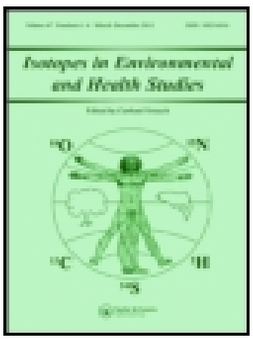

Isotopes in Environmental and Health Studies

ISSN: 1025-6016 (Print) 1477-2639 (Online) Journal homepage: http://www.tandfonline.com/loi/gieh20

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To cite this article: Alexander Heuser, Anton Eisenhauer, Katharina E. Scholz-Ahrens & Jürgen Schrezenmeir (2016): Biological fractionation of stable Ca isotopes in Göttingen minipigs as a physiological model for Ca homeostasis in humans, Isotopes in Environmental and Health Studies, DOI: 10.1080/10256016.2016.1151017

To link to this article: http://dx.doi.org/10.1080/10256016.2016.1151017

Published online: 21 Mar 2016.

ISOTOPES IN ENVIRONMENTAL AND HEALTH STUDIES, 2016
http://dx.doi.org/10.1080/10256016.2016.1151017

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Biological fractionation of stable Ca isotopes in Göttingen minipigs as a physiological model for Ca homeostasis in humans

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ABSTRACT

In order to investigate fractionation of calcium (Ca) isotopes in vertebrates as a diagnostic tool to detect Ca metabolism dysfunction we analyzed the Ca isotopic composition ($\delta^{44/40}Ca = [(^{44}Ca/^{40}Ca)_{sample}/(^{44}Ca/^{40}Ca)_{reference}]-1$) of diet, faeces, blood, bones and urine from Göttingen minipigs, an animal model for human physiology. Samples of three groups were investigated: 1. control group (Con), 2. group with glucocorticosteroid induced osteoporosis (GIO) and 3. group with Ca and vitamin D deficiency induced osteomalacia (−CaD). In contrast to Con and GIO whose average $\delta^{44/40}Ca_{faeces}$ values (0.39 ± 0.13‰ and 0.28 ± 0.08‰, respectively) tend to be lower than their diet (0.47 ± 0.02‰), $\delta^{44/40}Ca_{faeces}$ of −CaD (-0.27 ± 0.21‰) was significantly lower than their $\delta^{44/40}Ca_{diet}$ (0.37 ± 0.03‰), but also lower than $\delta^{44/40}Ca_{faeces}$ of Con and GIO. We suggest that the low $\delta^{44/40}Ca_{faeces}$ of −CaD might be due to the contribution of isotopically light Ca from gastrointestinal fluids during gut passage. Assuming that this endogenous Ca source is a common physiologic feature, a fractionation during Ca absorption is also required for explaining $\delta^{44/40}Ca_{faeces}$ of Con and GIO. The $\delta^{44/40}Ca_{urine}$ of all groups are high (>2.0‰) reflecting preferential renal reabsorption of light Ca isotopes. In Göttingen minipigs we found a Ca isotope fractionation between blood and bones ($\Delta^{44/40}Ca_{blood\text{-}bone}$) of 0.68 ± 0.15‰.

ARTICLE HISTORY
Received 22 July 2015
Accepted 19 December 2015

KEYWORDS
Animals; calcium-40; calcium-44; calcium metabolism; isotope application in medicine; isotope ecology; stable isotope tracer techniques

1. Introduction

Calcium is an essential earth alkaline metal and most important for the formation and maintenance of bones and teeth [1]. Calcium is also involved in processes like muscle contraction, signal transmission, cell apoptosis, cell reproduction, and the coagulation of blood. In larger concentrations, Ca is known to be cell poisoning and, hence, plasma Ca is held constant within very close limits (Ca homeostasis).

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The natural relative abundances of ^{40}Ca and ^{44}Ca are 96.97 and 2.06 atom%, respectively. During physiological transport, original Ca isotope ratios usually change at the transitions from one organ to the other due to phase transition and kinetic isotope fractionation processes. This observation has generated biomedical studies on Ca isotope fractionation [2–5] and on other heavy stable metal isotopes in vertebrates [6–9]. All of them pointed to the likelihood that the study of natural isotope variations in the human body may help to enhance the knowledge on metabolism functioning and may potentially serve for diagnosing mineral and trace element imbalances and related disorders.

Earlier observations showed that $\delta^{44/40}Ca$ (‰) = $[(^{44}Ca/^{40}Ca)_{sample}/(^{44}Ca/^{40}Ca)_{reference}]-1$ is lowest in bones and that the Ca isotopic composition of soft tissue and blood is similar to the dietary Ca isotopic composition [2]. According to a first simple model, Ca isotope fractionation takes place only during the formation of mineralized tissue (e.g. bone) where it becomes fractionated by about −1.3 ‰ ($\delta^{44/40}Ca$) relative to the diet [2]. In further simplification the authors stated that Ca isotope fractionation does not occur during metabolisation of dietary Ca and its uptake into the body nor during degradation of mineralized tissue or during excretion of Ca from blood to urine [2]. Following this approach, the Ca isotopic composition of urine appears to monitor the net bone mineral balance in humans [3–5]. In order to explain the isotope difference between diet and urine, Heuser and Eisenhauer [4] added a kidney box to the original model of Skulan and DePaolo [2] to account for the Ca reabsorption processes there and the enrichment of heavy Ca isotopes in urine relative to the corresponding diet. However, the refined model is not complete either, as it is also based on assumptions concerning Ca isotope fractionation between diet and soft tissue, as well as between blood and bones and on the assessment in urinary samples only [4].

In order to verify previous assumptions on the physiological Ca balance, we used Göttingen minipigs, which may serve as model organism for Ca homeostasis and mineral metabolism in the human body and which permit investigating Ca isotopic composition of all organ reservoirs, as well as the fractionation processes and factors between them [10]. For this purpose, Ca isotopes were measured in diet, blood plasma, urine, bones and faeces from Göttingen minipigs in which different osteopathies were induced.

2. Methods

2.1. Animal experiment

The study was approved by the *Ministerium für Umwelt, Natur und Forsten des Landes Schleswig-Holstein*, that regulates animal experiments. It was part of a larger intervention trial with primiparous Göttingen minipigs, in which the physiologic responses and the composition and properties of bone to various dietary or pharmacological treatments were investigated in a large-animal model. The purpose was to generate bone of different quantities (bone mineral densities – BMDs) and of comparable BMDs but with presumably different bone qualities, depending on the underlying bone disorders. These included glucocorticosteroid-induced osteoporosis (GIO) [10], therapy of GIO by bisphosphonate [11], and nutritionally induced osteomalacia (NIOM) which was achieved by a diet deficient in Ca and vitamin D (−CaD) [12]. The present Ca isotope study is restricted to (1) animals with GIO and (2) animals with −CaD compared metabolisation (3) animals of a healthy

control group (Con). The elaboration of the experimental methods of the animal trials have been described in detail before [10–12].

2.2. Animals, diets, housing and intervention

Eighteen primiparous sows of the Institute's own breeding herd were used ($n = 6$ per group). They were 20 months old at farrowing. In order to standardize the lactation productivity, litter size was standardized to five or six piglets and nursing time to six weeks. After lactation and before starting the intervention the sows were switched from the Institute's standard nutrition regimen for lactating minipigs to the Institute's semi-purified standard diet (SSD) for adult minipigs and an adaptation time of four weeks (pre-experimental). Then, at 30 months of age, sows were allocated to 1 of the 3 experimental groups Con, GIO and −CaD on the basis of their body weights.

Animals of Con and GIO continued on the SSD, while those of −CaD received the same SSD which, however, was deficient in Ca and free of vitamin D. The SSD contained (g/kg diet): corn starch, 290; sucrose, 240; casein, 150; cellulose, 80; lard, 75; margarine, 75; mineral and vitamin premix, 60; lactulose, 30, and 14 MJ metabolisable energy. The diet of Con and GIO contained 6 g/kg Ca, 7 g/kg P, and 160 µg/kg (6500 IU/kg) of vitamin D3 (Deutsche Vilomix Tierernährung GmbH, Neuenkirchen-Vörden, Germany). Their Ca was given as monocalcium phosphate plus calcium carbonate, and their vitamin D intake was 60 µg/d (2400 IU/d). The −CaD-diet contained 2 g/kg Ca, given as calcium carbonate and contained no vitamin D3.

For maintenance of body weights and to avoid fattening, individual feed intake was restricted to 370 g/d, all of which was completely consumed by all animals. Deionized water was available ad libitum. Animals were housed under controlled conditions on strawless floor cages in individual pens without daylight but artificial light between 06:00 h and 18:00 h, temperature between 20 °C and 22 °C and 60–70 % humidity. The glucocorticosteroid prednisolone (Lichtenstein Pharmazeutika, Mühlheim-Kärlich, Germany) was given orally at a dose of 1.0 mg/kg body weight/day for 8 weeks and was continued at a dose of 0.5 mg/kg body weight/day until the end of the experiment.

2.3. Sampling

At the beginning of the experiment (t_0) and after 2, 5, 7, 10, 13 and 15 months (t_2–t_{15}) blood, urine and faecal samples were collected, and BMD was monitored *in vivo*. Samples were stored at −18 °C until analysis. Then, animals were sacrificed by exsanguination after an anaesthesia with a combination of Dormicum® (Midazolam, 1 mg/kg BW; Roche, Switzerland) and Ketamin Hydrochlorid (5 mg/kg BW; CuraMed Pharma, Karlsruhe, Germany). Representative bone specimens were taken and stored until analysis at −18 °C. Here we report only results of analyses at t_{15} including the semisynthetic diets consumed at that period.

Bone samples were dried for 4 h at 105 °C to determine dry matter (%). The dried bone was ashed overnight in a muffle furnace at 450 °C, the ash was weighed, dissolved in 20 % (v/v) HCl. Blood samples were collected between 08:00 and 10:00 am after an overnight fast. Minipigs were individually transferred to metabolic cages for 7 days in total to allow the separate sampling of urine and faeces and to determine metabolic balances.

Urine and faeces were collected daily for 24 h and stored frozen at −18 °C until analysis. Calcium in urine was analysed as described above. Calcium absorption and retention were calculated on a mg/7-day basis as follows: absorption = intake − faecal excretion; retention = intake − (faecal excretion + urinary excretion) [12].

3. Calcium isotope analysis

3.1. Sample preparation

3.1.1. Blood (plasma)

About 2.5 ml double-distilled concentrated HNO_3 and 250 µl of concentrated $HClO_4$ (Merck Suprapure®, Merck KGaA, Darmstadt, Germany) were added to ~50–200 µl of plasma. The sample-acid mixture was placed on a hotplate at 100 °C for about 1.5 h and then placed for 12–14 h at 140 °C on the hotplate. Afterwards, the solution was evaporated to dryness, and the residue was dissolved in 2 ml of 2.5 mol L^{-1} HCl. To an aliquot of this solution containing 3 µg Ca, 240 µl of a $^{43}Ca/^{48}Ca$ double spike solution (Table 1) were added, evaporated and then dissolved in 250 µl of 10 mol L^{-1} HCl for further chemical purification.

3.1.2. Urine

To an aliquot of the urine sample (~3 µg of Ca) about 240 µl of the double spike solution, 2 ml double-distilled concentrated HNO_3 and 500 µl H_2O_2 (Merck Suprapure®, Merck KGaA, Darmstadt, Germany) were added. The mixture was then placed on a hotplate at 120 °C for 12–14 h. Finally, the solution was evaporated and the residue was dissolved in 250 µl of 10 mol L^{-1} HCl for further chemical purification.

3.1.3. Bones and faeces

These sample types were digested according to the method previously described [10,12]. From the solutions obtained after digestion we took an aliquot containing about 3 µg Ca and added our Ca double spike solution (240 µl). This mixture was dried down and redissolved in 250 µl of 10 mol L^{-1} HCl for further chemical purification.

3.2. Chemical purification

Following the digestion procedure, a chemical purification of the samples was performed in order to extract Ca from all other components/elements of the sample. For the chemical purification we followed the method of Wombacher et al. [13]. For the chemical separation, 1 ml of Biorad AG50W-X8 (200–400 mesh) cation exchange resin was placed in the columns and cleaned by rinsing with ~30 ml of 4 mol L^{-1} HCl. The column was

Table 1. Composition of the used $^{43}Ca/^{48}Ca$ double spike solution.

Isotope	Concentration (ng/µl)
^{40}Ca	0.0771
^{42}Ca	0.0043
^{43}Ca	0.4198
^{44}Ca	0.0258
^{48}Ca	0.5610

then equilibrated with 2 ml of 10 mol/L HCl, and the sample solution (0.25 ml of 10 mol L^{-1} HCl) was loaded onto the column. After washing with 2.5 ml of 10 mol L^{-1} HCl, the Ca-fraction was collected with 10.5 ml of a 7 mol L^{-1} HBr (Merck Suprapure®, Merck KGaA, Darmstadt, Germany). The eluate was dried down on a hot plate and diluted with 2 ml of a 2.25 mol L^{-1} HCl solution. Column blanks have been determined and were found to be about 30 ng of Ca. Compared to the 3000 ng of sample Ca loaded onto a column the blank contribution is 1 % and therefore is considered to be negligible.

For each measurement about 200 µl of the purified sample solution were evaporated, diluted with 1.5 µl of a 2.25 mol L^{-1} HCl solution and loaded onto a pre-outgassed Rhenium ribbon single filament with a Ta activator using a sandwich loading technique as previously described [14].

3.3. Mass spectrometry

Ca isotope measurements of double-spiked samples and of double-spiked standard materials were performed on a TRITON thermal ionization mass spectrometer (Thermo-Fisher Scientific, Bremen, Germany) at the mass spectrometer facilities of the GEOMAR Helmholtz Centre for Ocean Research Kiel, Germany. Details on the mass spectrometry, cup configuration and a detailed description of the method have been published [15]. Each sample was measured at least three to five times by repeated loading of the purified sample solution.

For data reduction and spike denormalisation we used an iterative algorithm [14]. The algorithm calculates the $^{44}Ca/^{40}Ca$ sample ratio from the measured $^{40}Ca/^{48}Ca$, $^{43}Ca/^{48}Ca$ and $^{44}Ca/^{48}Ca$ ratios. All Ca isotope data are reported as: $\delta^{44/40}Ca$ (‰) [16–18]. As standard material ('zero-delta' standard) we used the standard reference material NIST SRM 915a, which was measured together with the samples. The $\delta^{44/40}Ca$ sample values were calculated by averaging all $^{44}Ca/^{40}Ca$ calculated for every scan and using averaged $^{44}Ca/^{40}Ca$ of the SRM 915a measurements from the same turret for normalization. NIST SRM 1486 serves as a secondary standard and was measured like an unknown sample. NIST SRM 915a measurements were 0.00 ± 0.11 ‰ (±1 SD, $n = 64$). NIST SRM 1486 was measured to be -1.02 ± 0.13 ‰ (±1 SD, $n = 42$), which is in good agreement to the published value of −1.01 ‰ [15].

3.4. Statistics

For statistical analysis the program R (The R Foundation for Statistical Computing, http://www.r-project.org) was applied. Means and SD were given throughout. Correlations were regarded as significant at $P < .05$.

4. Results

The results of the Ca isotope measurements of blood, bones, faeces and urine are presented in detail in Table 2. Differences of excreta and tissues between the groups and their statistical significance are reported in Table 3. The analysed diet samples have slightly different Ca isotopic compositions. The diet for Con and GIO shows a $\delta^{44/40}Ca_{diet} = 0.47 \pm 0.02$ ‰ ($n = 3$) whereas the $\delta^{44/40}Ca_{diet}$ of −CaD is 0.37 ± 0.02 ‰ ($n = 7$).

Table 2. Measured Ca isotopic composition ($\delta^{44/40}$Ca, weighted) and weighted errors (±1 SD) in faeces, urine, bones and blood of Göttingen minipigs from the three investigated groups.

	faeces		urine		bones		blood (plasma)	
sample	$\delta^{44/40}$Ca (‰)	±1 SD	$\delta^{44/40}$Ca (‰)	±1 SD	$\delta^{44/40}$Ca (‰)	±1 SD	$\delta^{44/40}$Ca (‰)	±1SD
Control group (Con), $\delta^{44/40}Ca_{diet} = 0.47 \pm 0.02$ ‰								
1142	0.56	0.02	2.13	0.02	−0.53	0.02	0.08	0.02
1153	0.28	0.02	2.26	0.02	−0.17	0.02		
1161	0.46	0.02	1.77	0.02	−0.52	0.02		
1192	0.47	0.02	2.13	0.03	−0.44	0.02		
1233	0.22	0.02	1.61	0.02	−1.05	0.05		
1253	0.33	0.02	2.12	0.03	−0.61	0.02	0.07	0.02
Mean	0.39 ± 0.05 (SEM)		2.00 ± 0.10 (SEM)		−0.55 ± 0.12 (SEM)		0.07 ± 0.00 (SEM)	
Glucocorticosteroid-induced osteoporosis group (GIO), $\delta^{44/40}Ca_{diet} = 0.47 \pm 0.02$ ‰								
1127	0.25	0.03	2.00	0.04*	−0.11	0.02		
1136	0.16	0.02	2.67	0.02	0.04	0.02	0.53	0.02
1154	0.25	0.02	2.42	0.03	0.17	0.02		
1166	0.38	0.02	2.33	0.03	−0.20	0.02		
1217	0.27	0.03	2.07	0.04	−0.75	0.02		
1263	0.37	0.02	2.11	0.03	−0.21	0.02	0.70	0.02
Mean	0.28 ± 0.03 (SEM)		2.27 ± 0.10 (SEM)		−0.18 ± 0.13 (SEM)		0.62 ± 0.09 (SEM)	
Low Ca and no vitamin D diet group (−CaD), $\delta^{44/40}Ca_{diet} = 0.37 \pm 0.03$ ‰								
1130	−0.28	0.02	1.76	0.02	−0.57	0.02		
1137	−0.16	0.02	2.03	0.03	−0.49	0.02	0.15	0.02
1182	−0.54	0.02	1.79	0.03	−0.70	0.02		
1209	0.05	0.02	2.29	0.02	−0.41	0.02		
1226	−0.21	0.03	1.74	0.03	−0.56	0.02		
1244	−0.45	0.02	2.74	0.03	−0.34	0.02	0.44	0.02
Mean	−0.27 ± 0.09 (SEM)		2.06 ± 0.16 (SEM)		−0.51 ± 0.05 (SEM)		0.29 ± 0.15 (SEM)	

Note: The Ca isotopic composition of each sample have been measured at least three times by repeated sample loadings.
*Internal error as the sample was measured only once.

Table 3. Differences (Δ) of the Ca isotopic composition of analysed tissues and excreta between the three groups (Con, GIO and −CaD) and their statistical significances.

Δ	Faeces	Urine	Blood	Bone
Con−GIO	0.11 ‰ ($P = .437$)	−0.26 ‰ ($P = .330$)	−0.54 ‰ ($P = .059$)	−0.38 ‰ ($P = .057$)
−CaD−Con	−0.65 ‰ ($P < .001$)	−0.05 ‰ ($P = .951$)	0.22 ‰ ($P = .373$)	0.04 ‰ ($P = .954$)
−CaD−GIO	−0.54 ‰ ($P < .001$)	−0.21 ‰ ($P = .487$)	−0.32 ‰ ($P = .193$)	−0.33 ‰ ($P = .097$)

The individual $\delta^{44/40}Ca_{faeces}$ values range in total from −0.54 to 0.56 ‰ (Table 2 and Figure 1). The average $\delta^{44/40}Ca_{faeces}$ of Con (0.39 ± 0.13 ‰) and of GIO (0.28 ± 0.08 ‰) do statistically not differ and are relatively close to their corresponding diet $\delta^{44/40}Ca_{diet}$ (0.47 ± 0.02 ‰). However, the average $\delta^{44/40}Ca_{faeces}$ value of −CaD is significantly lower (−0.27 ± 0.21 ‰) than the average values of Con and GIO ($P = .0014$ and $P = .0017$, respectively). Also in contrast to the Con and GIO, the $\delta^{44/40}Ca_{faeces}$ value is significantly lower than the corresponding $\delta^{44/40}Ca_{diet}$ value (0.37 ± 0.02 ‰; $P = .00071$).

The individual Ca isotopic compositions of the analysed blood plasma samples ($\delta^{44/40}Ca_{blood}$) range from 0.07 to 0.70 ‰. In accordance with the data presented by Tacail et al., the Ca isotopic composition of blood plasma represents the Ca isotopic composition of whole blood with only small deviations [19]. The average $\delta^{44/40}Ca_{blood}$ of Con (0.07 ‰) is considerably isotopically lighter than the corresponding $\delta^{44/40}Ca_{diet}$ ($P = .0064$). In contrast, the average $\delta^{44/40}Ca_{blood}$ of GIO (0.62 ± 0.12 ‰) is slightly but statistically insignificantly higher than their corresponding $\delta^{44/40}Ca_{diet}$ (0.47 ± 0.02 ‰; $P = .34$) while the average $\delta^{44/40}Ca_{blood}$ of −CaD (0.29 ± 0.21 ‰) is close to its corresponding $\delta^{44/40}Ca_{diet}$ (0.37 ± 0.02; $P = .69$).

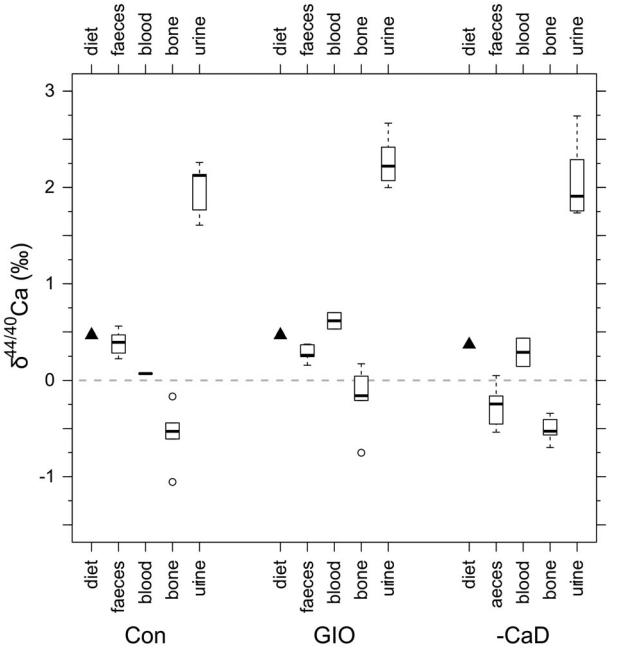

Figure 1. Box plot of measured Ca isotopic composition of diet ($\delta^{44/40}Ca_{diet}$), faeces ($\delta^{44/40}Ca_{faeces}$), blood ($\delta^{44/40}Ca_{blood}$), bone ($\delta^{44/40}Ca_{bone}$) and urine ($\delta^{44/40}Ca_{urine}$) from the three different investigated groups of the minipig trial: control group (Con), glucocorticosteroid-induced osteoporosis (GIO) and group with low Ca and no vitamin D diet (−CaD). Open circles indicate outliers.

The $\delta^{44/40}Ca_{urine}$ values range from 1.61 up to 2.74 ‰ with an average $\delta^{44/40}Ca_{urine}$ of 2.11 ± 0.32 ‰. Differences in the average $\delta^{44/40}Ca_{urine}$ between the three groups exist, but are less pronounced when compared to the observed differences in their $\delta^{44/40}Ca_{faeces}$ or $\delta^{44/40}Ca_{blood}$ values (cf. Table 3). While the average $\delta^{44/40}Ca_{urine}$ of Con (2.00 ± 0.28 ‰) and −CaD (2.06 ± 0.44 ‰) are similar, the average $\delta^{44/40}Ca_{urine}$ of GIO (2.27 ± 0.28 ‰) tends to be higher but is within error bars indistinguishable from the other two groups.

The individual $\delta^{44/40}Ca_{bone}$ values range from −1.06 to 0.17 ‰. While the average $\delta^{44/40}Ca_{bone}$ values are similar for groups Con (−0.56 ± 0.32 ‰) and −CaD (−0.51 ± 0.14 ‰), the average $\delta^{44/40}Ca_{bone}$ values of group GIO tend to be higher but are also within error bars indistinguishable from the others (−0.18 ± 0.35 ‰; $P = .05$).

5. Discussion

5.1. Fractionation between blood and bones

From our measured $\delta^{44/40}Ca_{bone}$ and $\delta^{44/40}Ca_{blood}$ values we determined the average difference between mineralized tissue (bone) and blood ($\Delta^{44/40}Ca_{blood-bone}$) to be 0.68 ± 0.15 ‰, which is in agreement of the value of 0.68 ± 0.23 ‰ calculated from the values of three individual samples (horse, chicken and fur seal) published by Skulan and DePaolo [2]. This can also be seen graphically from Figure 2. Both data sets are in general agreement and are complementing each other. The *y*-axis interception of the

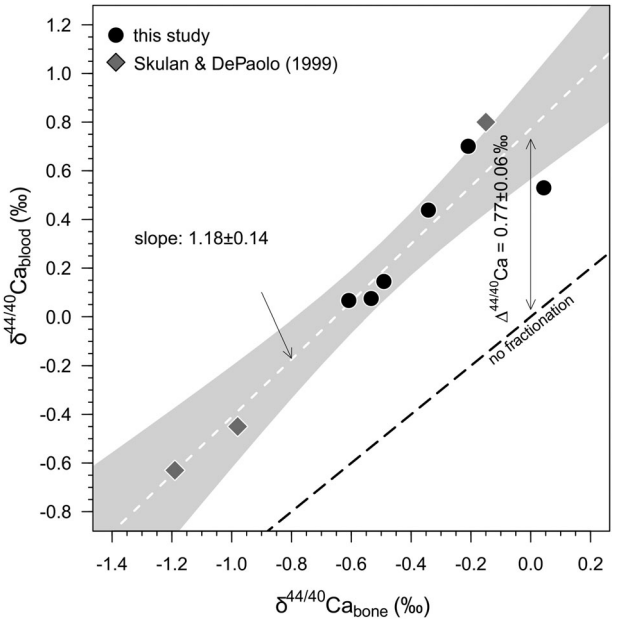

Figure 2. The plot of $\delta^{44/40}Ca_{blood}$ versus $\delta^{44/40}Ca_{bone}$ from this study and three individual samples (horse, chicken and fur seal) from the literature [2] illustrates the Ca isotope fractionation ($\Delta^{44/40}Ca_{bone-blood}$) during bone formation. The *y*-axis interception of 0.77 ± 0.06 directly reflects the Ca isotope fractionation factor $\Delta^{44/40}Ca_{bone-blood}$ for blood and bone ($P < .001$). The slope of the linear fit is close to unity (1.18 ± 0.14, $P < .001$) showing that the fractionation between blood and bones is independent from varying $\delta^{44/40}Ca_{blood}$ values. Grey shaded area represents 95 % confidence band of the linear regression trend (white dashed line).

linear correlation of the combined data (white dashed line) of 0.77 ± 0.06 ($P < .001$) directly gives the offset between blood and bones ($\Delta^{44/40}Ca_{blood-bone}$). Within error the slope of the linear fit is close to one (1.18 ± 0.14, $P < .001$), which indicates that $\Delta^{44/40}Ca_{blood-bone}$ is nearly constant over the whole range of $\delta^{44/40}Ca_{bone}$. The linear correlation of our data (best fit line not shown in Figure 2) gives only slightly different values. The *y*-axis interception is 0.66 ± 0.13 ($P < .01$), and the slope is 0.92 ± 0.30 ($P < .05$).

Concerning the isotopical difference between diet and bone ($\Delta^{44/40}Ca_{diet-bone}$) we calculated a value of 0.85 ± 0.25 ‰ which is statistically indistinguishable from the average $\Delta^{44/40}Ca_{blood-bone}$ value of 0.68 ± 0.15 ‰. This may indicate that the maximum Ca isotope fractionation between blood and diet is within the frame of the measured uncertainty and probably less than 0.2 ‰. Note that our $\Delta^{44/40}Ca_{diet-bone}$ value of 0.85 ± 0.25 ‰ reported here is lower than the one reported by the compilation of different species in Skulan and DePaolo calculated to be ~1.3 ‰. The difference in the two values is not a contradiction and rather may reflect the averaging across many species as done by Skulan and DePaolo (1999) [2] and the difference in the biomineralisation mechanisms active in mini pigs relative to other vertebrates.

5.2. Calcium isotope partitioning between bone, blood and urine

The Ca isotope values of all urine samples ($\delta^{44/40}Ca_{urine}$) are enriched in heavy Ca isotopes compared to all other compartments (Figure 1). Such an enrichment has already been reported for human urine [3–5,20] and was attributed to be a direct consequence of kidney-related mineral recycling processes and the preferential reabsorption of the light Ca isotopes from the primary urine resulting in an enrichment of the heavy Ca isotopes in the excreted secondary urine [4]. In support of this hypothesis a close positive relationship between $\delta^{44/40}Ca_{bone}$, $\delta^{44/40}Ca_{blood}$ and $\delta^{44/40}Ca_{urine}$ can be expected as has already be seen for the close relationship between $\delta^{44/40}Ca_{bone}$ and $\delta^{44/40}Ca_{blood}$ (Figure 2). Channon et al. [21] reported a correlation between $\delta^{44/42}Ca_{blood}$ and $\delta^{44/42}Ca_{urine}$ supporting the above hypothesis. The correlation ($r^2 = 0.89$, $P < .05$) between $\delta^{44/40}Ca_{blood}$ and $\delta^{44/40}Ca_{urine}$ of our study (Figure 3, neglecting the high $\delta^{44/40}Ca_{blood}$ value of one GIO sample) confirms the findings of Channon et al. [21] and further supports the above hypothesis.

In agreement with the close positive relationship between $\delta^{44/40}Ca_{bone}$, $\delta^{44/40}Ca_{blood}$ and $\delta^{44/40}Ca_{urine}$ (Figures 2 and 3), a correlation between $\delta^{44/40}Ca_{bone}$ and $\delta^{44/40}Ca_{urine}$ ($r^2 = 0.49$, $P < .005$) can be seen (Figure 4). However, a detailed analysis of the data indicates that the correlation between $\delta^{44/40}Ca_{bone}$ and $\delta^{44/40}Ca_{urine}$ is statistically significant for the Con ($r^2 = 0.67$, $P < .05$) and −CaD samples ($r^2 = 0.79$, $P < .05$) only. In contrast, for GIO samples the correlation is statistically insignificant ($r^2 = 0.36$, $P = .211$).

The general hypothesis that any changes in the Ca fluxes between bone, blood and urine are reflected in changes of the Ca isotope composition is confirmed by our data here. In particular, any flux of isotopically light Ca from the bones back to the blood is reflected also in a decrease of the $\delta^{44/40}Ca_{urine}$ values. Hence, a decrease of the $\delta^{44/40}Ca_{blood}$ and $\delta^{44/40}Ca_{urine}$ might in general be indicative for a change of the bone mineralization to demineralization ratio, as was previously suggested by Heuser and Eisenhauer [4]. This, however, is not supported by the data obtained in this study. The $\delta^{44/40}Ca_{blood}$ are even higher in GIO compared to Con and similar in

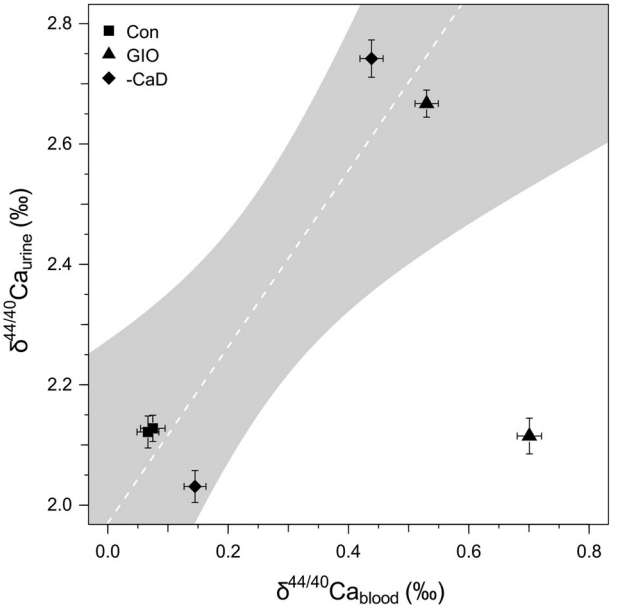

Figure 3. Plotted relation between of $\delta^{44/40}Ca_{blood}$ and $\delta^{44/40}Ca_{urine}$ also shows a significant correlation ($r^2 = 0.89$, $P = .017$) thereby neglecting a single $\delta^{44/40}Ca_{blood}$ value of +0.74 ‰ (GIO) as an outlier. Grey shaded area represents 95 % confidence band of the linear regression trend (white dashed line).

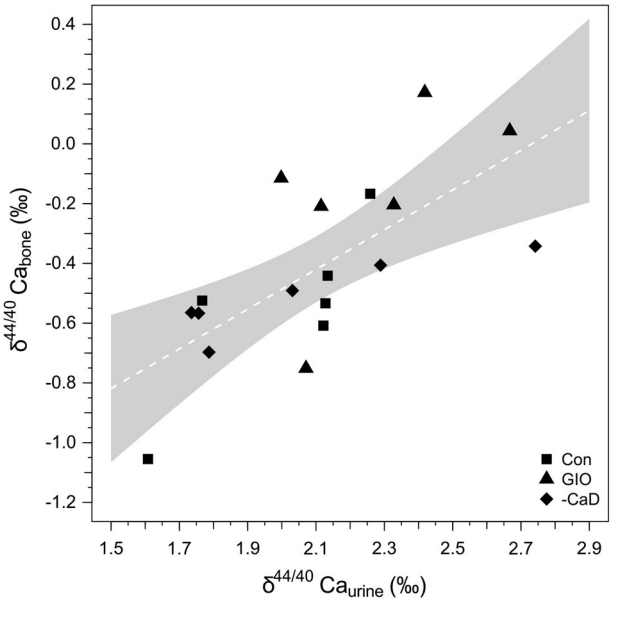

Figure 4. Taken all measured values together there is a positive but not significant trend for the $\delta^{44/40}Ca_{bone}$ and $\delta^{44/40}Ca_{urine}$ values ($r^2 = 0.49$). However, each group analysed individually, the $\delta^{44/40}Ca_{bone}$–$\delta^{44/40}Ca_{urine}$ relationship is significant for the Con and the −CaD group but insignificant for the GIO values. Grey shaded area represents 95 % confidence band of the linear regression trend (white dashed line).

−CaD compared to Con, although demineralization was documented in GIO and −CaD [10–12], and the fractionation between blood and bone remained similar through all three groups (Figure 2).

A shift of $\delta^{44/40}Ca_{blood}$ and $\delta^{44/40}Ca_{urine}$ to lower values cannot be detected here because this would require a properly determined baseline value of $\delta^{44/40}Ca_{blood}$ and $\delta^{44/40}Ca_{urine}$ before the onset of dietary change and drug intervention. Additionally, changes in the kidney mineral recycling rate may also trigger changes and superimposes the Ca isotope compositions of blood and urine, respectively.

5.3. Calcium isotope fractionation during intestinal Ca absorption

Usually, between 25% and 35% of total intestinal Ca is absorbed in the intestine [22,23] whereas the residue is excreted via faeces. If Ca isotopes become fractionated during intestinal absorption, it can be hypothesized that faeces should reflect this fractionation but with an inverse enrichment compared to the metabolized Ca. Thus, a deviation of $\delta^{44/40}Ca_{faeces}$ from the corresponding $\delta^{44/40}Ca_{diet}$ may indicate isotope fractionation during intestinal Ca absorption. A comparison of the $\delta^{44/40}Ca_{faeces}$ with their corresponding $\delta^{44/40}Ca_{diet}$ values shows that average $\delta^{44/40}Ca_{faeces}$ values of −CaD (−0.27 ± 0.21 ‰) are significantly lower ($P < .01$) by about 0.64 ‰ compared to their corresponding $\delta^{44/40}Ca_{diet}$. The difference between $\delta^{44/40}Ca_{faeces}$ and $\delta^{44/40}Ca_{diet}$ in GIO is less pronounced (0.19 ‰, $P < .01$) and average $\delta^{44/40}Ca_{faeces}$ of Con is not significantly different (0.08 ‰, $P = .19$) from its $\delta^{44/40}Ca_{diet}$.

In a first approach, one might assume that $\delta^{44/40}Ca_{faeces}$ values being lower than their corresponding $\delta^{44/40}Ca_{diet}$ might be due to a preferential absorption of heavy Ca isotopes in the intestine resulting in an enrichment of light Ca isotopes in the faeces. However, this enrichment is opposite to what would be expected, when comparing intestinal Ca absorption with renal Ca reabsorption. Ca transport during renal Ca reabsorption favours light Ca isotopes due to the kinetic type of fractionation resulting in an enrichment of heavy Ca isotopes in the excreted urine [3,4].

Both processes, renal Ca reabsorption and intestinal Ca absorption, make use of a mainly transcellular Ca transport, including transport via highly selective Ca channels of the same family (Transient receptor potential cation channel subfamily V, TRPV) as those of the apical part of the cell [23,24]. Therefore, also during intestinal Ca absorption the light Ca isotopes are expected to be taken up preferentially resulting in $\delta^{44/40}Ca_{faeces}$ being enriched in the heavy Ca isotopes. Following these considerations the average $\delta^{44/40}Ca_{faeces}$ values are actually expected to be isotopically heavier than their corresponding $\delta^{44/40}Ca_{diet}$. Our observations, however, stand in contrast to this.

In order to reconcile expectation with observation three approaches are conceivable:

(1) In mixed diets it is possible that dietary Ca reacts with other components of the diet like phytate or oxalate to form indigestible Ca phytate or Ca oxalate [25], respectively. In analogy to observations from calcium carbonates and phosphates, which are known to have a light Ca isotopic composition due to Ca isotope fractionation during precipitation [26,27], one could assume that Ca oxalates and Ca phytates are similarly enriched in the light Ca isotopes. Thus, light $\delta^{44/40}Ca_{faeces}$ could indicate Ca isotope

fractionation during formation of indigestible Ca minerals that would not be available during intestinal Ca absorption. However, the semisynthetic diet of the minipigs did not contain phytate or oxalate, but rather cellulose, which is considered to be less reactive. Only a compound-specific Ca isotope analysis of faeces may clarify this point.

(2) In addition to the transcellular Ca pathway a paracellular pathway also exists [23] to transport Ca from the gastrointestinal tract to the blood. Calcium may be fractionated differently during paracellular and transcellular pathway. At normal Ca intake the active transcellular pathway accounts for most of the absorbed Ca while the passive paracellular transport accounts for only 8–23 % of absorbed Ca [28]. It may be assumed that the passive paracellular pathway was proportionally increased in −CaD as they were fed with a low Ca and vitamin D free diet. Their plasma $1,25(OH)_2D_3$ concentrations were high, as a reaction of the low dietary Ca, while their low $25(OH)D_3$ values indicated vitamin D-deficiency and reduced active Ca absorption [12]. Whether or not a shift towards more pronounced passive Ca absorption could explain the low $\delta^{44/40}Ca_{faeces}$ of −CaD remains unclear pending future experiments.

(3) It may be speculated that an additional isotopically light Ca compartment exists which is secreted into the intestinal lumen. This additional compartment shifts the original dietary Ca isotopic composition towards isotopically lighter values. As a consequence, a preferential absorption of the light Ca isotopes then would shift the $\delta^{44/40}Ca_{faeces}$ back towards the $\delta^{44/40}Ca_{diet}$ resulting in $\delta^{44/40}Ca_{faeces}$ being close to the corresponding $\delta^{44/40}Ca_{diet}$ value as it is observed for Con samples. Based on the work of Mundy and Guise [29] it becomes obvious that gastrointestinal fluids are an additional source for Ca excreted into the intestinal lumen. These gastrointestinal fluids could possibly be a source of isotopically light Ca.

Unfortunately, there is no information on the Ca isotopic composition of such intestinal fluids available. Intestinal fluids such as digestive secretions/juices are produced in tissues like stomach, pancreas, liver (bile) and intestinal mucosa where active Ca transport with a transcellular pathway is involved. The fractionation between soft tissue and milk during lactation may serve as an analogue here. During lactation Ca is excreted into milk using a transcellular transport in the mammary glands [30,31]. Chu et al. [32] reported $\delta^{44/40}Ca$ values in human milk to be −2.0 to −2.9 ‰ which is lower by 1.0–1.9 ‰ than their corresponding $\delta^{44/40}Ca_{diet}$ value of a modern western diet of humans ($\delta^{44/40}Ca_{diet} \sim -1.0$ ‰). Therefore, it appears justified to assume that gastrointestinal fluids are isotopically lighter than their corresponding $\delta^{44/40}Ca_{diet}$ and even lighter by about 1–2 ‰ in their Ca isotope composition.

Interestingly, $\delta^{44/40}Ca_{blood}$ values of Con are lower than their $\delta^{44/40}Ca_{diet}$ by about 0.4 ‰ ($P < .001$) which generally supports possible fractionation of Ca during intestinal absorption. As expected from our basic assumption. Latter inferences are supported by the recent finding that blood (serum) and diet was different by about 0.2 ‰ per atomic mass unit (u) [19].

5.4. Influence of dietary Ca content on $\delta^{44/40}Ca_{faeces}$

When looking at the intestinal Ca absorption one has to keep in mind the total Ca content as well as the overall Ca isotopic composition in the gut. The total intestinal Ca consists of

the dietary Ca and the Ca entering the intestine via intestinal fluids (endogenous Ca). As the Ca isotopic composition of dietary Ca ($\delta^{44/40}Ca_{diet}$) and endogenous Ca ($\delta^{44/40}Ca_{endogenous}$) are different, variable amounts of dietary Ca relative to endogenous Ca results in a shift of the Ca isotopic composition of the total intestinal Ca and hence the $\delta^{44/40}Ca_{faeces}$ values. Indeed, the $\delta^{44/40}Ca_{faeces}$ values of −CaD fed with a diet containing 0.2 wt.-% Ca is significantly lower by 0.66 ‰ compared to the Con group fed with a diet containing 0.6 wt.-% Ca.

In addition to the differences in Ca content of the diet the amount of endogenous Ca is obviously different for the investigated groups which can be seen by comparing ingested with defaecated Ca. It has been reported earlier when investigating the whole set of 10 animals per experimental group that over a time period of 7 days group −CaD ingested 5180 mg Ca (Ca_{diet}) but excreted about 8373 ± 2258 (SEM) mg Ca via faeces (Ca_{faeces}) and 460 ± 85 mg via urine [12]. For comparison, in Con Ca intake (Ca_{diet}) was 15,540 mg per 7 days Ca intake, faecal excretion was 13,833 ± 1232 mg (SEM) [12] and urinary excretion was 391 ± 60 mg [12]. Like in the control group, Ca intake in the GIO group was 15,540 mg and the faecal excretion at baseline was 13,315 ± 1245 mg (SEM). At t_{15} faecal Ca excretion was 16,726 ± 2032 mg (SEM) and urinary Ca excretion was 341 ± 36 mg (SEM), resulting in negative Calcium absorption and retention [10].

From Figure 5 it can be seen that the ratio of excreted to dietary Ca (Ca_{faeces}/Ca_{diet}) is positively correlated ($r^2 = 0.749$, $P < .001$) with the Ca isotope difference between diet and faeces ($\Delta^{44/40}Ca_{diet-faeces}$). This correlation indicates a shift of $\delta^{44/40}Ca_{faeces}$ towards

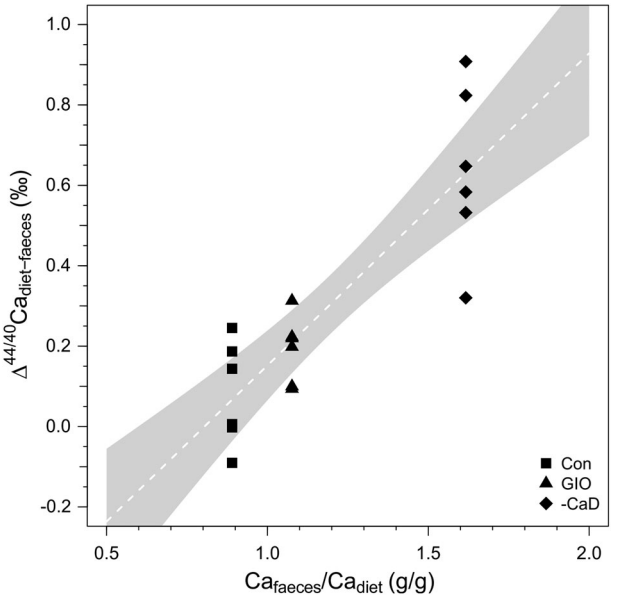

Figure 5. The calculated Ca isotope fractionation factors for diet and faeces ($\Delta^{44/40}Ca_{diet-faeces}$) of the three groups are plotted as function of the ratio of excreted to ingested Ca (Ca_{faeces}/Ca_{diet}). Both parameters are significantly correlated ($r^2 = 0.73$, $P < .001$) and show that the $\Delta^{44/40}Ca_{diet-faeces}$ values increase as a function of the Ca_{faeces}/Ca_{diet} ratio. Grey shaded area represents the 95 % confidence band of the linear regression trend (white dashed line).

$\delta^{44/40}Ca_{endogenous}$ as a consequence of a relative change in the proportion of dietary Ca and endogenous Ca.

Both, GIO and −CaD show a net Ca loss (−1527 and −3653 mg, respectively) which requires that Ca was liberated from the bones. Bone resorption should shift $\delta^{44/40}Ca_{blood}$ to lower values which then finally should cause lower $\delta^{44/40}Ca_{endogenous}$ and $\delta^{44/40}Ca_{urine}$ which cannot be detected here (cf. Section 5.2).

5.5. Limitations and shortcomings of the study

In the course of the study and the interpretation of the results several limitations and shortcomings arose. One of the most important shortcomings is the fact that the data represent a temporal snapshot only. To better constrain changes in the Ca isotopic composition of the different tissues and excreta the determination of baseline values unaffected by drug intervention or dietary changes would have been useful. The comparison of GIO and −CaD with Con samples gives no information on the temporal development of the observed differences between the groups.

The Ca isotopic composition of the diet and water has a big influence on how to interpret the data as diet and water represent the only external source of Ca to the body. It cannot be ruled out that $\delta^{44/40}Ca_{diet}$ is different between different batches of prepared food. Thus, $\delta^{44/40}Ca_{diet}$ may have changed in the course of the study. Blood and excreta should be affected to a larger extend by the actual dietary Ca isotopic composition because of their short turnover times. On the other hand, bones having longer turnover times may be affected to a larger but unknown extend by changes of $\delta^{44/40}Ca_{diet}$.

As extensively discussed the Ca isotopic composition of intestinal fluids may play an important role to explain $\delta^{44/40}Ca_{faeces}$. Although some lines of evidence exist to assume that $\delta^{44/40}Ca_{endogenous}$ are low a direct sampling of the intestinal fluids would have been needed to ensure that (and to which extend) intestinal fluids determine $\delta^{44/40}Ca_{faeces}$.

From a statistical point of view more samples from each group would have strengthened our interpretation of the investigated sample materials.

6. Conclusions

Our data show that the Ca isotope compositions of diet ($\delta^{44/40}Ca_{diet}$), faeces ($\delta^{44/40}Ca_{faeces}$), blood ($\delta^{44/40}Ca_{blood}$), bone ($\delta^{44/40}Ca_{bone}$) and urine ($\delta^{44/40}Ca_{urine}$) show distinct values which depend directly on their dietary treatment and drug intervention. Urinary Ca isotope values are always enriched in the heavy Ca isotopes whereas bones always show the isotopically lightest values.

The heavy isotope values in urine reflect fractionation of Ca in the kidney. Only a minor Ca fraction enriched in heavy Ca isotopes enters the urine whereas a much larger fraction slightly depleted in the heavy isotopes is recycled back to blood.

Contrary to our expectation the Ca isotope values of faeces ($\delta^{44/40}Ca_{faeces}$) tend to be isotopically light. Although different scenarios may be discussed, we favour the influx of isotopically light intestinal fluids as a source of light Ca into the gastrointestinal tract. The latter conclusion requires future confirmation.

The three groups Con, GIO and −CaD cannot be distinguished by their urine values alone ($\delta^{44/40}Ca_{urine}$). This may be due to enhanced recycling processes in the kidney superimposing the release of isotopically light Ca from the bones and the influence of the intestinal fluids. However, a distinction of the three groups Con, GIO and −CaD is possible by comparing the fractionation factor between diet and faeces ($\Delta^{44/40}Ca_{diet-faeces}$) with the ratios of the amount of Ca in the faeces to the one in the diet (Ca_{faeces}/Ca_{diet}). The clinical relevance of this observation has yet to be tested.

Acknowledgements

We thank A. Kolevica for lab support and K. Gonda for her analytical assistance (sample digestions and chemical Ca analyses). J. Kunze, H. Fischer and D. Siewertsen are acknowledged for their thoroughly performed animal care. We are thankful for the constructive and helpful input given by two anonymous reviewers. The editor is thanked for the handling of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the Deutsche Forschungsgemeinschaft (DFG) Project No. Ei-272/23-1 and Schr 430/5-1,5-2 and by GEOMAR internal funding.

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