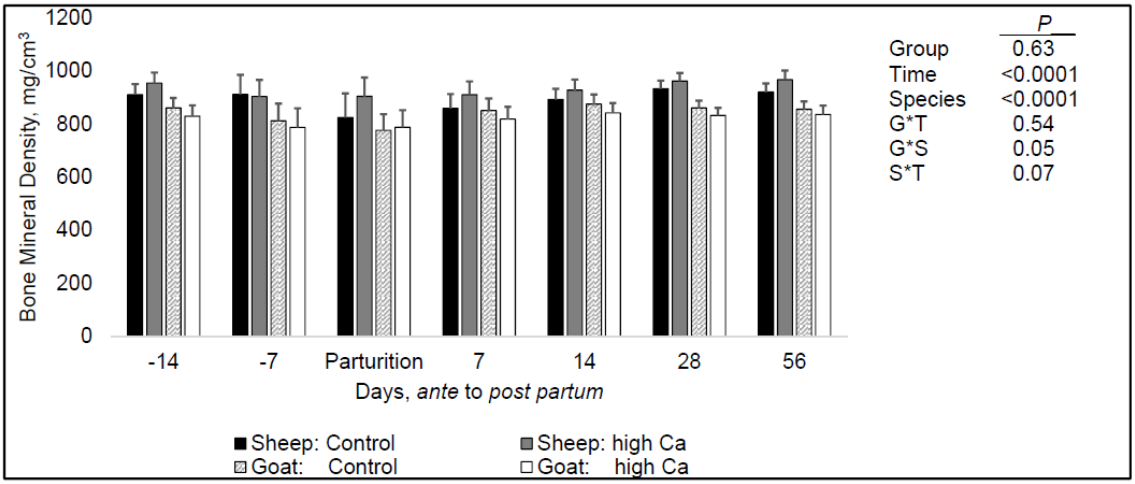

Figure 4. Effects of varying dietary Ca (0.6 vs. 1.3% DM) of dairy sheep and goats during the last three weeks *ante partum* as well as time and animal species on bone mineral density from two weeks *ante partum* to 56 d *post partum*. Effective sample size was five sheep and six goats per group. Data is presented as least square mean values with Tukey-adjusted upper 95% confidence limits based on repeated measures linear mixed model analysis. G*T, G*S and S*T refer to statistical interactions between feeding group 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.

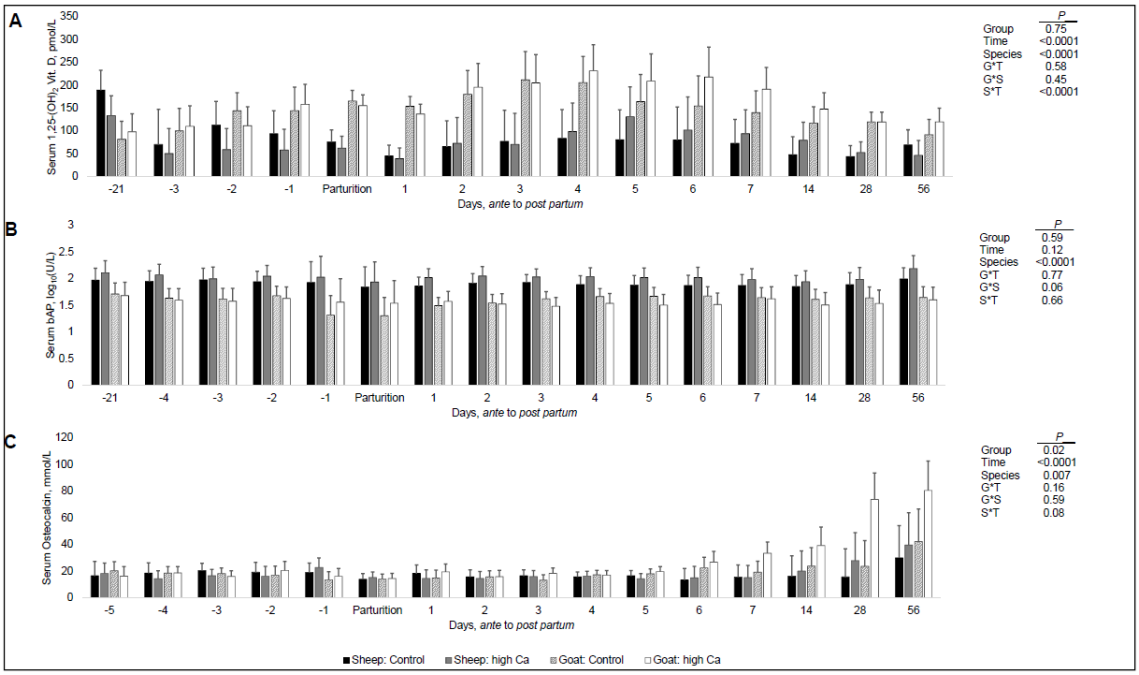

Figure 5. Effects of varying dietary Ca (0.6 vs. 1.3% DM) of dairy sheep and goats during the last three weeks *ante partum* as well as time and animal species on serum 1,25$(OH)_2$ Vitamin D (A), serum bone specific alkaline phosphatase activity (B), and serum osteocalcin (C) from three weeks and 5 d *ante partum*, respectively, to 56 d *post partum*. Effective sample size was five sheep and six goats per group. Data is presented as least square mean values (Log10-scale in case of bone specific alkaline phosphatase activity) with Tukey-adjusted upper 95% confidence limits based on repeated measures linear mixed model analysis. G*T, G*S and S*T refer to statistical interactions between feeding group 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.

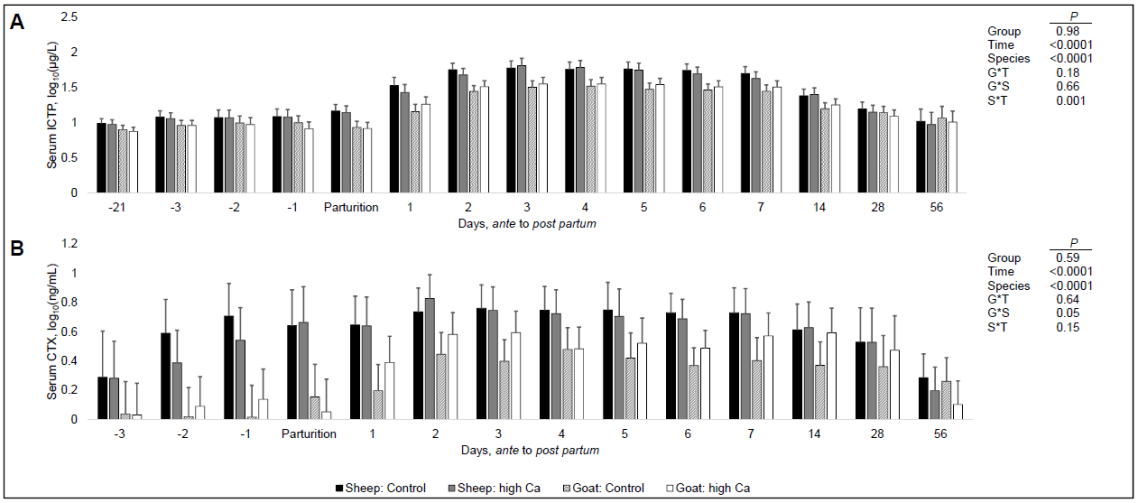

Figure 6. Effects of varying dietary Ca (0.6 vs. 1.3% DM) of dairy sheep and goats during the last three weeks *ante partum* as well as time and animal species on serum ICTP (A) and serum CTX (B) from three weeks and 3 d *ante partum*, respectively, to 56 d *post partum*. Effective sample size was five sheep and six goats per group. Data is presented as least square mean values (Log10-scale in case of bone specific alkaline phosphatase activity) with Tukey-adjusted upper 95% confidence limits based on repeated measures linear mixed model analysis. G*T, G*S and S*T refer to statistical interactions between feeding group 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.

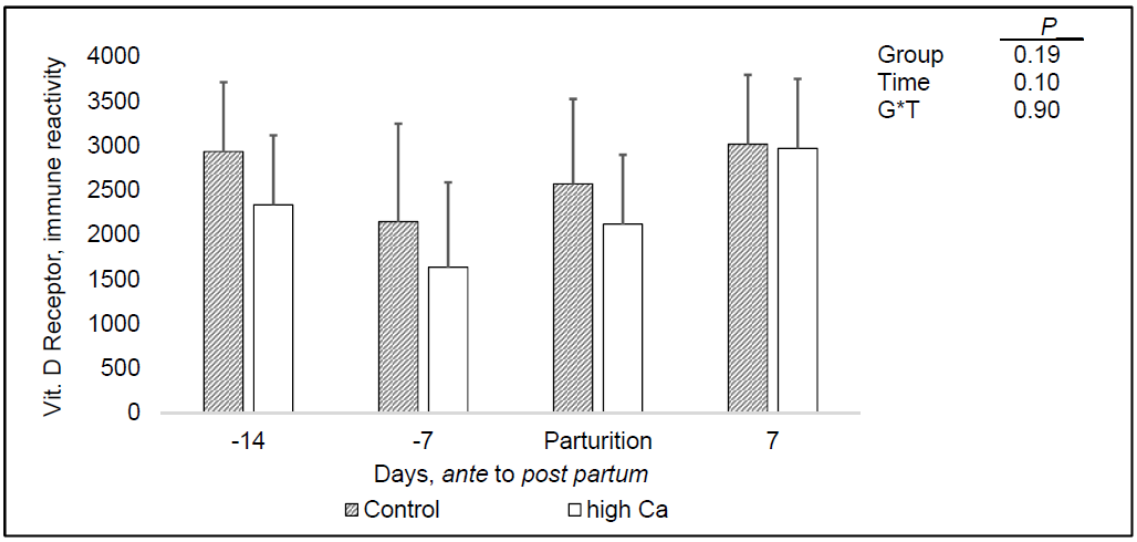

Figure 7. Effects of varying dietary Ca (0.6 vs. 1.3% DM) of dairy sheep and goats during the last three weeks *ante partum* as well as time on caprine vitamin D receptor abundance in colonic mucosa from two weeks *ante partum* to 7 d *post partum*. Effective sample size was six goats per group. Data is presented as least square mean values with Tukey-adjusted upper 95% confidence limits based on repeated measures linear mixed model analysis. G*T, refers to statistical interaction between feeding group and time. $P \leq 0.05$ was defined as statistical type I error threshold. See Figure 8 for exemplary immunohistochemical images.

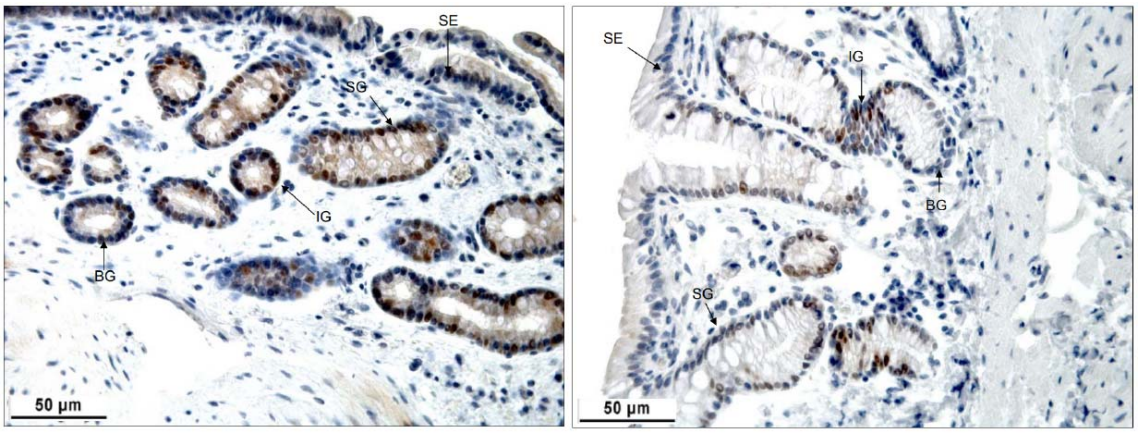

Figure 8. Exemplary immunohistochemical staining of the caprine Vitamin D Receptor in the colon of goats at parturition, fed 0.6 % DM (left) or 1.3 % DM (right) dietary calcium during the last three weeks prepartum. Arrows mark SE, surface epithelium; SG, surface gland sections; IG, intermediate gland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