High calcium to sheep and goats antepartum

Antepartum high dietary supply of calcium affects bone homeostasis and offspring growth in dairy sheep and dairy goats

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Interpretive Summary

Many studies have focused on deficient Ca supply to small dairy ruminants, and little is known about high dietary Ca during the last days antepartum. 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 postpartum 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 the final 21 days antepartum 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} antepartum for both species; 2.9 and 3.1 kg DM/d*animal^{-1} for sheep and goats postpartum, respectively) according to recommendations with the exception of Ca during antepartum, where 1 group received a high Ca diet with $CaCO_3$ (1.3% in DM; 2.49fold recommendations) based on hay and concentrate 3 weeks antepartum, 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 antepartum to 56 days postpartum. 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 antepartum 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 antepartum 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 1,25-$(OH)_2$ vitamin D (**calcitriol**) as well as markers of bone formation and

resorption were monitored, revealing significant increases in serum osteocalcin postpartum in goats and sheep fed high Ca antepartum. 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 stresses the need for further research on balanced dietary strategies to optimize health and productivity of lactating animals 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;; ICTP = crosslinked carboxyterminal teleopeptide of type I collagen; 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 a key factor to consider. For example, sheep and goats have been demonstrated to respond quite differently to Ca-restricted feeding with the sheep tending quicker to bone mobilization while the latter will initially 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 antepartum 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 hypocalcemia, 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 postpartum though a higher tendency for carrying twin or even triplet fetuses may increase the risk of developing a hypocalcemia during the last trimester given an increasing fetal Ca requirement during the last 4 weeks antepartum 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 antepartum 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 antepartum 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 antepartum diet (Lean et al., 2006). There has been some debate regarding the proper dietary Ca concentration antepartum, 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 2 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 exceptionally low and very high dosages showing protective effects. Model I included antepartum DCAD, as well as diet Ca, P, Mg, and diet exposure time as variables while Model II included antepartum dietary K and S in replacement of DCAD. Both models suggested 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 inconsistency between available studies on the interaction of dietary Ca dosage and milk fever incidence. This raises an 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 antepartum 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 antepartum 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 antepartum, providing 1.15 and 2.54 times the Swiss daily Ca intake 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 not have benefited our research. Observations continued until 56 days

postpartum, 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 on the higher Ca diet antepartum (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. A total of 10 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 ± SD1.5 kg and 101 ± SD2.22 kg, respectively, and 78.3 ± SD2.22 kg and 81.0 ± SD2.31 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 Swiss recommendations for energy and nutrient intake 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 Swiss feeding recommendations which accounted for 17.6 and 19.0 g

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

177 Collection and handling of samples and growth parameters

178 Colostrum was hand milked from each animal immediately after parturition, and yield ranged
179 between 0.5 – 1.2 L per animal in both species. After sampling 50 mL for subsequent analysis,
180 colostrum was distributed equally over nursing bottles and supplied to the neonates.

181 Sheep and goats were weighed before morning feeding the day before and directly after
182 parturition. Due to the suckling of the offspring, daily milk yield could not be assessed. The
183 lambs and kids were weighed directly after parturition and once between 33-59 days
184 postpartum, to determine the average daily weight gain during the suckling phase. The variation
185 in the time between 1st and 2nd weighing was because lambs and kids were weaned at different
186 ages to be subject to a different research purpose. To account for this variation in observational
187 periods, we included the time interval between the 1st weighing at birth and the 2nd weighing
188 (the age of each lamb and kid at 2nd weighing) as a continuous variable in the statistical analysis
189 of weight gain. This approach controls for potential age effects that could bias the estimation of
190 treatment effects (see the subsection on statistical analysis below).

191 For this experiment, a closed batch of grass hay (2nd cut) and concentrates was ordered and 3
192 representative samples each were taken according to published standard methods 1.3

193 ("Regulations of the German Agricultural Society on the Sampling of Feed and Sample
194 Treatment") and 1.5 ("Sampling of Hay") (VDLUFA, 2012) in order to investigate the feed
195 value by chemical analysis. Feed samples were directly subject to chemical analysis after
196 sample collection.

197 Blood was collected in daily frequency during 28 days before estimated parturition and on days
198 1, 2, 3, 4, 5, 6, 7, 14, 28, and 56 postpartum, in any case before morning feeding at 8 a.m. in
199 serum monovettes (9 and 6 mL, Vacuette®, Greiner Bio-One Vacuette Schweiz GmbH) and
200 subject to serum collection (1580 x g, 10 min) after allowing coagulation at room temperature.
201 The serum was stored at -80°C until further usage. After the actual parturition, samples of days
202 -21, -4, -3, -2, -1, 0 (parturition), 1, 2, 3, 4, 5, 6, 7, 14, 28, and 56 were reserved for further
203 analysis.

204 Urine was collected in a beaker upon spontaneous urination, in parallel to the blood sampling
205 antepartum and on days 7 and 14 postpartum, and homogenized to be separated into 2 equal
206 subsamples. The 1st of the subsamples was acidified (12 mol/L HCl, pH 1-2) for later Ca
207 determination, the other was subject to the later determination of creatinine. Both subsamples
208 were stored at -20°C. After parturition, samples of days -21, -14, -7, 0, 7, and 14 were reserved
209 for further usage. Fecal grab samples of each animal were collected in parallel to blood
210 sampling antepartum and days -21, -2, -1, and 0 were subject to further analysis. Samples were
211 stored at -20°C until further usage.

212 All goats were subject to colonic mucosal biopsies by veterinary personnel on days -21, -7, 0,
213 and 7. An endoscope (PCF-20, Olympus) equipped with biopsy forceps was inserted 40 cm into
214 the large intestine via the anus to collect 4 samples (1.5-2.0 mm diameter) per animal*time^{-1}; 2
215 of said samples were immediately put into 4% neutral buffered formalin and the remaining 2
216 samples were snap frozen in liquid nitrogen and stored at -80°C.

217 Feed analysis

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

243 protocol by measuring solution not via ICP-OES but by flame atomic absorption spectrometry
244 (flame-AAS) (ContrAA 700, Analytik Jena).

245 Analysis of blood serum parameters of bone and mineral homeostasis

246 Blood serum was analyzed for osteocalcin, 1,25-$(OH)_2$ vitamin D (**calcitriol**), bone-specific
247 alkaline phosphatase (**bAP**), crosslinked carboxyterminal teleopeptide of type I collagen
248 (**ICTP**), and crosslaps (**CTX**).
249 Serum osteocalcin was quantified with a commercial EIA test kit (MikroVue Osteocalcin EIA
250 Kit; Quidel Corporation). Intra- and interassay CVs were 5.00% and 5.75% with a sensitivity
251 of 0.45 ng/mL. Serum calcitriol determination was conducted with an RIA kit (1,25VitD RIA,
252 sheep-anti 1,25VitD antibody; Immunodiagnostics Systems GmbH). 9.0, 9.3 <8pmol/L Intra-
253 and interassay CVs were 9.0% and 9.3% with a sensitivity an 8 pmol/L. Serum bAP was
254 analyzed with an immunoassay via a monoclonal anti-bAP and para-nitrophenylphosphate as a
255 substrate (Alkphase-BTM^{TM}; Metra Biosystems). Intra- and interassay CVs were 0.58 and 0.52.
256 Serum ICTP was measured by a RIA immunoassay (UniQ® ICTP RIA, DiaSource) with an
257 intra- and interassay CV of 5.6 and 10.0 and a sensitivity of 0.6 µg/L. Serum CTX was assayed
258 by a commercial ELISA assay (Serum CrossLaps (CTX-I) ELISA; Immunodiagnostics
259 Systems GmbH) with intra- and interassay CVs of 1.7% and 2.5% at a sensitivity of 0.02 ng/mL.

260 For CTX, ICTP, and calcitriol, data gaps at -4 days and for CTX additionally for -21 days, as
261 depicted in Figures 5 and 6, respectively, were due to compromised sub-samples. All other
262 sampling decisions were intentional, based on a balance between data relevance, workload, and
263 monetary considerations.

264 Analysis of Ca and creatinine in biological matrices

265 Serum Ca was measured via flame-AAS after microwave wet digestion of 2 mL serum per
266 technical replicate in 65% HNO_3 and 30% H_2O_2 (Ethos, MLS GmbH) against a dilution

267 gradient (0.2, 0.4, 0.6, 0.8, 1.0 mg/L) of a commercial AAS-certified standard (ROTI®Star 1
268 000 mg/l Ca, in 2 % HNO_3; Roth).
269 Dried Feces (105°C, 4 h) was milled (0.5 mm) and 500 mg per technical replicate were subject
270 to Ca analysis as described for serum above.
271 Colostrum were defatted by centrifugation (4000 x g, 4°C, 30 min), and subject to the same
272 procedures of sample preparation and Ca analyzes as described for feces and blood serum.
273 Urine was analyzed for total Ca with the method described for serum and further analyzed for
274 creatinine by a kinetic color test using a commercial kit (Crea Jaffe DIA00540, Diatools AG)
275 on the Cobas Mira autoanalyzer with an intra- and interassay CV of 0.83% and 0.85% at a
276 sensitivity of 18 µmol/L. The urinary Ca excretion was expressed as ratio to urinary creatinine
277 since the material could not be sampled quantitatively. Creatinine is generally excreted at a
278 relatively constant rate, making it a useful reference compound to normalize mineral
279 concentrations for differences in urine dilution caused by hydration status, collection timing or
280 other individual factors (Sullivan et al., 2012).

281 Vitamin D receptor immunoreactivity

282 Goat colonic mucosal biopsies were subject to semi-quantitative immunohistochemical
283 determination of vitamin D receptor (**VDR**) abundance as originally described by Liesegang et
284 al. (2008) and Sidler-Lauff et al. (2010). Caprine colonic VDR was labelled after rinsing the
285 tissue with TBS buffer and incubation for 80 min with a biotinylated rat monoclonal antibody
286 (9A7γ, ThermoScientific) at a 1:200 dilution in Tris-buffered saline against unspiked Tris-
287 buffered saline on an adjacent colonic section as negative control and duodenal cross sections
288 of pigs (obtained from the in-house slaughtering facility of the Vetsuisse-Faculty, University of
289 Zurich) (aa 89-105) as positive control based on earlier reported identical cross-reactivity
290 (Milde et al., 1989). For sheep, no suitable antibody was available at the time of study.

291 Nuclear immunoreactivity was assessed with a binocular microscope (DMLB®, Leica) with a
292 digital camera (DC480, Leica) (magnification 400x, 5-7 microscopic fields). For each sample,
293 500 cells of the following cell types were evaluated for nuclear staining intensities: surface
294 epithelial cells, intermediate glandular/crypt epithelial cells, superficial glandular/crypt
295 epithelial cells, basal glandular/crypt epithelial cells. Staining intensities of nuclei were scored
296 0, 0.5, 1, 2, 3 for negative, very weak, weak, intermediate, and strong, which is correlated to
297 the absence of brown staining (only blue counter staining), light brown, brown, and dark brown,
298 respectively. From this an immune reactivity score was calculated with the following formula:

299 $$\text{Immune Reactivity}$$
300 $$= 0 \times \text{n(negative)} + 0.25 \times \text{n(very weak)} + 1 \times \text{n(weak)}$$
301 $$+ 4 \times \text{n(intermediate)} + 9 \times \text{n(strong)}$$

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

305

306 Bone mineral density

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

312

313 Statistical analysis

314 The individual animal was the smallest experimental unit. All data analysis was performed with
315 SAS 9.4 (SAS Institute Inc.) applying the procedure MIXED. Repeated measures mixed models
316 were applied to analyze the effects of the fixed variables "diet Ca", "species", "time", and
317 interactions considering the individual animal nested under the respective feeding group as a
318 random factor. Repeated measures mixed modelling involved in any case the mapping of
319 covariance structures appropriate for the respective data structure following guidance by Wang
320 and Goonewardene (2004). Due to the uneven spacing of times of observation for respective
321 datasets, covariance structures like first-order autoregressive (AR(1)) and related structures
322 (e.g., Toeplitz), which require an equal spacing of time, were not considered. The Bayesian
323 Information Criterion (BIC) served as model fit statistic, and we chose the candidate yielding
324 the lowest BIC. The respectively mapped covariance structure for different repeated measures
325 datasets were as follows: compound symmetry (serum Ca, urinary Ca), heterogeneous
326 compound symmetry (BMD), first-order ante-dependent (serum bAP, serum ICTP, serum CTX,
327 serum calcitriol, serum osteocalcin, fecal Ca). Due to the limited availability of suitable
328 antibodies, the VDR protein expression over time was only analyzed in colonic biopsies of
329 goats, therefore no species effect could be estimated.

330 The colostral Ca concentration of sheep and goats was analyzed with an endpoint mixed model
331 with the fixed effect of "diet Ca", "species", and interaction (diet Ca*species), again including
332 the individual animal nested under the respective feeding group as random effect. Birth weights
333 and daily weight gain of the offspring were also analyzed with an endpoint mixed model
334 accounting for the fixed effects of "diet Ca", "species", "sex" and interactions (diet Ca*species,
335 diet Ca*sex) including the individual animal nested under respective mother as random effect.
336 For the analysis of average daily weight gain, the animals' age at the 2nd weighing was included
337 as a continuous control variable to account for potential age-related bias in estimating average
338 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 (Cl[upper, lower]). $P \leq 0.05$ 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 postpartum 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). The BMD 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). The effect sizes and minimum number of replicates required to achieve statistical significance (with a minimum statistical power of $1-\beta = 0.8$ at $\alpha = 0.05$) (McDonald, 2014) were estimated using R version 4.4.2 (The R Project for Statistical Computing). This analysis employed the SIMR package following the approach outlined byGreen and MacLeod (2016), with the modification of using the previously published datasets mentioned earlier 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.

358 **Results**

359 Animal health and development

360 All sheep and goats remained healthy throughout the entire study period with no signs of
361 pathology and hypocalcemia, based on continuous veterinary surveillance. Sheep showed 5
362 cases of triplets, 4 cases of twins and 1 single birth (n = 24 lambs in total) whereas most goats
363 gave birth to twins with 1 case of triplets and 2 cases of single births (n = 23 kids in total). The
364 sex ratio of sheep and goat offspring was (m/f) 0.71 and 1.30, respectively.

365

366 All lambs and kids were born with species-dependent birth weights (4.62 Cl[4.22, 5.01] vs. 5.15
367 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
368 Cl[4.71, 5.41] kg for female vs. male; $P = 0.16$) and unaffected by the mothers' dietary Ca
369 supply antepartum (4.89 Cl[4.53, 5.23] vs. 4.88 Cl[4.54, 5.22] kg at 0.6% vs. 1.3% in DM,
370 respectively; $P = 0.94$) (Figure 1A). However, lambs and kids of mothers fed high Ca
371 antepartum showed significantly lower daily weight gain from milk intake than animals fed low
372 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;
373 $P = 0.02$) irrespective of sex and species, which expressed distinct effects on the animals'
374 growth performance (0.19 Cl[0.17, 0.21 vs. 0.21 Cl[0.19, 0.23] kg/d for female vs. male; $P =$
375 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).
376 Supplementary Table 2 highlights all LSmeans and associated Tukey-adjusted 95% confidence
377 limits of birth weights and daily weight gain of offspring as related to species, sex, and dietary
378 Ca supply of the mothers antepartum.

379

380 Quantitative markers of calcium metabolism

381 Analyzed fecal Ca concentrations during the observation period ranged between 1.46 and
382 5.19 % in DM in sheep as well as 1.17 and 14.9 % in DM in goats whereas the ranges for urinary

383 Ca excretion were 0.005 and 0.41 mmol/mmol creatinine in sheep as well as 0.004 and 1.55
384 mmol/mmol creatinine in goats. Fecal and urinary data had to be log_{10}-transformed to meet the
385 precondition of normality, accounting for fecal Ca ranges of 0.16 and 0.71 log_{10}(% in DM) for
386 sheep as well as 0.07 and 1.17 log_{10}(% in DM) for goats. The respective log-transformed urinary
387 Ca excretion ranges were -2.31 and -0.38 log_{10}(mmol/mmol creatinine) in sheep as well as
388 -2.42 and -0.19 log_{10}(mmol/mmol creatinine) in goats. Supplementary Table 3 highlights all
389 LSmeans and associated Tukey-adjusted 95% confidence limits of fecal Ca concentrations and
390 urinary Ca excretion, expressed in the original measurement scale as related to species, time,
391 and dietary Ca supply antepartum.
392 Higher supply with dietary Ca antepartum significantly increased fecal log_{10}-Ca concentrations
393 until parturition by 1.43fold vs. 2.87fold in sheep vs. goats and in any case significantly above
394 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
395 Cl[0.53, 0.79] log_{10}(% in fecal DM) in 0.6% vs. 1.3% in DM of sheep and goats, respectively;
396 $P < 0.0001$) (Figure 2A). On average, sheep showed lower fecal Ca than goats (0.41 Cl[0.36,
397 0.46] vs. 0.47 Cl[0.43, 0.52] log10(% in fecal DM) for sheep vs. goats; $P = 0.05$) but the
398 response patterns over time were different. Sheep in the low Ca group slightly declined fecal
399 Ca until parturition to 0.64fold of the level at -21 days antepartum, while sheep in the in the
400 high Ca group responded opposite to this as mentioned before (1.43fold increase until
401 parturition). In contrast, both control and high Ca goats increased the levels until parturition but
402 in different magnitudes (1.75fold vs. 2.87fold for low vs. high Ca). This difference in time
403 patterns between species was reflected by a strong statistical interaction between species and
404 time ($P < 0.0001$).
405 In contrast, the feeding regime had no effect on the urinary Ca excretion (-1.04 Cl[-1.22, -0.86]
406 vs. -0.96 Cl[-1.14, -0.79] log10(mmol/mmol creatinine) at 0.6% vs. 1.3% in DM; $P = 0.56$)
407 (Figure 2B). Values fluctuated over time but a clear trend in adaptation of urinary losses was
408 not evident ($P = 0.61$). The only significant effect was in comparison between sheep and goats

409 irrespective of treatment or time, with goats excreting on average 3.26fold the levels of sheep
410 (-1.26 Cl[-1.44, -1.07]vs. -0.75 Cl[-0.91, -0.58] $\log_{10}$(mmol/mmol creatinine) in sheep vs.
411 goats; $P = 0.0003$).

412 Figure 3 shows the response of serum Ca and colostral Ca to varying Ca feeding antepartum.
413 Serum Ca was not affected by the antepartum feeding (2.47 Cl[2.35, 2.59] vs. 2.49 Cl[2.37,
414 2.61] mmol/L at 0.6% vs. 1.3% in DM; $P = 0.80$) and gradually and significantly ($P < 0.0001$)
415 decreased independent of animal species towards the initial 4 days postpartum to gradually
416 increase to previous levels during the further course of the observation period (Figure 3A). In
417 any case, sheep showed on average 1.16fold higher serum Ca levels than goats (2.29 Cl[2.17,
418 2.40] vs. 2.67 Cl[2.54, 2.79] mmol/L in goats vs. sheep; $P = 0.0003$).
419 The colostral Ca concentration (Figure 3B) was 2.35fold higher in sheep than in goats (1.44
420 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
421 the antepartum Ca feeding regime (2.36 Cl[1.58, 3.14] vs. 2.46 Cl[1.67, 3.24] g/L at 0.6% vs
422 1.3% Ca in DM; $P = 0.86$) (Figure 3B).
423 Supplementary Table 4 highlights all LSmeans and associated Tukey-adjusted 95% confidence
424 limits of serum Ca and colostral Ca as related to species, time, and dietary Ca supply
425 antepartum.

426

427 Figure 4 highlights the BMD of dairy sheep and dairy goats from -14 day antepartum to 56 days
428 postpartum, as affected by Ca feeding during the last 3 weeks antepartum, time and animal
429 species. Higher Ca feeding antepartum did not affect BMD (868 Cl[845, 891] vs. 877 Cl[854,
430 900] mg/cm^3 at 0.6% vs. 1.3% in DM; $P = 0.63$), which significantly declined towards
431 parturition from 932 Cl[904, 960] to 864 Cl[807, 922] mg/cm^3 in sheep and 845 Cl[817, 873]
432 to 782 Cl[738, 826] mg/cm^3 in goats, to gradually increase postpartum until day 56 of lactation
433 where it roughly approached in both species the initial level at the beginning of the observation

434 (Sheep: 944 Cl[920, 967] mg/cm^3, Goats: 846 Cl[823, 868] mg/cm^3 $P < 0.0001$). On average,
435 sheep showed 1.08fold higher BMD than goats (839 Cl[808, 853] vs. 913 Cl[888, 938] mg/cm^3
436 in goats vs. sheep; $P < 0.0001$).
437 Supplementary Table 5 highlights all LSmeans and associated Tukey-adjusted 95% confidence
438 limits of BMD as related to species, time, and dietary Ca supply antepartum.

439

440 Serum calcitriol and markers of bone formation

441 Serum calcitriol, bAP and osteocalcin of sheep and goats over time in response to varying Ca
442 feeding antepartum are shown in Figure 5. Serum calcitriol was 1.98fold higher in goats than
443 in sheep (70.2 Cl[56.8, 83.6] vs. 139 Cl[128, 149] pmol/L in sheep vs. goats; $P < 0.0001$) but
444 not different between antepartum feeding groups (103 Cl[90.1, 117] vs. 105 Cl[92.3, 119]
445 pmol/L at 0.6% vs. 1.3% in DM; $P = 0.75$) (Figure 5A). Serum levels significantly changed
446 over time ($P < 0.0001$) which interacted with species ($P < 0.0001$) as follows. In sheep, serum
447 levels reduced towards parturition and fluctuated throughout, whereas in goats serum levels
448 increased until 4 days postpartum to turnaround and approach initial levels until 56 days
449 postpartum.
450 Serum activity of bAP ranged between 38 and 251 U/L in sheep as well as 21 and 117 U/L in
451 goats. After Log_{10}-transformation to meet the precondition of normality, these ranges were 1.58
452 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
453 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
454 no significant effect on bAP (Figure 5B). This was also true for time, though control goats
455 showed a transient numerical drop by ~22% of serum activity around parturition ($P = 0.12$).
456 Sheep showed on average 1.25fold higher serum activities than goats (1.58 Cl[1.49,, 1.68] vs.
457 1.97 Cl[1.888, 2.07] log_{10}(U/L) in goats vs. sheep; $P < 0.0001$).

458 Serum osteocalcin remained stable and comparable between species from -5 days antepartum
459 to 5 days and 7 days postpartum, respectively, after which it gradually increased on average by
460 2.27fold in sheep and 3.23fold in goats until 56 days postpartum ($P < 0.0001$) (Figure 5C). This
461 difference in magnitude was significant between species ($P = 0.007$). Most notably, this increase
462 in serum osteocalcin was significantly higher in those sheep and especially goats who received
463 higher dietary Ca during the last 3 weeks antepartum (29.9 Cl[5.62, 54.1] vs. 39.6 Cl[15.3, 63.8]
464 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,
465 respectively; $P = 0.02$).
466 Supplementary Table 6 highlights all LSmeans and associated Tukey-adjusted 95% confidence
467 limits of serum calcitriol, bAP, and osteocalcin, expressed in the original measurement scale
468 also in the case of bAP, as related to species, time, and dietary Ca supply antepartum.

469

470 Serum markers of bone resorption

471 Serum concentrations of ICTP ranged between 4.87 and 90.2 µg/L in sheep as well as 5.75 and
472 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
473 ng/mL in goats. After Log_{10}-transformation to meet the precondition of normality, these ranges
474 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
475 0.76 and 1.76 log_{10}(ng/mL) as well as -0.46 and 0.94 log_{10}(ng/mL). Supplementary Table 7
476 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of serum ICTP
477 and CTX, expressed in the original measurement scale, as related to species, time, and dietary
478 Ca supply antepartum. Serum ICTP showed no marked changes in both species until parturition,
479 afterwards it significantly increased over the initial 2 days postpartum (1.49fold vs. 1.60fold in
480 sheep vs. goats) and held a plateau until 7 days postpartum from which on the levels gradually
481 reduced over the following weeks to approach initial values at 56 days postpartum ($P < 0.0001$)
482 (Figure 6A). Average serum levels were significantly higher for sheep than goats (1.19 Cl[1.14,

483 1.23] vs. 1.30 Cl[1.26, 1.35] $\log_{10}$(µg/L) in goats vs. sheep; $P < 0.0001$) with a significant
484 interaction between species and time reflecting fluctuations in differences between species over
485 given times ($P = 0.001$). The feeding antepartum did not express any significant effect on serum
486 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
487 1.3% vs. 0.6% in DM; $P = 0.98$).
488 Sheep showed markedly higher serum CTX levels during this study than goats ($P < 0.0001$)
489 (Figure 6 B). In sheep serum levels significantly increased from -3 days antepartum until 2 days
490 postpartum and held a plateau until day 7 postpartum with a subsequent decline during the
491 following weeks approaching initial values at day 57 postpartum. In contrast goats showed not
492 much response until parturition after which it peaked at 2 days postpartum and fluctuated
493 around that plateau for the next 14 days to decline until day 56 with the high Ca group showing
494 the steepest decline. These responses over time were significant ($P < 0.0001$) but did not
495 statistically interact with other variables like diet Ca or species. Neither on average (0.35
496 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
497 time (Diet Ca*Time: $P = 0.64$) did the feeding antepartum affect serum CTX levels in a
498 significant manner.
499

500 Caprine colonic abundance of mucosal vitamin D receptor

501 Figure 7 highlights the statistical evaluation of VDR immune reactivity in caprine colonic
502 mucosa in goats fed 0.6% in DM and 1.3% in DM dietary Ca antepartum. Figure 8 presents
503 exemplary immunohistochemical images from both groups at parturition. Supplementary Table
504 8 highlights all LSmeans and associated Tukey-adjusted 95% confidence limits of VDR
505 immune reactivity, as related to time and dietary Ca supply antepartum.
506 Vitamin D receptor abundance in caprine colonic mucosa was not significantly affected by diet
507 Ca antepartum (2268 Cl[1856, 2680] vs. 2669 Cl[2214, 3125] immune reactivity at 1.3% vs.

508 0.6% in DM; $P = 0.19$). In both groups, the scores showed a curvilinear trend over time which

509 could not be statistically confirmed ($P = 0.1$).

510

511

512 **Discussion**

513 An earlier meta-analysis concluded that the risk for hypocalcemia in dairy cows significantly
514 increases if dietary Ca during the antepartum phase is between 1.1-1.5% in DM (Lean et al.,
515 2006). This hypothesis has yet not been tested in dairy sheep and goats, therefore the present
516 study investigated whether Ca fed at 1.3% in DM (compared to 0.6% in DM) during the last 3
517 weeks antepartum increases the incidence of hypocalcemia antepartum and/or postpartum by
518 dysregulating Ca homeostasis.

519 Effects on quantitative Ca homeostasis and the incidence of hypocalcemia

520 Sheep and goats showed in any case significant differences in the expression of observed
521 measures of quantitative Ca homeostasis (fecal Ca, urinary Ca excretion, colostrum Ca, serum
522 Ca, BMD). Differences in magnitude of these parameters between both species have also been
523 observed in several earlier comparative studies. Our observation of higher values in sheep with
524 respect to urinary Ca excretion and colostral Ca and BMD are in line with earlier studies
525 (Liesegang et al., 2006, 2007, Liesegang, 2008). We made the same observation with serum Ca,
526 which agrees to Kohler et al. (2013) and Liesegang (2008), though Wilkens et al. (2014)
527 observed the opposite with goats expressing higher values than sheep. The reason for this
528 discrepancy is unclear at the moment. Interestingly, both species exhibited a drop in serum Ca
529 at 3 and 4 days postpartum, respectively. Similar response patterns have recently been described
530 in dairy cows, particularly for Zn balance and serum levels (Daniel et al., 2023). It is possible
531 that the serum Ca pattern observed in the present study mirrors a transient drop in Ca retention
532 at the onset of lactation, comparable to the patterns observed for Zn in dairy cows. However,
533 due to lack of data this interpretation remains speculative for the present experimental
534 conditions and requires confirmation in future studies.

535 The feeding showed mostly no or just numerical effects on quantitative measures. Increasing
536 dietary Ca feeding during the antepartum phase significantly increased fecal Ca concentration

537 by at least 2fold during the last 3 weeks but did not significantly affect serum and colostral Ca
538 concentration and urinary Ca excretion in both species at any given point during the study. Most
539 notably, no signs of hypocalcemia or any pathology were noted antepartum or postpartum,
540 respectively, although according to Oetzel (1988), dairy sheep and goats are expected to
541 develop milk fever symptoms comparable to cows but at 1-3 weeks postpartum. However, since
542 1988, advancements in milk yields and the associated calcium losses suggest that feeding
543 regimes promoting milk fever may now be more likely to trigger the condition and potentially
544 earlier than originally described by Oetzel. Literature on the effects of Ca oversupply in sheep
545 and goats is scarce, particularly with respect to the antepartum phase. However, the only study
546 on sheep by Braithwaite (1979)agrees with our observations. The colleague studied gradual
547 differences in dietary Ca intake in adult sheep and observed increasing fecal Ca losses.
548 Fractional Ca absorption (% of intake) was at 31.8 % at the lowest Ca supply (0.22 % in DM)
549 and dropped to a plateau around ~9-10% with gradually increasing diet Ca to 0.6, 1.2, and 2.4%
550 in dietary DM, respectively. In parallel, bone Ca resorption dropped inversely to the increase in
551 Ca intake and inevitable unregulated absorption of surplus Ca was increasingly redirected into
552 the gastrointestinal tract. Given the response of our sheep and goats in the present study, it may
553 be concluded that for our animals and under the present study conditions, we can reject our
554 initial hypothesis of increased milk fever risk when feeding higher Ca in the range proposed by
555 Lean et al. (2006) for dairy cows. It remains to be tested what the outcome would be when
556 feeding a different Ca source, for example a source with a higher water solubility like $CaCl_2$
557 (Goff and Horst, 1993). Neither sheep nor goats showed any relevant response in urinary Ca
558 excretion in response to the feeding regime over time, confirming once more results from earlier
559 studies suggesting livestock ruminants do regulate Ca primarily via the gastrointestinal tract
560 (Herm et al., 2015, Wild et al., 2021). We assume that the animals were able to dampen uptake
561 from the gastrointestinal tract and, in parallel, effectively rerouted unavoidable unregulated Ca
562 uptake towards excretory pathways, as described by Braithwaite (1979). Future studies must

563 identify the precise physiological mechanisms behind this effective compensation of dietary Ca
564 overload. See in this context also our discussion below, on potentially confounding factors
565 associated with our study. Furthermore, the functional basis for the differences in fecal Ca
566 accumulation between the 2 species remains to be investigated. Initially, goats exhibited lower
567 fecal Ca levels at -21 days antepartum, but this trend reversed until parturition. The underlying
568 causes are currently speculative and may involve differences in dry matter digestibility or the
569 regulation of Ca absorption at the gut barrier. Future studies should explore the effects of high
570 Ca feeding on gastrointestinal pH, as well as the digestive and absorptive capacities for dry
571 matter and minerals, to provide a more comprehensive understanding of this phenomenon.
572

573 Effects on serum calcitriol and markers of bone formation and resorption

574 We observed a core set of serum bone formation and resorption markers, which have been
575 earlier established in ruminants from gestation into lactation (Liesegang et al., 2000, Liesegang
576 et al., 2007). Observed measures of bone formation and resorption showed changes over time,
577 that have earlier been attributed to the species-dependent adaption towards parturition and the
578 onset and further course of lactation (Liesegang et al., 2006, 2007, Liesegang, 2008, Kohler et
579 al., 2013, Wilkens et al., 2014). However, none of the observed parameters showed a significant
580 response to the feeding treatment during the final weeks of the antepartum and the majority of
581 the postpartum period. Consequently, the BMD was not affected and suggests the feeding
582 intervention with 1.3% in DM Ca in the diet caused no obvious dysregulation of Ca and bone
583 homeostasis in either species as compared to the lower Ca feeding. However, serum osteocalcin
584 showed a significant gradual increase in concentration from d7 to d56 of lactation in animals
585 receiving the high dietary Ca antepartum. This response was true for both species but most
586 pronounced in the dairy goats. Serum osteocalcin is considered a marker of bone formation,
587 with increasing levels pointing towards rising embedding of Ca in the skeleton (Liesegang et

588 al., 2000). This observation was puzzling given the lack of response of calcitriol as well as
589 markers of bone formation (calcitriol, bAP) and resorption (ICTP, CTX)) and especially since
590 the effect occurred 8d after the suspension of the high Ca feeding. To the best of our knowledge
591 such a response pattern has not yet been reported for serum osteocalcin and it raises questions
592 on its physiological purpose. The rise in serum osteocalcin was not associated with changes in
593 BMD (or mineral concentration; data not shown) nor did other markers of bone formation show
594 an orchestrated response that would suggest any feeding-associated changes in bone
595 metabolism. Therefore, it may be necessary to have a look at osteocalcin from a different
596 perspective. Biomedical research efforts in recent years suggest a hormonal role of
597 uncarboxylated osteocalcin beyond bone metabolism, involving among others the regulation of
598 systemic energy metabolism (Lee et al., 2007), muscle energy metabolism (Mera et al., 2016),
599 and brain development (Obri et al., 2018). At this point, the meaning of these results is up to
600 speculation and more targeted research on the matter is urgently needed.
601

602 Effects on offspring development

603 High Ca treatment of mothers antepartum was associated with decreased average daily weight
604 gain of suckling lambs and kids. Since the offspring showed no differences in birth weight that
605 were associated to the feeding of the mothers antepartum, we rule out quantitative effects on
606 placental nutrient transfer. The most plausible explanation for reduced offspring growth during
607 the suckling phase is an impairment of milk yield and/or quality. Feed intake was not affected
608 in the present study and all animals consumed the necessary dietary dry matter according to
609 recommendations. At the same time, we noted differences in serum osteocalcin in the higher
610 Ca groups, pointing towards a distinct physiological conditioning. This likely has affected the
611 quantity and/or quality of secreted milk and therefore the growth response of their offspring,
612 but this needs yet to be confirmed. To the best of our knowledge there is yet no data available

613 in dairy sheep and goats that allows us a more informed discussion on this matter and future
614 studies must put a focus on the potential effects of the present experimental conditions on milk
615 yield and quality as well as lactation physiology. In addition, a further hypothesis may deserve
616 some attention in future studies. High Ca feeding during the last 3 weeks antepartum and the
617 associated physiological adaption of the mothers may have promoted fetal programming in the
618 offspring that impaired their digestive capacity for Ca (and maybe other nutrients) postpartum
619 and growth response. The possibility of mineral nutrition during gestation to induce fetal
620 programming in ruminants either for the benefit or disadvantage of the offspring has recently
621 been reviewed by Anas et al. (2023) for cattle, highlighting a multitude of already established
622 interactions as well as future routes for research on the matter. In this context, an earlier study
623 in rats tested the effects of varying dietary Ca supply during gestation including higher supply
624 at 1.2% in DM (Li et al., 2018). The colleagues observed abnormal expression of hepatic and
625 adipose genes in the offspring as well as dyslipidemia and hepatic lipid accumulation in
626 response. Unfortunately, no data on Ca homeostasis in rat mothers and their offspring was
627 obtained. Nevertheless, it may be argued that high supply of a mineral over several weeks may
628 induce epigenetic changes in the mother, which could be inherited to the offspring. It may
629 therefore be interesting to have a closer look on the epigenetic consequences of high dietary Ca
630 as well as opposite feeding regimes during gestation and their potential to imprint Ca
631 homeostatic regulation and development of the offspring postpartum.
632

633 <u>The geographical region and other potentially confounding factors</u>

634 Depending on the geographic location, Ca levels in the environment are quite variable. In
635 Switzerland, animals are often confronted with high levels of native Ca in diets, originating
636 from the Ca-rich geological formations, which shape the chemical composition of soil in certain
637 areas. For example an earlier survey of Schlegel et al. (2016) found a mean Ca concentration

638 of 7 g/kg DM in 236 samples of mixed grassland from Posieux, Switzerland, and our own
639 experience with grass hay qualities delivered to our animal facility in the Zurich area are in line
640 with this observation. This leads to quite relevant intake levels of native Ca from feed
641 particularly when grass-based roughage is fed, even when mineral Ca supplements are not
642 applied. Since the organism attempts to maintain Ca homeostasis by fine-tuning absorption,
643 losses, and mobilization of skeletal reserves according to the environmental conditions (Martín-
644 Tereso and Martens, 2014, Wilkens and Muscher-Banse, 2020), it may be argued that sheep and
645 goats raised predominantly on Ca-rich roughage may be better suited to deal with high dietary
646 Ca challenge. However, it may also suggest that during the feeding of high dietary Ca due to
647 active supplementation of inorganic Ca to such raw materials, a metabolic imprinting as
648 hypothesized earlier may more likely promote the observed adverse effects on daily weight gain
649 of suckling lambs and goat kids. Therefore, the present results must be considered with caution
650 when it comes to the general translation into dairy sheep and goat feeding systems. More data
651 is urgently needed also from regions with less Ca in the soil.

652 This study is among the earliest to compare high Ca responses between sheep and goats. While
653 many biomarkers in our findings align with previous research on bone metabolism and calcium
654 homeostasis principles within and across these species, our study is too preliminary to draw
655 definitive conclusions regarding species-specific effects under the current treatment conditions.
656 Given that "species" is an intrinsic characteristic, it cannot be effectively randomized within a
657 single study limited to 1 herd per species. However, our findings represent an initial step toward
658 further research in this area, underscoring the need for not only replication of these experimental
659 conditions but also replication across multiple herds to strengthen the reliability of observed
660 responses.

661 The current Swiss feeding recommendations for sheep and goats suggest comparable dietary
662 Ca levels during the last 3 weeks antepartum (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 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 antepartum Ca feeding of the mothers. Nevertheless, this is the 1st 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 hypocalcemia in sheep and goats challenged with higher Ca during the last 21 days antepartum. However, serum osteocalcin showed increasing concentrations in lactating sheep and goats fed higher Ca antepartum 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 antepartum 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 antepartum 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.

Supplemental material for this article is available at

http://dx.doi.org/10.13140/RG.2.2.32486.33601.

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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Table 1. Chemical composition and daily intake levels of feedstuffs fed during the last 21 days antepartum 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
Ether extract, 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
antepartum				
Grass hay (2nd cut)	2.00	2.00	2.00	2.0
[3,4]Concentrate	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
postpartum				
Grass hay (2nd cut), kg/d	2.40	2.40	2.40	2.40
[3,4]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 $CaCO_3$.

[4]During antepartum 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.

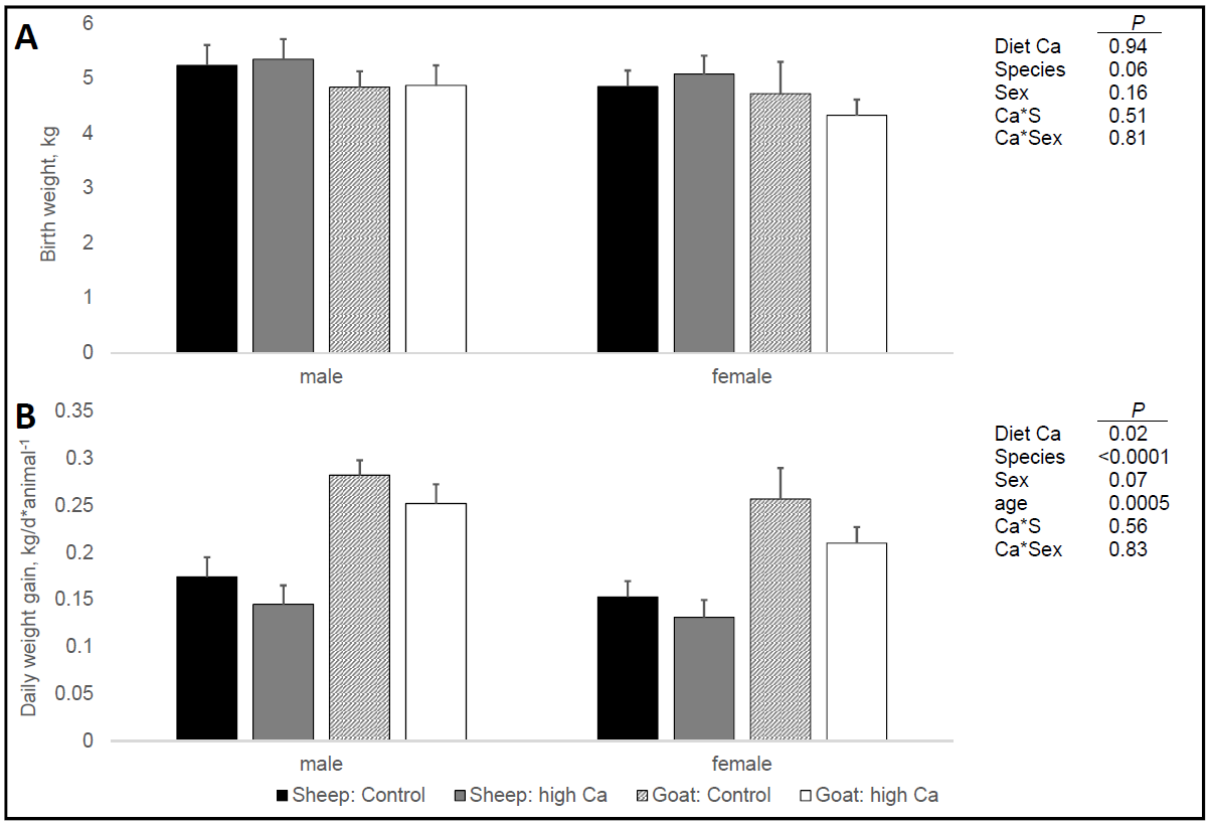

Figure 1. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks antepartum as well as sex and species of offspring on the birth weight (A) and daily weight gain during the suckling phase (B). Effective sample size was 5 sheep and 6 goats per group, which 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 2nd weighing during the suckling phase, which differed between individual animals due to management reasons (range: 33-59 days postpartum) 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.

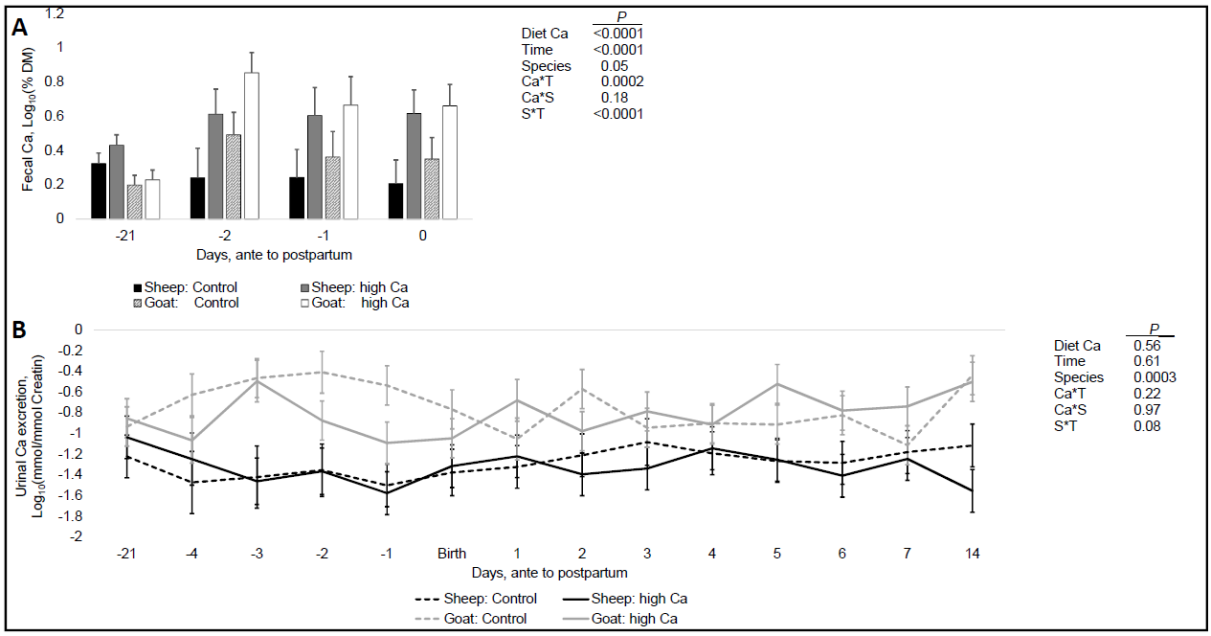

Figure 2. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks antepartum as well as time and animal species on the fecal Ca concentration (A) and urinary Ca excretion (B) from 3 weeks antepartum to parturition and 14 days postpartum, respectively. Effective 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.

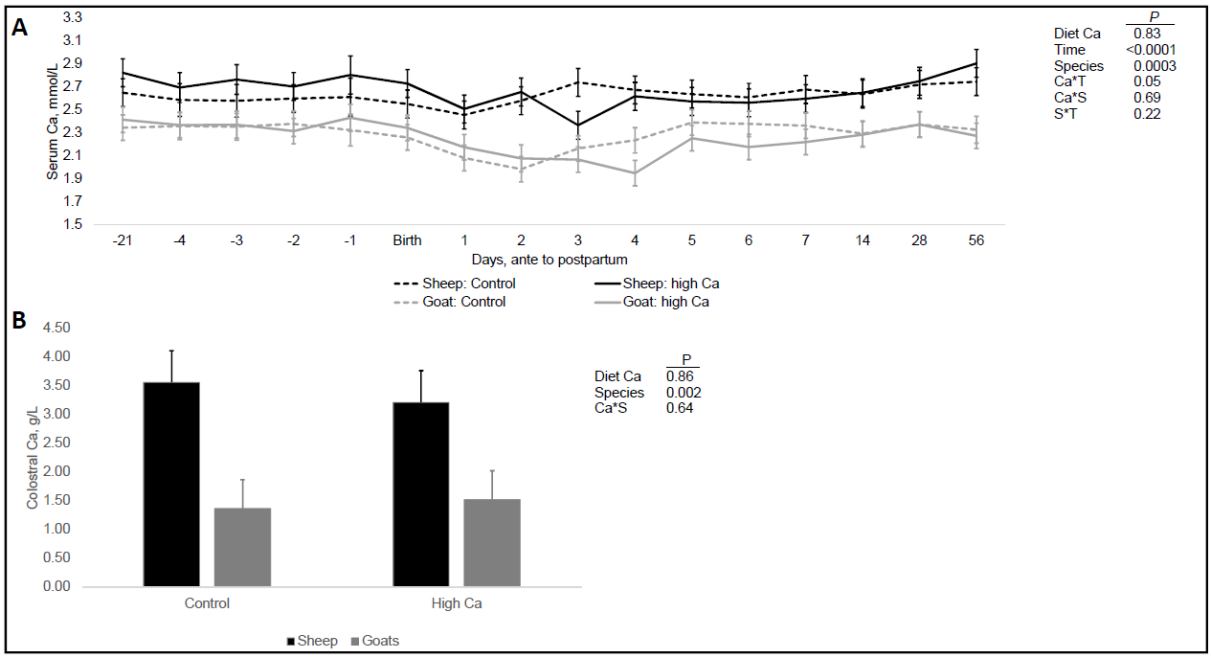

Figure 3. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks antepartum as well as time and animal species on serum Ca (A) and colostral Ca (B) from 3 weeks antepartum to parturition and 56 days postpartum, respectively. Effective sample size was 5 sheep 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.

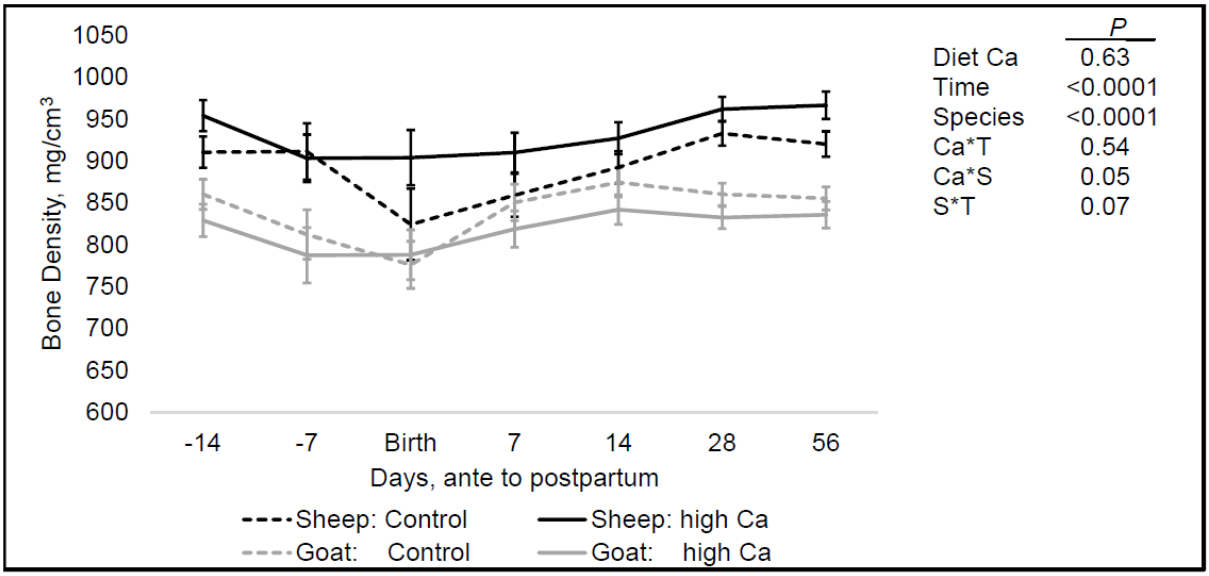

Figure 4. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks antepartum as well as time and animal species on BMD from 2 weeks antepartum to 56 days postpartum. Effective sample size was 5 sheep and 6 goats per group. Data is presented as least square 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.

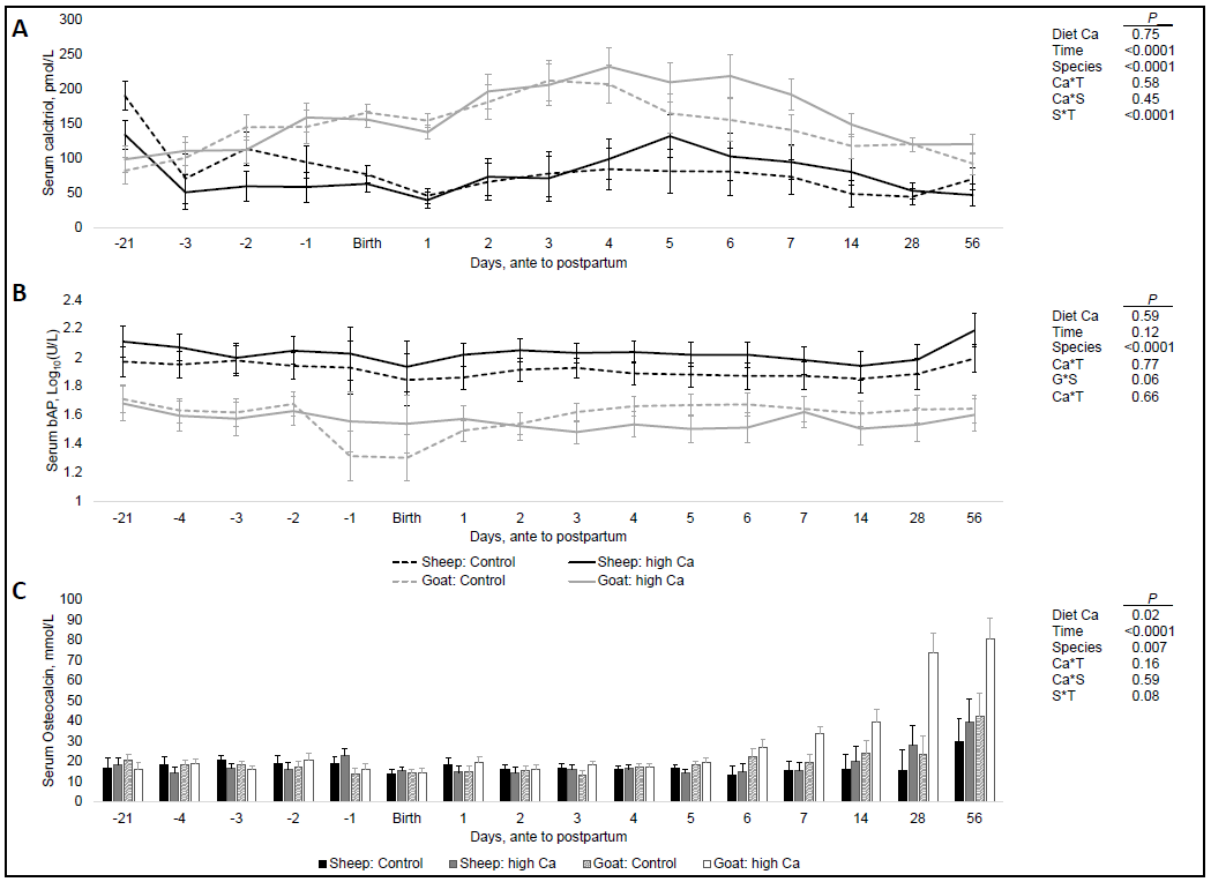

Figure 5. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks antepartum as well as time and animal species on serum calcitriol (A), serum bAP (B), and serum osteocalcin (C) from 3 weeks and 5 days antepartum, respectively, to 56 days postpartum. Effective sample size was 5 sheep and 6 goats per group. Data is presented as least square mean values (Log_{10}-scale in case of bAP 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 for analysis of calcitriol at -4 days antepartum, we cannot provide the respective data.

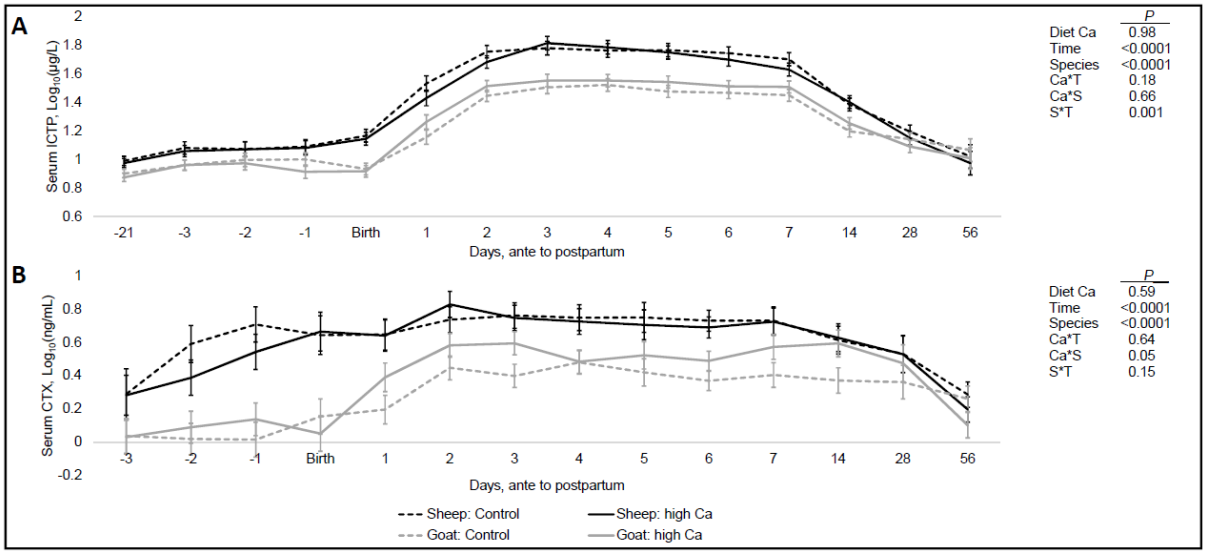

Figure 6. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks antepartum as well as time and animal species on serum ICTP (A) and serum CTX (B) from 3 weeks and 3 days antepartum, respectively, to 56 days postpartum. Effective sample size was 5 sheep and 6 goats per group. Data is presented as least square mean values (Log_{10}-scale in case of bAP 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 CTX at -21 days antepartum and for ICTP and CTX at -4 days antepartum, we cannot provide the respective data.

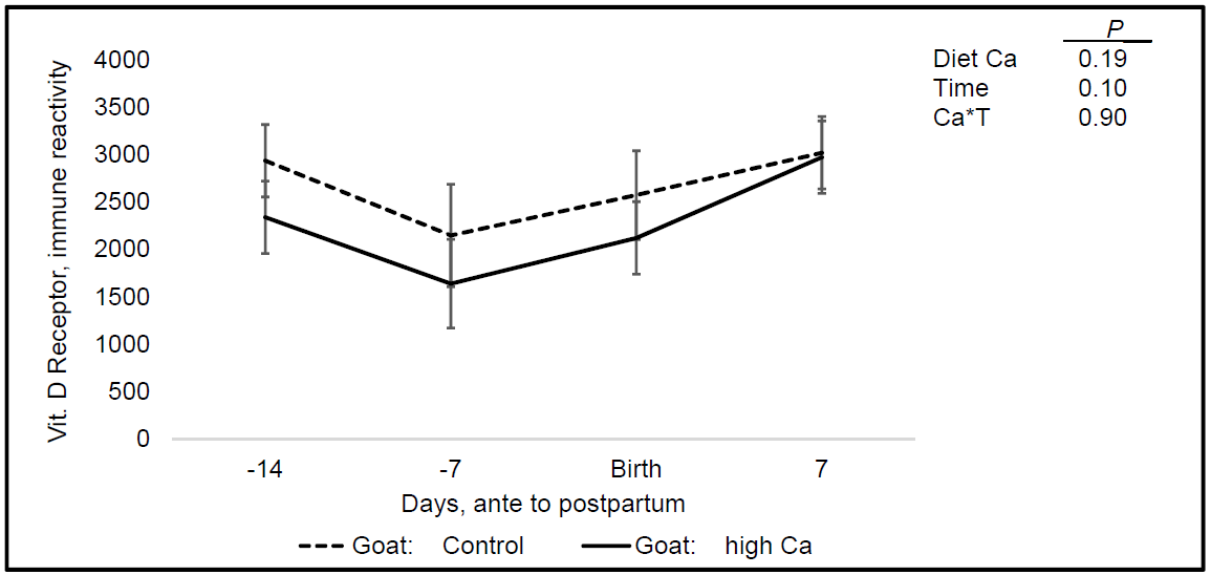

Figure 7. Effects of varying dietary Ca (0.6 vs. 1.3% in DM) of dairy sheep and goats during the last 3 weeks antepartum as well as time on caprine vitamin D receptor abundance in colonic mucosa from 2 weeks antepartum to 7 days postpartum. Effective sample size was 6 goats per group. Data is presented 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.

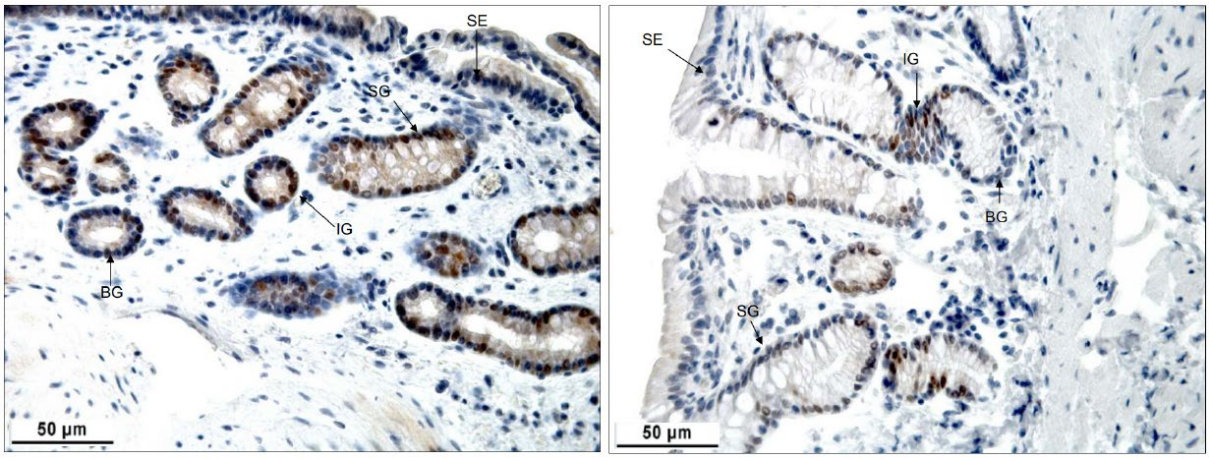

Figure 8. Exemplary immunohistochemical staining of the caprine VDR in the colon of goats at parturition, fed 0.6 % in DM (left) or 1.3 % in DM (right) dietary Ca during the last 3 weeks antepartum. Arrows mark SE, surface epithelium; SG, surface gland sections; IG, intermediate gland sections; BG, basal 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 antepartum showed reduced immune reactivity to VDR staining, though this could not be statistically confirmed. Figure 7 shows the statistical analysis of VDR immune reactivity over time from -14 days antepartum to 7 days postpartum.