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**Effects of Diets differing in Dietary Cation-Anion Difference and Calcium
Concentration on Calcium Homeostasis in Neutered Male Sheep**

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Summary

The aim of the present study was to demonstrate basic effects of negative dietary cation-anion difference (DCAD) diets against a background of different calcium (Ca) supplies in a sheep model. Twenty-three male castrated East Friesian milk sheep were randomly assigned to four different feeding groups: The ration of each group was either high or low in DCAD (highDCAD/lowDCAD) combined with adequate or restricted Ca supply (nCa/lowCa). At baseline, serum and urine was collected from all sheep and a peripheral quantitative computed tomography (pQCT) of the left metatarsus was performed. After a 14-day adaptation period to the different diets, urine, faeces and serum were collected on days 0, 4, 7, 14, and 22, and pQCT was performed on days 0 and 22. LowDCAD was significantly associated with lower urine pH, higher urinary Ca excretion, higher ionized Ca in blood, and higher serum Ca concentrations. Blood pH and bone parameters did not differ significantly between groups. It is unclear from which compartment the high amounts of Ca excreted with urine in the lowDCAD groups originated. Interestingly, lowDCAD resulted in higher renal mRNA abundance of parathyroid hormone receptor (PTHrP) but unaffected mRNA abundance of Ca transporters. As neither renal abundance of these transporters nor Ca excretion were influenced by dietary Ca supply, our results support the hypothesis that increased urinary Ca observed with low DCAD diets represents a loss rather than an excretion of surplus Ca.

Key words: calcium, dietary cation-anion difference, hypocalcaemia, parathyroid hormone receptor, sheep

Zusammenfassung

Das Ziel der vorliegenden Studie war, grundlegende Effekte von Rationen mit negativer Kationen-Anionen Bilanz (DCAD) in Abhängigkeit von der Kalzium(Ca)-Versorgung in einem Schafmodell zu demonstrieren. Dreiundzwanzig männlich kastrierte Ostfriesische Milchschafe wurden zufällig vier verschiedenen Fütterungsgruppen zugeordnet: highDCAD und lowDCAD kombiniert mit bedarfsdeckender oder eingeschränkter Ca-Versorgung (nCa/lowCa). Von allen Schafen wurde Serum und Urin gewonnen und eine periphere quantitative Computertomographie (pQCT) des linken Metatarsus durchgeführt. Nach einer 14-tägigen Anfütterungsphase wurden Urin, Kot und Serum an den Tagen 0, 4, 7, 14 und 22 gesammelt. pQCT wurde an den Tagen 0 und 22 durchgeführt. LowDCAD war mit einem signifikant niedrigeren Urin-pH, einer höheren Urin-Ca-Ausscheidung, höheren ionisiertem Ca und Serum-Ca-Konzentrationen verbunden. Blut-pH und Knochenparameter unterschieden sich nicht signifikant zwischen den Gruppen. Es ist unklar, woher die hohen mit lowDCAD in den Urin ausgeschiedenen Ca-Mengen stammten. LowDCAD führte zu einer höheren renalen mRNA-Abundanz des Parathormonrezeptors (PTHrP), aber zu keiner Veränderung der mRNA-Abundanz von Ca-Transportern. Da weder die renale Abundanz dieser Transporter noch die Ca-Ausscheidung durch die Ca-Aufnahme beeinflusst wurde, unterstützen unsere Ergebnisse die Hypothese, dass das mit lowDCAD in den Urin ausgeschiedene Ca eher einen Verlust als eine Ausscheidung von überschüssigem Ca darstellt.

Schlüsselwörter: Kalzium, negative Kationen-Anionen Bilanz, Hypocalcämie, Parathormonrezeptor, Schafe

Manuskript

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Effects of Diets differing in Dietary Cation-Anion Difference and Calcium Concentration on Calcium Homeostasis in Neutered Male Sheep

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ABSTRACT

Feeding low dietary cation-anion difference (DCAD) diets is one strategy to prevent milk fever in cows. The mechanism of action as well as whether the calcium (Ca) supply of such diets combined with this feeding regimen should meet the requirements, are still unclear. Small ruminants are commonly used as models for cows. The goal of the present study was to demonstrate basic effects of DCAD against a background of different Ca supplies in a sheep model. Twenty-three male castrated East Friesian milk sheep, aged 11-12 months, were randomly assigned to four different feeding groups. The ration of each group was either high or low in DCAD (highDCAD/lowDCAD) combined with adequate or restricted Ca supply (nCa/lowCa). At baseline, serum and urine was collected from all sheep and a peripheral quantitative computed tomography (pQCT) of the left metatarsus was performed. After a 14-day adaptation period to the different diets, the experiment started (day 0). Urine, faeces and serum were collected on days 0, 4, 7, 14, and 22, and pQCT was performed on days 0 and 22. On day 22, the sheep were sacrificed and sampled for functional studies. LowDCAD was significantly associated with lower urine pH, higher urinary Ca excretion, higher ionized Ca in blood, and higher serum Ca concentrations. Blood pH and bone parameters did not differ significantly between groups. It is unclear from which compartment the high amounts of Ca excreted with urine in the lowDCAD groups originated. Interestingly, lowDCAD resulted in higher renal mRNA abundance of parathyroid hormone receptor (PTHrP) but unaffected mRNA abundance of Ca transporters. As neither renal abundance of these transporters nor Ca excretion were influenced by dietary Ca supply, our results support the hypothesis that increased urinary Ca observed with low DCAD diets represents a loss rather than an excretion of surplus Ca.

Key words: calcium, dietary cation-anion difference, hypocalcaemia, parathyroid hormone receptor, sheep

INTRODUCTION

Adapting to the enormous calcium (**Ca**) demand and maintaining required Ca plasma concentrations in late gestation, or at the initiation of lactation, is challenging for many mammals including ruminants. The challenge mainly arises in late gestation due to the calcification of the foetal skeleton, and/or after birth due to excessive Ca losses via milk; the former is more often observed in sheep, and the latter in dairy cows and goats (Oetzel et al. 1988, Pugh 2002, Brozos et al., 2011). Nevertheless, at the time of suddenly increased Ca demand, Ca homeostasis mechanisms are not fully effective. Some studies suggest that cows require adaptation periods of 48 h (Goff et al., 1986; Martin-Tereso and Verstegen, 2011). Consequently, with a prevalence of 49 % (resp. 5.7%) in the animals starting the 3rd lactation, the risk of subclinical (resp. clinical) hypocalcaemia (milk fever, parturient paresis) remains high in German dairy herds (Venjakob et al., 2017). Hypocalcaemia is known to be a gateway disorder predisposing the animals for different secondary diseases such as mastitis, retained fetal membranes, displaced abomasum, dystocia, and ketosis (Curtis et al., 1983; Goff, 2008; Martinez et al., 2012), resulting in animal welfare issues as well as large financial expenses.

In the last decades, many strategies have been proposed to prevent milk fever and subclinical hypocalcemia. The method of feeding anion-rich diets, first recommended by Ender et al. (1971) has received considerable attention in the past (Block, 1984; DeGaris and Lean, 2008; Goff, 2008). Feeding a diet with dietary cation-anion difference (**DCAD**) values between -50 and -150 mEq/kg DM during the last 10-28 days of gestation results in a higher intestinal absorption of the “strong” anions in comparison with their corresponding cations (Horst et al., 1997; DeGaris and Lean, 2008). This causes a compensated metabolic acidosis, increasing the sensitivity of target tissues to parathyroid hormone (**PTH**), thus increasing the available Ca in blood (Goff et al., 1991; 2014). Furthermore, the metabolic acidosis generates a hypercalciuria as a result of an increased Ca clearance from the blood, and thus Ca fluxes are increased systemically. Another strategy is feeding Ca restricted diets 3 weeks prepartum and is based on

the principle above: low Ca intake creates a Ca deficit and prompts metabolic adaptation of renal, intestinal and bone tissue via PTH and 1,25-dihydroxyvitamin D to restore Ca homeostasis (Martin-Tereso and Verstegen, 2011). However, many details of the basic mechanisms caused by low DCAD diets, as well as restrictive Ca diets are still unclear in ruminants. Moreover, different studies considering the incidence of hypocalcaemia and analyzing the influence of dietary Ca levels on DCAD came to controversial conclusions (Horst et al., 1997; Lean et al., 2006; Liesegang et al., 2007; 2008). Additionally, there are conflicting reports of the apparent Ca digestibility in cows fed diets low in DCAD (Leclerc and Block, 1989; Abu Damir et al., 1994; Liesegang et al., 2007), which might be associated with different dietary Ca levels (Lomba et al., 1978).

It was the purpose of our experiment to study basic effects of different DCADs along with varying Ca supply on Ca homeostatic mechanisms in a sheep model. The sheep serves as a small model animal and represents the dairy cow in this experiment, where milk fever is of greater economic interest. Our main objective was to prove the hypotheses that feeding a diet low in DCAD increases the fractional excretion (**FE**) of Ca independently of dietary Ca supply, while Ca transport mechanisms and apparent digestibility are altered by both factors, DCAD and dietary Ca. It was of particular interest to test if the abundance of receptors and transport proteins crucial for Ca homeostatic mechanisms are affected by the different experimental diets. In order to test these hypotheses, we created four different sheep feeding groups, which were provided diets differing in DCAD (high/low) and Ca (normal/low), measured relevant Ca associated parameters including ruminal and jejunal Ca fluxes via Ussing chamber technique and analyzed renal and intestinal Ca homeostasis mechanisms on RNA level. To avoid any interferences with the reproductive cycle and in order to focus the DCAD research on functional basic effects, we used wethers: neutered, male sheep.

MATERIALS AND METHODS

This study was approved by the Cantonal Veterinary Office of Zurich, based on the animal welfare law of Switzerland (approval ZH 092/16).

Animals

Twenty-three male castrated East Friesian milk sheep, aged 11-12 months, were randomly assigned to four different feeding groups. The concentrate of each group was modified for DCAD and Ca content. Groups were kept in pens on wood shavings with free access to water and a salt lick stone (AGROSAL D-Heilbronn; saline including 38.5% sodium, additive iodine 100 mg/kg) with a photoperiod from 7 am to 7 pm. Prior to the experiment (from weaning until the start of the experiment) all sheep were fed a conventional diet consisting of hay and concentrate (Melior 9 2921C Combifloc, Meliofeed AG, Herzogenbuchsee, Switzerland) according to the requirements. DCAD was not calculated for that diet. Day-14 represents the start of the 14-day adaptation period, in which the amount of experimental concentrate and straw was slowly increased to the experimental level for each group. After the 14 day adaptation period, the experiment started (day0). Day-14 was used as the baseline throughout the experiment, as all animals received the same diet until that time. The sheep were sacrificed after 21 days on the full experimental diet. Weight (**W**) and Body Condition Score (**BCS**) were checked once per week. The sheep were shorn before the experiment started.

Feeding Regimen

The diet consisted of straw and concentrate. After lab analysis of the contents of straw, four special concentrates were designed, containing different DCAD values and Ca concentrations (Table 1) by using the following formula for the DCAD: $DCAD = [(Na^{++} K^{+}) - (Cl^{-} + SO_4^{2-})]$ in mEq/kg DM (Wang and Beede, 1992).

Each group's diet met the minimum nutritional requirement recommended by Agroscope (Agroscope, 2016), except Ca in the low Ca groups (38.5% of recommended requirement, Table 1).

Table 1. Daily allowance (kilogram dry matter) and chemical composition (g/kg dry matter) of the feed mixtures used (complete diet). Different sheep feeding groups received either a diet high or low in dietary cation-anion difference (highDCAD/ lowDCAD) combined with normal or low Calcium (Ca) supply (nCa/ lowCa)

Intake [g/kg DM]	highDCADnCa	highDCADlowCa	lowDCADnCa	lowDCADlowCa
Daily allowance				
NEL (MJ/kg TS)	9.42	9.26	9.52	9.50
Straw	718.7	718.7	718.7	718.7
Concentrate	625.3	491.9	622.6	571.2
Barley flakes	124.0	212.5	141.7	177.1
Anionic salt (g/d) (ammonium chloride)			3.0	3.0
Chemical composition (complete diet)				
Dry matter [g/kg oS]	924.3	924.9	891.1	922.7
Crude ash	89.1	74.2	96.1	83.5
Crude protein	110.4	104.1	115.5	110.0
Crude fat	23.9	22.5	24.8	23.7
Crude fibre (CF)	236.7	241.4	250.5	244.4
ADF ²	312.1	317.4	327.8	320.8
NDF ³	509.7	517.1	531.5	521.8
Ca	6.8	2.6	6.9	2.6
Na	2.6	2.8	2.3	2.2
K	24.6	22.7	12.0	11.5
P	3.3	3.4	3.4	3.3
S	8.8	8.1	9.1	8.4
Cl	1.6	1.6	6.2	5.5
DCAD [meq/kg DM] ¹	150	152	-335	-289
VitD3 (IE/kg DM)	508.8	400.1	506.4	459.2
ADL ⁴ (% DM)	4.33	4.32	4.18	4.35

¹ DCAD, dietary cation–anion difference, calculated as [(meq Na⁺/kg DM + meq K⁺/kg DM) - (meq Cl⁻/kg DM + meq SO₄²⁻/kg DM)] (Wang and Beede, 1992).

² ADF, acid detergent fibre [g/kg DM] = 1.14 CF [g/kg DM] + 42.2, (r = 0.93) (Kamphues et al., 2004).

³ NDF, neutral detergent fibre [g/kg DM] = 1.58 CF [g/kg DM] + 135.7, (r = 0.88) (Kamphues et al., 2004).

⁴ ADL, acid detergent lignin (van Soest et al., 1991).

The concentrate was manufactured at Agroscope, Posieux, Switzerland. The sheep were fed individually according to weight and group, twice per day out of plastic buckets tied up during feed intake. The diet was calculated such that the daily weight gain of the sheep was 150 g.

Sampling Regimen

Before the adaptation period (**day-14**), serum and urine were collected from all sheep. Furthermore, a peripheral quantitative computed tomography (**pQCT**) of the left metatarsus was performed. After the 14-day adaptation period to the new diet, the experiment started (**day0**). Urine, faeces and serum were collected on days 0, 4, 7, 14 and 22 and pQCT was performed on days 0 and 22.

Spontaneous urine and faecal samples were collected in the morning (before feeding) in blank plastic cups and frozen at -20°C until analysis. The urine pH was measured directly after sampling by a pH-meter (827 pH lab, Metrohm AG, Herisau, Switzerland).

Blood (2x 5ml) was drawn using a Vacutainer system (BD Vacutainer, SST II Advance, Becton, Dickinson and Company, NJ, USA) after urine sampling from a vena jugularis externa, allowed to clot at room temperature and centrifuged 30 min after withdrawal (Sigma Laborzentrifugen Type 2-15, 3,000 x g, 15 min, room temperature). Serum was pipetted into plastic containers and stored at -20°C and -80°C until analyses were performed.

On **day22** the animals were sacrificed at the slaughterhouse of the Tierspital Zurich. Different samples were collected immediately post mortem: the rumen was dissected and a piece of the ventral rumen wall was manually stripped from the underlying submucosal and muscle layers, rinsed in physiological saline (0.9%), and placed in buffer solution (please refer to Wilkens et al., 2011) until it was finally mounted into Ussing chambers. Jejunum (central part) was longitudinally cut, washed in physiological saline, and was also mounted into Ussing chambers. Directly after opening the rumen a sample of ruminal fluid was collected, centrifuged (3,000 x g, 15 min, room temperature), and stored at -20°C. Renal cortex, duodenal and jejunal mucosa were collected, frozen in liquid nitrogen, and stored at -80°C until further analysis.

The left metatarsus was isolated from the adherent tissue and stored at -20°C. The ionized blood Ca and blood pH levels were measured by i-STAT® Analyzer (Abbott Point of Care Inc., Princeton, NJ, USA) according to manufacturer's instructions immediately after sacrifice.

Analyses of Serum, Urine and Ruminal Fluid

Concentrations of (**Ca**) in serum, urine and ruminal fluid were analyzed by flame technique of atomic absorption spectrometry using High- Resolution Continuum Source Atomic Absorption Spectrometer ContrAA 700 (Analytik Jena, Germany). The measurements were performed at $\lambda = 422.7$ nm.

Analysis for phosphate (**PHOS**) and creatinine (**Crea**) was performed via colorimetry using an autoanalyzer (Cobas Mira Roche-autoanalyzer, F. Hoffman-La Roche Ltd., Basel, Switzerland) and commercial test kits (PHOS, testkit PHOS DIA00620, ammonium molybdate, Diatools AG, Villmergen, Switzerland, intra- and interassay coefficients of variation 0.86 and 1.07%, sensitivity 0.07 mmol/L; Crea, testkit CREA JAFFE DIA00540, Diatools AG, Villmergen, Switzerland, intra- and interassay coefficients of variation 0.83 and 0.85%, sensitivity 18 $\mu\text{mol/L}$).

The following parameters were analyzed by using commercial kits as described by Liesegang et al. (2003): Osteocalcin (**OC**, MikroVue Osteocalcin EIA Kit; Quidel Corporation, San Diego, CA, intra- and interassay coefficients of variation 4.8 and 4.8%, sensitivity 0.45 ng/mL), serum Crosslaps (**SCL** Serum CrossLaps (CTX-I) ELISA, Immunodiagnostic Systems GmbH, intra- and interassay coefficients of variation 1.7 and 2.5%, respectively, sensitivity 0.020 ng/mL), **1,25vitD** (1,25-Dihydroxy Vitamin D RIA, Immunodiagnostics Systems GmbH, intra- and interassay coefficients of variation 9.1 and 9.6%, respectively, sensitivity <8 pmol/L; cross reactivity with 1,25-dihydroxyvitamin D₃ 100%, with 1,25-dihydroxyvitamin D₂ 97%, with 24,25-dihydroxyvitamin D₃ and 25-hydroxyvitamin D₃ <0.01%), **25vitD** (25-Hydroxy Vitamin D RIA, Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany, intra- and interassay coefficients of variation 5.3 and 8.1%, respectively sensitivity <3 nmol/L; cross reactivity with

25-hydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃ 100%, with 25-hydroxyvitamin D₂ 75%, with vitamin D₃ <0.01%, and with vitamin D₂ <0.3%).

Fractional excretion (**FE**) of Ca was calculated using the following formula: $FE\ (Ca) = (Crea\ serum\ [\mu M] \times Ca\ urine\ [\mu M]) / (Crea\ urine\ [\mu M] \times Ca\ serum\ [\mu M]) \times 100\ [\%]$. FE of PHOS was calculated the same way, but with PHOS concentrations instead of Ca.

Analysis of Feed and Faeces

To determine DM and for further analyses, feed and faeces were dried at 103°C in aluminium bowls in a drying oven until weight was constant, ashed at 550°C in a muffle furnace, grinded with a feed mill (Retsch, Type ZM200, 10000 rpm; Retsch GmbH, Haan, Germany) and hydrolyzed in hydrochloric acid (0.8%). The mineral contents (Ca, P) of feed and faeces were analyzed using the same technique as in serum, urine and ruminal fluid.

Acid detergent lignin (**ADL**) was analyzed as described by van Soest et al. (1991). Acid detergent fiber (**ADF**) and neutral detergent fiber (**NDF**) were calculated as described in Kamphues et al., 2004.

Feedstuffs were analyzed for crude ash, crude protein, crude fat and crude fiber by the standardized proximate analysis, according to the VDLUFA method book III (Naumann and Bassler, 1997).

Apparent digestibility (**AD**) of Ca and PHOS was estimated by indicator method, using ADL as internal marker and following formula (Stangl 2014): $AD\ (\%) = 100 - [(\% \text{ indicator in feed} / \% \text{ indicator in faeces}) \times (\% \text{ mineral in faeces} / \% \text{ mineral in feed}) \times 100]$.

Ussing Chamber Technique

Modified Ussing chamber technique as described by Sidler-Lauff et al. (2010) with four different buffer solutions were used: one for each side (serosal or mucosal) of rumen and intestine. For buffer solutions see Wilkens et al. (2011).

An automatic voltage clamping device (Aachen Microclamp, AC Copy Datentechnik, Aachen, Germany) was used to continuously short-circuit the epithelium with correction for the solution

resistance. The tissue conductance (**Gt**) was calculated according to Ohm's law after applying bipolar current pulses (± 100 mA) and recording the resulting voltage across the tissue. The values for Gt and the continuously applied short circuit current (**Isc**) were recorded. Ten minutes after fixation of the tissues in the chambers, 70kBq was added as $^{45}\text{CaCl}_2$ (specific activity 687.82 MBq/mg; PerkinElmer Life Sciences, Rodgau-Jürgensheim, Germany) on the mucosal (resp. serosal) side to measure Ca fluxes from mucosal to serosal (resp. serosal to mucosal) side of the intestinal wall. After an additional 60 min to allow isotope flux rates to reach a steady state and Isc to stabilize, unidirectional ion flux rates were determined in sequential 20-min periods (Sidler-Lauff et al., 2010). From the mean of determined unidirectional Ca fluxes (**Jms**, fluxes from the mucosal to the serosal side; **Jsm**, fluxes from the serosal to the mucosal side), net flux of calcium was calculated by: **Jnet**= Jms - Jsm (nmol/h per cm^2).

Analysis of Bone Samples

Before starting (day-14), when the sheep were completely switched to the experimental diet (day0) and at the end of the experiment (day22, post mortem) the left metatarsus of every sheep was scanned in the diaphysis (50% of the metatarsus length) and in the distal metaphysis (10% of the metatarsus length) by using peripheral quantitative computed tomography (pQCT; XCT 960 A bone scanner; Stratec Medizinaltechnik, Pforzheim, Germany) as described by Liesegang and Risteli (2005).

The total and cortical bone mineral density (**BMD**) as well as cortical bone thickness (**CBT**) were determined in the diaphysis (cortical mode 2, threshold for cortical bone >640 mg/cm^3) and trabecular and total BMD in the distal metaphysis (peel mode 2, threshold for trabecular bone >710 mg/cm^3). Bone turnover (**BT**) was estimated by: $\text{BT} = \text{SCL}/\text{OC} \times 1000$ (Liesegang et al., 2000).

RNA Isolation and Quantitative RT-PCR

Isolation of total RNA of the renal cortex was performed using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA integrity and quality (**RNA IQ**) of all isolated RNA was checked by using a Qubit 4 fluorometer and a Qubit RNA IQ assay kit (cat. no. Q33222, Thermo Fisher Scientific, Wilmington, DE, USA) in accordance with the manufacturer's protocol. The RNA concentrations were measured by UV spectrophotometry (Thermo Fisher Scientific NanoDropTM Spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). 1 µg of isolated RNA was reverse transcribed for qPCR by use of QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol.

To quantify abundance of the calcium-sensing receptor (**CaSR**), the apical calcium channel transient receptor potential cation channel subfamily V member 5 (**TRPV5**), the cytosolic Ca binding protein calbindin D28k (**CaBP_{D28k}**), the basolateral sodium/Ca exchanger 1 (**NCX1**), and the reference genes **GAPDH** (glyceraldehyde-3-phosphate dehydrogenase) and **β-actin**, gene specific hydrolysis primers and probes were synthesized by TIB MOLBIOL (Berlin, Germany; Table 2). Reaction mixtures (20 µl each) included TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Darmstadt, Germany), 25 ng reverse transcribed RNA, 0.3 µM specific primers and 0.1 µM of specific probe. Polymerase chain reaction products were amplified (95°C, 20 s; 95°C; 45 cycles of 95°C, 3 s and 60°C, 30 s) and analyzed on a real-time PCR cycler (CFX96TM, Bio-Rad, Munich, Germany).

Table 2. Primers and probes used for hydrolysis assays

Gene ¹	Accession no.	Hybridisation position	Sense and antisense primers, probes (5' → 3')	Source
GAPDH	NM_001190390.1	516 - 537 594 - 610 564 - 586	CAAGGTCATCCATGACCACTTT CGGAAGGGCCATCCACA FAM- TGTCCACGCCATCACTGCCACCC- TMR	(Wilke ns et al., 2009)
β-actin	NM_001009784.3	259 – 279 348 – 369 287 - 308	CTCAGAGCAAGAGAGGCATCC GCAGCTCGTTGTAGAAGGTGTG FAM- CAAGTACCCCATTTGAGCACGGC-TMR	(Elfers et al., 2014)
CaBP _{D28k}	XM_004011846.3	397 – 420 452 – 477 422 - 450	GTGGATCAGTATGGGCAGAGAGAT GAAGTTCTCTTCTGTGGGTAACACAT FAM- AGCCAACTCTACAATTCCTATTTTTCC AT-TMR	(Herm et al., 2015)
CaSR	XM_027956314.1 and XM_027956319.1	2243 - 2262 2216 - 2235 2506 - 2526 2479 - 2499 2352 – 2378 2325 - 2351	ATCGGGTCCTCCTCGTGTTT GGACTTGAAGGCGAAGAAGAA FAM- TGTCATCTGTGCCATTTGGCTCAACA C-TMR	(Elfers et al., 2014)
NCX1	XM_012173106.3 to XM_027966903.1	2630 – 2649 2479 – 2498 2511 – 2530 2508 – 2527 2476 – 2495 2481 – 2500 2546 – 2565 2520 – 2539 2744 – 2763 2593 – 2612 2625 – 2644 2622 – 2641 2590 – 2609 2595 – 2614 2660 – 2679 2634 – 2653 2654 – 2680 2503 – 2529 2535 – 2561 2532 – 2558 2500 – 2526 2505 – 2531	AGAAGCTGCCCTCCTGTTTT GCCAATCATGAGGATGGAGA FAM- ACGTGATGCACTTTCTGACTGTGTTCT -BBQ	(Wilke ns et al., 2011)

2570 – 2596
2544 – 2570

TRPV5	XM_0279	1967 – 1986	AGGAGGAAGGACAAGGGCAT	(Wilke ns et al., 2011)
	69122.1	2051 – 2067	GGACAGGGAAGGGGTCG	
		2006 - 2027	FAM-CGGTGACTGAAAGCGGGCTTCT- BBQ	

¹GAPDH, glyceraldehyde 3-phosphate dehydrogenase; CaBP_{D28k}, calbindin-D28K; CaSR, Calcium-sensing receptor; NCX1, sodium/Ca exchanger type 1; TRPV5, transient receptor potential vanilloid type 5.

Abundance of parathyroid hormone receptor (**PTHR**), 1 α -OHase (**CYP27B1**), 24-OHase (**CYP24A1**) and reference gene ribosomal protein S9 (**RPS9**) were analyzed by SYBR Green PCR assays using the SensiFAST™ SYBR® No-ROX Kit (Bioline, London, UK). Gene specific primers were purchased by TIB MOLBIOL (Berlin, Germany; Table 3). Reaction mixtures (20 μ l each) contained SensiFast Mastermix (Bioline, London, UK), 25 ng reverse transcribed RNA and 0.2 μ M specific primers. The PCR products were amplified using the real-time PCR cyclers mentioned above, but another program (95°C, 3 min; 40 cycles of 95°C, 10 s and 60°C, 30 s). The thermal profile for determining the melting curve began with an incubation of 10 min at 55°C with a gradual increase in temperature (0.5°C/10 s).

Table 3. Primers used for SYBR Green assays

Gene ¹	Accession no.	Hybridisation position	Sense and antisense probes (5' → 3')	Source
RPS9	XM_027978858.1 and XM_027978859.1	188 – 208 250 – 270 231 – 252 293 – 314	CGCCTCGACCAAGAGCTGAA G CTCCAGACCTCACGTTTGTTC C	(Sacco et al., 2012)
CYP24A1	XM_027976458.1 and XM_027976459.1	230 – 249 457 – 476 both	GAGGCCTCAAGAAACAGCAC CTGACCCTCTGCCAGTCTTC	(Hermes et al., 2015)
CYP27B1	XM_027967507.1	1701 – 1720 2033 – 2052	ACCTGGAAATTCCCGTGTCC GATGCTTCTCTCAGGCACCA	(Hermes et al., 2015)
PTHr	XM_027957724.1 to XM_027957728.1	1416 – 1435 1377 – 1396 2060 – 2079 1076 – 1095 1245 – 1264 1666 – 1685 1627 – 1646 2310 – 2329 1326 – 1345 1495 – 1514	ATCAACATCGTGCGGGTTCT AGCCTGTACCTCACCATTGC	(Hermes et al., 2015)

¹RPS9, ribosomal protein S9; CYP24A1, 24-hydroxylase; CYP27B1, 1 α -hydroxylase; PTHr, parathyroid hormone receptor.

Absolute copy numbers were determined using calibration curves generated with cloned PCR fragment standards (Wilkens et al., 2009). The expression stability of the reference genes was tested using RefFinder (Xie et al., 2012). Abundance of genes of interest was normalized to a mean of three reference genes: GAPDH, β -actin and RPS9. Reactions were performed in duplicate and included cDNA samples, genomic standards and a no-template control with water. Analysis of renal vitamin D receptor (**VDR**) and apical calcium channel transient receptor potential cation channel subfamily V member 5 and 6 (**TRPV5/6**) mRNA abundance was performed differently. The differences are as follows: RNA was extracted by using TRI Reagent protocol (TRI Reagent Solution, Ambion, Rotkreuz, Switzerland). Primers and probes were synthesized by Mircosynth AG (Balgach, Switzerland; Table 4). The reaction mixture (20 μ l

each) included Promega GoTaq Probe qPCR Master Mix (Promega, Madison, USA), 10 ng reverse transcribed RNA, 0.6 μ M specific primers and 0.25 μ M of specific probe. Polymerase chain reaction products were amplified (95°C, 2 min; 45 cycles of 95°C, 15 s and 60°C, 60 s) and analyzed on ABI 7500 Fast real-time PCR System (Applied Biosystems, Darmstadt, Germany).

Table 4. Primers and probes used for VDR and TRPV5/6 (hydrolysis assays)

Gene ¹	Accession no.	Hybridisation position	Sense and antisense primers, probes (5' → 3')	Source
GAPDH	NM_00119039	85 - 104	GTCACCAGGGCTGCTTTTAA	(Nemeth et al., 2017)
H	0.1	257 – 276	ATCTCGCTCCTGGAAGATGG	
		196 – 215	FAM-AAGTTCCACGGCACAGTCAA-BHQ-1	
β -actin	NM_00100978	319 - 338	ACTGGGACGACATGGAGAAG	(Nemeth et al., 2017)
	4.3	426 – 445	GTCATCTTCTCACGGTTGGC	
		350 - 369	FAM-CACCTTCTACAACGAGCTGC-BHQ-1	
RPS9	XM_02797885	516 - 534	GTCACATCAGGGTCCGCAA	This study
	8.1	591 – 608	GGGAGCGCAGGGAGAAGT	
	and XM_02797885	561-587 and 578 - 596	FAM-TCGTGCGCCTGGACTCCCAGAA	
	9.1	653 – 670	GCACA-BHQ-1	
		623-649		
VDR	XM_02796740	1101 - 1120	CAGATCGTGCTGCTGAAGTC	(Nemeth et al., 2017)
	3.1	1260 - 1279	ACCTGGAACCTTGATGAGGGG	
		1172 - 1191	FAM-CGACATGTCCTGGACCTGTG-BHQ-1	
TRPV5 /6	XM_02796912	608 – 625	TCGTCCCTCCAGCCCAACA	This study
	2.1 and XM_00400812	730 – 747	CACTCCGGCCAGCTTGAA	
	9.4	638 - 658	FAM-GCCAGATGTACAACCTGCTGC-BHQ-1	
		both		

¹GAPDH, glyceraldehyde 3-phosphate dehydrogenase; VDR, vitamin D receptor; RPS9, ribosomal protein S9; VDR, vitamin D receptor; TRPV5/6, transient receptor potential vanilloid type 5 and 6.

The used primers derived either from ovine, caprine or bovine sequences. Efficiency of all primers included in this study was tested using dilution series. Amplification products were sequenced (GATC biotech AG, Konstanz, Germany resp. Microsynth AG, Balgach, Switzerland) and sequence specificity of amplicons was verified by NCBI Blast database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical Analysis

All statistical analyses were conducted with Excel (Microsoft Excel Professional Plus 2016, Microsoft Corporation, Redmond, WA, USA) and the statistical software R version 4.0.2 (R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria. URL: www.R-project.org) using a generalized linear mixed model using Template Model Builder (glmmTMB) for all analyses with repeated measurements and a linear or general linear model (lm/glm) for measurements without repetitions. When the data were not normally distributed, a gamma (log) regression model was used as this gave the best fit compared to a linear model or a lognormal/gaussian glm/glmmTMB by maximum likelihood. The association of the outcomes on various factors was tested with multiple independent variables. The independent variables were DCAD (categorical variable, high or low), Ca (categorical variable, normal or low) and day (categorical variable, day-14, day0, day4, day7, day14, day22; continuous variable of the *P* values for “day” reported in the tables). In the models, day was analyzed as a factor, excluding the *P* values for day reported in the tables (see section tables) which were obtained by treating day as a numeric variable. HighDCAD, and nCa were releveled as the references and day-14 as the baseline of the experiment. When there were repeated measurements, sheep and group were used as grouping variables or random effects. The nested random effect of sheep within group was denoted by (1|Group/Sheep) and deals with any differences between both sheep and group at baseline (day-14). Independent variables were tested for multiple comparisons (including interactions between the independent variables). To remove non-significant variables in the model, backward selection was used for

all regression models until the model with the lowest BIC remained. Parameters with a $P < 0.05$ (i.e., the probability of the null hypothesis of no association between the variables) in the final model were considered significant. All data are reported as means $\pm$ standard error (SE). Non-significant variables of the models are not reported.

RESULTS

Bodyweight and BCS

Due to normal growth, the body weight of all groups increased from 49.6 ± 4.45 kg before starting the experiment up to 57.0 ± 4.41 kg at the end of the experiment (mean daily weight gain of 0.2 ± 0.02 kg). The range of BCS was 2.59 ± 0.21 before starting the experiment and 2.68 ± 0.14 at the end of the experiment. Between groups, no significant differences in body weight and BCS were found at any point in time.

pQCT

Bone parameters analyzed by pQCT, changed due to the normal growth of the young sheep, but did not differ significantly between groups at any point in time: Total BMD in the diaphysis showed no significant changes over time in all groups. It was from 821.26 ± 45.39 before the start of the experiment and 841.03 ± 50.57 mg/cm³ at the end of the experiment ($P = 0.07$). Cortical BMD in the diaphysis increased from 1081.20 ± 30.57 to 1112.31 ± 18.95 mg/cm³ ($P = 0.003$). CBT in the diaphysis slightly decreased from 4.00 ± 0.32 to 3.92 ± 0.29 mg/cm³ ($P = 0.05$). Trabecular BMD in the metaphysis increased from 383.82 ± 44.91 to 438.47 ± 32.66 mg/cm³ ($P < 0.001$). Total BMD in the metaphysis increased from 443.59 ± 41.56 to 502.25 ± 29.50 mg/cm³ ($P < 0.001$).

Blood and Serum Parameters

Blood and serum parameters significantly associated with the different diets are listed in Table 5. LowDCAD showed a slight tendency to be associated with a decrease in blood pH ($P = 0.103$). Furthermore, lowDCAD was significantly associated with higher iCa in blood and

higher serum Ca concentrations ($P = 0.046$ and $P = 0.002$ resp.). Serum Ca concentrations showed an overall decrease over the entire observation period (between day-14 and day22; $P < 0.001$). Serum PHOS concentrations were not associated with the different diets.

LowCa was associated with significantly higher serum SCL concentrations ($P < 0.001$). SCL concentrations in serum were higher on all days compared to day-14, but did not change throughout the trial. LowDCAD was associated with lower serum OC concentrations ($P < 0.001$), but only days 14 and 22 differed significantly from day-14. Increased bone turnover (SCL: OC*1000) was associated with both dietary factors ($P < 0.001$ both), and was higher on all days compared to day-14, but did not differ significantly throughout the trial. It is important to note that bone turnover rates indicated different starting points at baseline. However, the nested mixed model was appropriate to deal with this. Serum 25vitD concentration was not associated with the different diets, but with time. On day0, serum 25vitD concentrations were significantly higher compared to day-14. During the trial, 25vitD dropped and reached significant lower values on day22 compared to day-14. In contrast, lowCa was associated with increased 1,25vitD serum levels ($P < 0.001$). 1,25vitD was increased on all days compared to day-14, but did not change throughout the trial.

Table 5. Blood and serum parameters of sheep fed either high or low Dietary cation-anion difference diets (highDCAD/lowDCAD) combined with normal or low calcium supply (nCa/lowCa). For statistical analysis, a glmmTMB gaussian resp. gamma(log) family model was used with day as a categorical variable, excluding the *P* values for day reported in the tables which were obtained by treating day as a numeric variable. For pH and ionized calcium in blood, a lm model was used instead of glmmTMB as these variables were measured only on one day (day of sacrifice)

Dependent variable	Group (means $\pm$ SE)				Independent variable	<i>P</i> -value
	highDCAD nCa	highDCAD lowCa	lowDCAD nCa	lowDCAD lowCa		
Blood pH	7.39 $\pm$ 0.02	7.34 $\pm$ 0.07	7.30 $\pm$ 0.06	7.33 $\pm$ 0.05	n.s. ⁵	
day22						
iCa ¹ , mmol/L	1.18 $\pm$ 0.04	1.15 $\pm$ 0.09	1.23 $\pm$ 0.07	1.23 $\pm$ 0.10	lowDCAD ⁶	0.046
day22						
Calcium, mmol/L						
day-14	2.52 $\pm$ 0.20	2.49 $\pm$ 0.10	2.59 $\pm$ 0.15	2.55 $\pm$ 0.15	lowDCAD	0.002
day0	2.43 $\pm$ 0.07	2.32 $\pm$ 0.10	2.53 $\pm$ 0.20	2.50 $\pm$ 0.11	day ⁸	< 0.001
day4	2.47 $\pm$ 0.07	2.29 $\pm$ 0.07	2.45 $\pm$ 0.19	2.43 $\pm$ 0.13		
day7	2.39 $\pm$ 0.11	2.34 $\pm$ 0.14	2.46 $\pm$ 0.14	2.40 $\pm$ 0.17		
day14	2.31 $\pm$ 0.08	2.27 $\pm$ 0.07	2.45 $\pm$ 0.17	2.47 $\pm$ 0.14		
day22	2.35 $\pm$ 0.14	2.23 $\pm$ 0.09	2.54 $\pm$ 0.23	2.37 $\pm$ 0.10		
Phosphate, mmol/L						
day-14	2.01 $\pm$ 0.22	1.98 $\pm$ 0.19	1.98 $\pm$ 0.22	1.93 $\pm$ 0.23	n.s.	
day0	1.90 $\pm$ 0.20	2.12 $\pm$ 0.53	1.74 $\pm$ 0.16	1.72 $\pm$ 0.30		
day4	1.87 $\pm$ 0.45	2.06 $\pm$ 0.40	1.74 $\pm$ 0.25	2.05 $\pm$ 0.57		
day7	2.01 $\pm$ 0.16	2.03 $\pm$ 0.45	1.65 $\pm$ 0.44	1.80 $\pm$ 0.48		
day14	2.13 $\pm$ 0.30	2.06 $\pm$ 0.39	1.82 $\pm$ 0.42	1.81 $\pm$ 0.38		
day22	2.18 $\pm$ 0.21	2.08 $\pm$ 0.58	1.69 $\pm$ 0.35	1.77 $\pm$ 0.49		
Serum Crosslaps, ng/mL						

day-14	1.33 ± 0.25	1.60 ± 0.40	1.56 ± 0.32	1.75 ± 0.54	lowCa day	< 0.001
day0	1.51 ± 0.28	2.17 ± 0.60	1.64 ± 0.25	2.16 ± 0.47		0.008
day4	1.49 ± 0.25	2.21 ± 0.45	1.63 ± 0.37	1.97 ± 0.31		
day7	1.50 ± 0.39	2.32 ± 0.49	1.76 ± 0.08	2.10 ± 0.50		
day14	1.25 ± 0.30	2.12 ± 0.79	1.68 ± 0.42	2.11 ± 0.27		
day22	1.34 ± 0.33	1.91 ± 0.45	1.82 ± 0.21	2.32 ± 0.29		
Osteocalcin, ng/mL						
day-14	152.5 ± 23.2	152.1 ± 19.5	154.7 ± 9.9	148.8 ± 12.2	lowDCAD day	< 0.001
day0	156.4 ± 7.0	164.8 ± 13.9	134.4 ± 24.7	134.8 ± 16.1		0.005
day4	155.8 ± 6.5	160.9 ± 13.2	132.5 ± 19.0	139.8 ± 18.7		
day7	165.2 ± 13.3	159.9 ± 14.0	145.2 ± 32.0	138.3 ± 20.4		
day14	149.1 ± 30.9	159.4 ± 15.2	132.3 ± 26.1	125.9 ± 15.3		
day22	144.8 ± 17.7	159.9 ± 19.0	122.2 ± 17.1	134.4 ± 14.9		
Bone turnover ratio ⁴						
day-14	8.83 ± 1.85	10.70 ± 3.00	10.09 ± 2.03	11.84 ± 3.89	lowDCAD lowCa day	< 0.001
day0	9.59 ± 1.48	13.10 ± 3.23	12.41 ± 2.33	16.02 ± 2.52		< 0.001
day4	9.59 ± 1.53	13.69 ± 2.41	12.45 ± 3.18	14.09 ± 1.01		< 0.001
day7	9.02 ± 1.98	14.53 ± 2.72	12.58 ± 2.76	15.27 ± 3.53		
day14	8.62 ± 2.26	13.44 ± 5.12	12.76 ± 2.60	16.85 ± 2.44		
day22	9.35 ± 2.55	11.93 ± 2.44	15.15 ± 2.88	17.32 ± 1.70		
25vitD ² , nmol/L						
day-14	135.1 ± 20.2	125.3 ± 9.9	134.2 ± 16.2	138.1 ± 21.6	day	0.001
day0	170.5 ± 29.4	137.7 ± 14.7	149.7 ± 19.4	143.3 ± 18.5		
day4	180.2 ± 22.5	136.8 ± 11.8	145.5 ± 16.8	135.8 ± 26.8		
day7	169.4 ± 13.6	131.0 ± 13.7	132.9 ± 13.9	137.9 ± 25.1		
day14	156.7 ± 13.8	127.6 ± 9.6	127.7 ± 12.9	128.1 ± 18.4		
day22	148.2 ± 19.8	120.2 ± 13.1	117.4 ± 14.1	117.7 ± 22.3		
1,25vitD ³ , pmol/L						

day-14	70.97 ± 14.68	68.02 ± 12.85	61.22 ± 16.48	69.10 ± 10.39	lowCa ⁷	< 0.001
day0	62.37 ± 24.78	125.67 ± 37.6	62.52 ± 20.26	147.05 ± 53.0	day	< 0.001
day4	61.59 ± 19.60	140.21 ± 15.6	63.50 ± 16.03	158.53 ± 71.7		
day7	62.18 ± 24.44	118.67 ± 40.4	55.32 ± 17.65	176.87 ± 77.2		
day14	71.61 ± 18.61	128.02 ± 18.1	70.38 ± 21.54	172.58 ± 81.4		
day22	69.32 ± 24.71	120.89 ± 21.2	57.87 ± 7.51	185.05 ± 55.1		

¹iCa, ionized Ca; ²25vitD, 25-Hydroxy Vitamin D; ³1,25vitD, 1,25-dihydroxyvitamin D; ⁴bone turnover, calculated as Serum Crosslaps: Osteocalcin*1000; ⁵n.s., not significant (P > 0.05); ⁶compared to highDCAD; ⁷compared to nCa; ⁸day as a numeric variable

Urine Parameters

Significant urine parameters of the sheep as associated with the different diets are displayed in Table 6. LowDCAD was associated with lower urine pH and higher FE of Ca ($P < 0.001$ both). LowCa was also associated with higher FE of Ca, albeit less so than DCAD ($P < 0.001$). LowDCAD and lowCa in interaction slightened the associated increase of each dietary factor on FE of Ca. Urine pH was lower on all days compared to day-14 and decreased during the trial. FE of Ca was higher on all days compared to day-14 and did not change throughout the trial. FE of PHOS was not associated with the different diets, but with time. FE of PHOS was higher on all days compared to day-14 and increased throughout the trial ($P < 0.001$).

Table 6. Urine parameters as well as fractional excretion (FE) of calcium and phosphate in sheep fed either high or low Dietary cation-anion difference diets (highDCAD/lowDCAD) combined with normal or low Ca supply (nCa/lowCa). For statistical analysis, a glmmTMB gaussian resp. gamma(log) family model was used with day as a categorical variable, excluding the *P* values for day reported in the tables which were obtained by treating day as a numeric variable

Dependent variable	Group (means $\pm$ SE)				Independent variable	<i>P</i> -value
	highDCAD nCa	highDCAD lowCa	lowDCAD nCa	lowDCAD lowCa		
Urine pH						
day-14	8.72 $\pm$ 0.16	8.73 $\pm$ 0.23	8.64 $\pm$ 0.08	8.65 $\pm$ 0.09	lowDCAD ²	< 0.001
day0	8.61 $\pm$ 0.15	8.32 $\pm$ 0.4	6.84 $\pm$ 1.51	7.09 $\pm$ 1.59	day ⁴	< 0.001
day4	8.54 $\pm$ 0.15	7.78 $\pm$ 1.37	6.71 $\pm$ 1.43	5.84 $\pm$ 0.93		
day7	8.57 $\pm$ 0.25	7.27 $\pm$ 1.47	6.05 $\pm$ 1.41	6.16 $\pm$ 1.55		
day14	8.53 $\pm$ 0.08	7.83 $\pm$ 1.12	6.13 $\pm$ 1.53	5.61 $\pm$ 0.53		
day22	8.62 $\pm$ 0.10	8.41 $\pm$ 0.09	5.51 $\pm$ 0.82	5.61 $\pm$ 0.53		
FE ¹ Calcium, %						
day-14	0.08 $\pm$ 0.04	0.36 $\pm$ 0.35	0.35 $\pm$ 0.20	0.29 $\pm$ 0.10	lowDCAD	< 0.001
day0	0.07 $\pm$ 0.04	0.27 $\pm$ 0.20	2.92 $\pm$ 3.40	1.53 $\pm$ 1.45	lowCa ³	< 0.001
day4	0.06 $\pm$ 0.03	0.55 $\pm$ 0.97	1.81 $\pm$ 1.51	1.96 $\pm$ 1.59	lowDCAD*lowCa	< 0.001
day7	0.14 $\pm$ 0.09	0.29 $\pm$ 0.24	3.03 $\pm$ 1.60	2.10 $\pm$ 3.03	day	< 0.001
day14	0.07 $\pm$ 0.03	0.32 $\pm$ 0.28	2.85 $\pm$ 1.31	3.09 $\pm$ 2.70		
day22	0.11 $\pm$ 0.12	0.17 $\pm$ 0.09	3.79 $\pm$ 2.85	4.01 $\pm$ 4.39		
FE ¹ Phosphate, %						
day-14	0.39 $\pm$ 0.45	0.49 $\pm$ 0.65	0.23 $\pm$ 0.05	0.34 $\pm$ 0.39	day	< 0.001
day0	0.52 $\pm$ 0.81	3.26 $\pm$ 7.45	0.18 $\pm$ 0.04	1.81 $\pm$ 3.75		
day4	0.88 $\pm$ 1.47	1.93 $\pm$ 2.35	0.21 $\pm$ 0.04	1.70 $\pm$ 3.15		
day7	0.74 $\pm$ 0.76	1.26 $\pm$ 2.24	0.21 $\pm$ 0.04	1.22 $\pm$ 2.45		

day14	0.25 ± 0.25	2.00 ± 3.11	0.26 ± 0.08	2.93 ± 4.91
day22	0.62 ± 0.89	1.96 ± 4.27	0.42 ± 0.43	2.42 ± 3.30

¹FE, Fractional Excretion; ²compared to highDCAD; ³compared to nCa; ⁴day as a numeric variable

Rumen Parameters

Significant rumen parameters as associated with the different diets are presented in Table 7. Ca mineral concentration analyzed in ruminal fluid on the day of sacrifice was not associated with the different diets. PHOS in ruminal fluid was, compared to a diet high in DCAD and normal in Ca, associated with a lower concentration only when DCAD was high and Ca was low ($P = 0.028$).

Table 7. Effect of different diets on the analysed parameters in ruminal fluid, net calcium (Ca) ion fluxes (Jnet) through ruminal and jejunal mucosa and apparent digestibility (AD) of calcium and phosphate (mean $\pm$ SE). Different sheep feeding groups received either a diet high or low in Dietary cation-anion difference (highDCAD/ lowDCAD) combined with normal or low Ca supply (nCa/ lowCa). For statistical analysis, a lm resp. glm gamma(log) family model was used, except for AD where a glmmTMB gaussian family model was used with day as a categorical variable, excluding the *P* values for day reported in the tables which were obtained by treating day as a numeric variable

Dependent variable	Group (means $\pm$ SE)				Independent variable	<i>P</i> -value
	highDCAD nCa	highDCAD lowCa	lowDCAD nCa	lowDCAD lowCa		
Rumen fluid						
Calcium, mmol/L	1.07 $\pm$ 0.24	1.05 $\pm$ 0.18	1.17 $\pm$ 0.17	1.10 $\pm$ 0.27	n.s. ²	
Phosphate, mmol/L	25.77 $\pm$ 1.76	21.59 $\pm$ 3.04	24.87 $\pm$ 4.17	27.94 $\pm$ 4.36	highDCADlowCa ³	0.028
Ussing Chamber						
Rumen, nmol/h/cm ²						
mucoserosal	13.31 $\pm$ 2.41	12.20 $\pm$ 3.72	12.87 $\pm$ 1.98	14.90 $\pm$ 4.26	n.s.	
seromucosal	1.36 $\pm$ 0.76	1.76 $\pm$ 0.46	2.26 $\pm$ 1.19	4.15 $\pm$ 5.16	n.s.	
Jnet ¹	11.96 $\pm$ 2.46	10.44 $\pm$ 3.53	10.60 $\pm$ 1.32	10.75 $\pm$ 5.00	n.s.	
Jejunum, nmol/h/cm ²						
mucoserosal	28.66 $\pm$ 7.17	33.89 $\pm$ 3.68	32.08 $\pm$ 7.45	32.40 $\pm$ 6.92	n.s.	
seromucosal	25.02 $\pm$ 5.71	18.50 $\pm$ 3.84	21.15 $\pm$ 4.52	15.31 $\pm$ 7.90	lowCa ⁴	0.020
Jnet ¹	3.64 $\pm$ 2.96	15.39 $\pm$ 6.03	10.93 $\pm$ 5.23	17.09 $\pm$ 5.34	lowDACD ⁵	0.003
					lowCa	< 0.001
					lowDCAD*lowCa	0.039
Apparent digestibility Calcium, %						

day0	-13.69 ± 20.50	-8.26 ± 30.22	6.74 ± 8.15	-11.83 ± 12.66	lowDCADnCa ⁶	0.027
day4	-17.79 ± 23.70	-20.19 ± 23.54	1.51 ± 11.14	-19.02 ± 30.96		
day7	4.9 ± 11.12	-7.85 ± 8.89	3.95 ± 10.27	-11.24 ± 10.71		
day14	-16.32 ± 22.24	-7.13 ± 46.58	0.32 ± 23.22	-0.72 ± 17.42		
day22	-11.47 ± 18.16	-36.24± 48.45	25.99 ± 31.28	-3.83 ± 6.02		
<hr/>						
Apparent digestibility						
Phosphor, %						
day0	-25.85 ± 26.37	-1.69 ± 27.24	-0.55 ± 9.33	-16.78 ± 19.04	n.s.	
day4	-18.40 ± 18.36	-25.07 ± 30.69	-7.33 ± 13.43	-22.68 ± 17.14		
day7	-7.53 ± 20.04	-6.54 ± 15.04	-2.26 ± 12.60	-15.96 ± 8.42		
day14	-17.15 ± 22.53	-1.44 ± 25.68	-17.62 ± 15.81	-10.45 ± 16.30		
day22	-12.18 ± 14.80	-6.95 ± 15.20	-0.68 ± 10.60	-20.49 ± 23.96		

¹From the mean of determined unidirectional Ca fluxes (Jms, fluxes from the mucosal to the serosal side; Jsm, fluxes from the serosal to the mucosal side), net flux of calcium was calculated by: $J_{net} = J_{ms} - J_{sm}$ (nmol/h per cm²); ²n.s., not significant ($P > 0.05$); ³compared to a diet high in DCAD and normal in Ca., effect only when DCAD is high and Ca is low; ⁴compared to nCa; ⁵compared to highDCAD;; ⁶compared to a diet high in DCAD and normal in Ca., effect only when DCAD is low and Ca is normal

Ussing Chamber Experiment

Ruminal and jejunal net fluxes of Ca as associated with the different diets are presented in Table 7. While no associations of the different diets on unidirectional or net Ca absorption could be found in rumen, significant differences in jejunum were found. Indeed, mucoserosal Ca fluxes did not differ in the jejunum. LowCa was associated with higher seromucosal Ca fluxes in the Jejunum ($P = 0.020$). LowDCAD and lowCa were associated with higher jejunal Ca net fluxes ($P = 0.003$ and $P < 0.001$ resp.; Table 7). The associated increases of each dietary factor on jejunal Ca net fluxes were, however, weakened in interaction ($P = 0.039$).

Apparent Digestibility

Apparent digestibility of Ca was, compared to a diet high in DCAD and normal in Ca, associated to be higher only when DCAD was low and Ca was normal ($P = 0.027$; Table 7). Apparent digestibility of PHOS was not associated with the different diets.

RNA IQ and mRNA Abundance of renal and intestinal Ca regulating parameters (PCR)

The RNA IQ of the isolated RNA was on average 8.97 ± 1.38 for the kidney samples (except the isolated RNA for determination of VDR in the kidney, which was 9.43 ± 0.37), 9.34 ± 0.41 for the duodenal samples and 9.40 ± 0.30 for the jejunal samples.

The mRNA abundance of the analyzed receptors and proteins involved in Ca homeostasis as associated with the different diets are displayed in Table 8. Renal CaSR was, compared to a diet high in DCAD and normal in Ca, associated with a lower mRNA abundance only when DCAD and Ca were low ($P = 0.010$). Furthermore, lowCa and lowDCAD in interaction were associated with higher renal mRNA abundance levels of CYP27B1 ($P = 0.032$). LowDCAD was associated with a higher renal mRNA abundance of PTHR ($P < 0.001$). LowCa was associated with a higher jejunal TRPV5/6 mRNA abundance ($P = 0.049$). No differences in the mRNA abundance levels of CYP24A1, CaBPD28k, NCX1, TRPV5 and VDR could be found in the kidney. Additionally, no differences could be found for VDR in the duodenal and jejunal mucosa.

Table 8. Effect of different diets on messenger Ribonucleic acid (mRNA) abundance of receptors and transport proteins in renal cortex resp. duodenal and jejunal mucosa (mean $\pm$ SE). Different sheep feeding groups received either a diet high or low in Dietary cation-anion difference (highDCAD/ lowDCAD) combined with normal or low Ca supply (nCa/ lowCa). mRNA abundance was normalized to a mean of 3 different reference genes: GAPDH (glyceraldehyde-3-phosphate dehydrogenase), β -actin, and RPS9 (Ribosomal protein S9). For statistical analysis, a lm resp. glm model gamma(log) family was used

Dependent variable	Group (means $\pm$ SE)				Independent variable	P-value
	highDCAD nCa	highDCAD lowCa	lowDCAD nCa	lowDCAD lowCa		
Renal cortex						
CaBP _{D28k} ¹	1.00 $\pm$ 0.53	0.90 $\pm$ 0.52	0.66 $\pm$ 0.22	0.84 $\pm$ 0.11	n.s. ¹⁰	0.008
CaSR ²	1.00 $\pm$ 0.65	1.03 $\pm$ 0.47	0.77 $\pm$ 0.23	0.38 $\pm$ 0.26	lowDCADlowCa ¹¹	
CYP24A1 ³	1.00 $\pm$ 0.49	1.38 $\pm$ 2.16	0.83 $\pm$ 0.50	0.84 $\pm$ 0.45	n.s.	
CYP27B1 ⁴	1.00 $\pm$ 1.40	0.50 $\pm$ 0.50	0.79 $\pm$ 0.26	2.51 $\pm$ 1.51	lowDCAD*lowCa	0.032
NCX1 ⁵	1.00 $\pm$ 0.40	0.67 $\pm$ 0.38	0.67 $\pm$ 0.37	0.56 $\pm$ 0.13	n.s.	< 0.001
PTHr ⁶	1.00 $\pm$ 0.28	0.93 $\pm$ 0.28	1.68 $\pm$ 0.42	1.94 $\pm$ 0.93	lowDACD ¹²	
TRPV5 ⁷	1.00 $\pm$ 0.49	0.58 $\pm$ 0.22	0.86 $\pm$ 0.39	0.94 $\pm$ 0.20	n.s.	
VDR ⁸	1.00 $\pm$ 0.20	1.12 $\pm$ 0.19	1.13 $\pm$ 0.22	1.03 $\pm$ 0.24	n.s.	
Duodenum						
VDR ⁸	1.00 $\pm$ 0.10	0.86 $\pm$ 0.11	0.86 $\pm$ 0.10	0.96 $\pm$ 0.24	n.s.	
Jejunum						
VDR ⁸	1.00 $\pm$ 0.16	1.13 $\pm$ 0.17	0.80 $\pm$ 0.30	0.90 $\pm$ 0.20	n.s.	0.049
TRPV5/6 ⁹	1.00 $\pm$ 0.96	1.68 $\pm$ 0.81	1.34 $\pm$ 0.91	2.19 $\pm$ 1.58	lowCa ¹³	

¹CaBPD28k, calbindin D28k; ²CaSR, calcium-sensing receptor; ³CYP24A1, 24-OHase; ⁴CYP27B1, 1 α -OHase; ⁵NCX1, sodium/Ca exchanger type 1; ⁶PTHr, parathyroid hormone receptor; ⁷TRPV5, transient receptor potential vanilloid type 5; ⁸VDR, vitamin D receptor; ⁹TRPV5/6, transient receptor potential vanilloid type 5 and 6; ¹⁰n.s., not significant ($P > 0.05$); ¹¹compared to a diet high in DCAD and normal in Ca., effect only when DCAD and Ca are low; ¹²compared to highDCAD; ¹³compared to nCa

DISCUSSION

Animal model

In order to induce metabolic acidosis and effectively control hypocalcaemia, various urinary pH values in the range of 5.5 and 7.0 have been recommended for cows (Jardon, 1995; Moore et al., 2000; Charbonneau et al., 2006). For sheep, experiments were carried out using urinary pH values in the range of 5.0 and 7.0 (Braithwaite 1972, Las et al., 2007, Liesegang et al., 2008). In this study a diet very low in DCAD was fed (-300 mEq/kg DM) in order to investigate the underlying physiological mechanisms in a low number of animals under experimental conditions. The lowDCAD diets resulted in urinary pH values between 5.5-6.0 without affecting blood pH. Alterations of Ca homeostasis could be shown by effects on both, ionized and total Ca. Although none of the results obtained indicated that the sheep suffered from clinical or subclinical hypocalcemia (serum Ca < 2.0 mmol/L; Bickhardt et al. 1998), the animals responded to the new diets with remarkable changes such as differences in urine pH levels and FE of Ca, blood levels of iCa, serum levels of total Ca, mRNA abundance of PTHR, and AD of Ca. Thus, we find that this animal model was appropriate to study basic effects of DCAD and to test our hypotheses, which are discussed below. However, the results presented may not necessarily be transferable to female reproducing sheep or other species, but can be seen as a first step to address the basic physiological pathways correlated with these feeding regimes in order to understand their effects in the prevention of milk fever in other animals such as dairy cows where milk fever is of greater economic interest.

Transporter mediated renal Ca excretion

While other species, such as rats easily adapt to very low Ca diets by increasing apparent absorption, diminishing renal Ca excretion, and enhancing renal PHOS excretion (Zhang et al., 2008), there are no comparable effects of a dietary Ca restriction on renal Ca or PHOS excretion reported in ruminants (Martz et al., 1999; Taylor et al., 2009; Wilkens et al., 2010; 2012). These

findings could be verified in the current study. Despite differences in serum 1,25vitD concentrations, the lowCa diets did not result in a compensatory upregulation of TRPV5 and CaBP_{D28K} in renal tissue, which has been reported for monogastric animals (Hoenderop et al., 2002; Ko et al., 2009).

An increase in urinary Ca excretion associated with low DCAD was evident in the present trial. As urinary Ca concentration could be diminished by an EDTA infusion in cows kept on an anion-rich diet, Schonewille et al. (1999) concluded that the large amounts of Ca excreted via the kidney represent a surplus that can be used when Ca homeostasis is challenged. In the present study, the increased excretion of Ca in urine/ FE of Ca was nearly independent of the dietary Ca intake indicating that renal Ca excretion is an unregulated process under these conditions. An inhibitory effect on tubular Ca absorption in acidotic states has been postulated (Yeh et al., 2003). In fact, further research reports that TRPV5 is only half as effective in a state of metabolic acidosis as it is in normal states (Suzuki et al., 2008). In the present study, the TRPV5 mRNA abundance in the kidney was not affected by the different diets supporting the hypothesis that the activity of TRPV5 rather than the abundance was influenced by low DCAD.

Ca mobilization from bone

In the present study, the pQCT analysis of sheep metatarsi revealed changes in BMD in all groups due to a physiological aging or growing process in the young sheep (Liesegang et al., 2013). At no point in time, differences between the groups were observed. In addition, intensive bone monitoring was carried out throughout the experiment by measuring specific bone parameters in the serum. SCL and OC are both proteins that are released during bone resorption and bone formation, respectively, and have been established as suitable markers for several animal species in previous studies (Liesegang et al., 1998; 2000; Russell, 2001; Liesegang and Risteli, 2005). The SCL, indicating enhanced osteoclast activity, increased during the trial with lowCa, but not with lowDCAD. Interestingly, the lowDCAD diets seemed to reduce serum concentrations of OC, which suggests decreased bone formation, while dietary Ca does not

exert any effects on this parameter. Bone tissue is involved in maintaining acid-base balance (Krieger et al., 2004), probably mediated by a proton sensing receptor expressed in osteoblasts, osteoclasts and osteocytes (Jorgetti et al., 2016). Chronic metabolic acidosis induced by NH_4Cl administration in rats resulted in reduced plasma OC concentrations and an increase in plasma TRAP5c activity indicating enhanced bone mobilization (Gasser et al., 2014). The observation that only bone formation seemed to be affected by the metabolic acidosis in our study might be related to the rather moderate challenge of acid-base balance. The increase in bone resorption with the lowCa diets is probably mediated by a rise in PTH that also explains the stimulated production of $1,25\text{vitD}_3$. The additive effects of the two factors Ca and DCAD are represented by the bone turnover rate that was numerical highest in the lowDCADlowCa group. In contrast to these findings, changes of BMD and CBT were not evident. Increased bone turnover rates normally lead to reduced bone mineral content (Lips and van Schoor, 2011). The fact that only the metatarsus was measured by pQCT is a possible explanation for these conflicting results between pQCT and bone marker analysis (Liesegang, 2008), as bone markers show the turnover of the whole skeleton. Additionally, due to the temporal shift in the occurrence of changes in bone, no differences might have been detected in pQCT.

PTH sensitivity and vitamin D metabolism

A diet highly positive in DCAD reduced the renal sensitivity of PTHR to PTH in cows (Gaynor et al., 1989; Goff et al., 1991), while cows fed a negative DCAD diet responded better to exogenous PTH (Goff et al., 2014). It has been discussed that metabolic alkalosis results in a condition of pseudo hypoparathyroidism caused by a change in the tertiary structure of the PTH receptor-G-protein complex and a reduction in its affinity for PTH (Goff et al., 2014). In the present study, renal PTHR abundance was clearly stimulated by low DCAD at the mRNA level while dietary Ca had no effect as previously reported (Herm et al., 2015). An acidosis associated increase in PTHR RNA expression and affinity of the receptor to PTH was shown in vitro in osteoblast-like cells and in the renal cortex of cattle (Disthabanchong et al., 2002; Rodriguez et

al., 2016). Interestingly, renal CaSR mRNA abundance was lower in the lowDCADlowCa group. On the one hand, PTH and 1,25vitD can modulate the expression of the CaSR. On the other hand, CaSR activation interferes with renal responses to 1,25vitD and PTH (Riccardi and Brown, 2010). As the plasma concentration of Ca determined via the CaSR can directly inhibit the synthesis of 1,25vitD (Bushinsky et al., 1985), a downregulation of CaSR might lead to a desensitization towards plasma Ca and thus favor the activation of 1,25vitD production. This assumption is supported by the observation that the CYP27B1 mRNA abundance was significantly increased by the interaction of low DCAD and low Ca. Of course, 1,25vitD plasma concentrations were also increased in the highDCADlowCa group (1.8fold), but the rise was numerically more pronounced in the lowDCADlowCa group (2.7fold). Unfortunately, PTH itself was not measured because there is no reliable test kit for sheep on the market. But the fact that 1,25vitD was stimulated only by lowCa, and not by lowDCAD, indicates that PTH secretion was not induced by lowDCAD.

Gastrointestinal Ca absorption

Different in vitro and in vivo studies presented very well the essential role of ruminal Ca absorption in small ruminants. The role of ruminal Ca transport in vivo probably depends on Ca intake since an increase in Ca supply leads to increased preduodenal Ca net absorption in sheep and in cattle (Khorasani et al., 1997; Schroeder and Breves, 2006). We expected the rumen net flux rates to be increased by the low DCAD diets. Experiments presented in Wilkens et al. (2016) and Leonhard-Marek et al. (2007) led to the assumption that by diets low in DCAD apical transport systems are functionally changed due to alterations of surface and intracellular pH as other studies reported changes of ruminal epithelia due to the diet's energy content (Lodemann and Martens, 2006; Etschmann et al., 2009; Klevenhusen et al., 2013). However, ruminal flux rates were not affected by the different diets in the present study. It is important to note that in contrast to other studies (e.g., Wilkens et al., 2012, 2016) the animals were not fed before sacrifice, meaning that both the epithelium as well as the sample of rumen fluid were

acquired long after the last feeding. This difference in the experimental design also explains the observation that rumen Ca concentrations were apparently not altered by dietary Ca. It might be that the effects observed before are not as long-lasting as we expected them to be. The lack of any adaptation of the rumen epithelium to the dietary Ca content has been shown before (Wilkens et al., 2012). In the small intestine, a low dietary Ca content increased TRPV6 gene transcription and thus Ca transport probably via increased 1,25vitD (van Abel et al., 2003).

In vitro experiments only give information on mechanistic aspects. In addition, Schroeder and Breves (2006) hypothesized a negative correlation between the net absorption in the forestomachs and the intestinal net absorption of Ca in cows as part of a compensatory effect. Therefore, we also addressed total tract digestibility in vivo.

Outcomes of different studies concerning the effect of lowDCAD diets on total apparent digestibility of Ca are inconsistent; while some studies report a higher apparent Ca digestibility with diets low in DCAD (Abu Damir et al., 1994; Schonewille et al., 1999; Roche et al., 2007), others found no or negative effects on apparent Ca digestibility or gastrointestinal Ca absorption when reducing the diet's DCAD (Leclerc and Block, 1989; Oehlschlaeger et al., 2014). These conflicting results could depend on the dietary formulation, particularly with regard to the dietary Ca supply. Positive effects of anionic diets on Ca absorption are expected only when the dietary Ca concentration is sufficient (Lomba et al., 1978; Wilkens et al., 2016).

Based on the findings mentioned above, we assumed an increase in apparent Ca digestibility to occur in group lowDCADnCa, but not in group lowDCADlowCa, which is consistent with our results. It is obvious that the calculated values of AD in this experiment are mostly negative. There were two sources of error in the calculation: on the one hand, an indicator method (ADL) was used to calculate the AD. On the other hand, the faecal sample once a day represents only a snapshot. A total collection method of the faeces would only have been possible if the animals had been kept in metabolic cages, which could not be performed for animal welfare reasons. However, a comparison of the values obtained shows that AD of Ca was highest in group

lowDCADnCa. As increased intestinal absorption indicated by the results on intestinal flux rates and gene abundance was observed with the low Ca but not with the low DCAD diets, we assume that the rumen contributed to the increased overall AD in the lowDCADnCa group although we were not able to prove an effect of the low DCAD feeding regime on rumen function in vitro.

CONCLUSIONS

The hypothesis that lowDCAD increases the FE of Ca regardless of dietary Ca intake was confirmed by this study. It is still unclear where the excess calcium that is excreted with lowDCAD in the urine comes from. Since no significant differences between the groups could be detected in the BMD, a significant bone participation in the Ca homeostasis of our sheep is unlikely. However, we can not exclude bone participation in the long term as bone marker analysis indicated. Our main hypothesis of an increased Ca net flux in the rumen could not be confirmed by this experiment. A significantly increased apparent Ca digestibility was shown in sheep that were fed lowDCADnCa. The jejunal Ca net fluxes in Ussing chamber were increased by both lowDCAD and lowCa. Mean serum Ca concentrations in group highDCADlowCa decreased slightly below the known reference range despite increased TRPV5/6 and jejunal Ca flux rates. Renal PTHR mRNA abundance, which is higher in groups with low DCAD, likely plays an important role in this process. Future studies should be conducted with female sheep or cows around parturition to verify whether the results shown can be reproduced under the influence of female sex hormones.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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