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ORIGINAL PAPER

Combining collagen extraction with mineral Zn isotope analyses from a single sample for robust palaeoecological investigations

Jeremy McCormack[1,2] · Nicolas Bourgon[1,3] · Virginie Sinet-Mathiot[1] · Zeljko Rezek[4] · Geoff M. Smith[1,5] · Jean-Jacques Hublin[1,4] · Mohammed Dabain[6] · Helen Fewlass[1]

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Abstract

Collagen extraction from bones or dentine, commonly used for radiocarbon (^{14}C) dating and stable carbon and nitrogen isotope (δ^{13}C and δ^{15}N) analyses, involves the dissolution of the bioapatite of skeletal elements. This fraction is typically disposed of during pretreatment. Here, we test the possibility of utilising this dissolved mineral solution for analysis of the bioapatite zinc isotope composition (δ^{66}Zn). Bioapatite δ^{66}Zn is a novel trophic level indicator similar to collagen δ^{15}N but with isotopic fractionation independent from nitrogen, thus providing additional dietary information. We tested ways to minimise Zn contamination of the dissolved mineral phase during collagen extraction. We then used archaeological bone samples from Ain Difla (Jordan) and Ranis (Germany) to compare δ^{66}Zn values of dissolved bioapatite following our collagen extraction protocol with δ^{66}Zn values from the same sample material dissolved in a metal-free cleanroom. Our results demonstrate that with only minor adjustments to minimise Zn contamination, the dissolved mineral solution from collagen extraction protocols commonly employed for ^{14}C dating and (palaeo)dietary analysis can be used for additional δ^{66}Zn analyses even when collagen extraction does not take place in a cleanroom. Our protocol allows us to gain an additional dietary proxy to complement δ^{15}N trophic level interpretations and perform more robust (palaeo)ecological investigations without further destructive sampling.

Keywords Zinc isotopes · Collagen extraction · Carbon and nitrogen isotopes · Palaeodiet · Trophic level

Introduction

For decades, stable isotope analysis of carbon and nitrogen (δ^{13}C and δ^{15}N) from bulk bone or dentine collagen has been one of the most powerful tools in archaeology and ecology to reconstruct the diet of an organism. Because of the relatively large biological fractionation, δ^{15}N values can be used as a trophic level indicator with an average of 3 to 5‰ higher values with increasing trophic level (e.g. Hobson and Welch 1992; Vander Zanden and Rasmussen 2001; Bocherens and Drucker 2003). In contrast to nitrogen, δ^{13}C values behave much more conservatively and are commonly used as a tracer of the primary production source(s) of a food web (e.g. Sponheimer et al. 2006; De la Vega et al. 2019).

The application of this approach relies on the assumption of a predictable isotopic fractionation factor between diet and consumer tissue; i.e. metabolic processes governing isotopic fractionation within an organism are mostly constant (Warinner and Tuross 2010). However, some physiological factors may also influence diet-tissue δ^{15}N fractionation in consumers, including nutritional stress (Hobson et al. 1993; Fuller et al. 2005) and growth rate (Warinner and Tuross 2010). Other than physiological factors, environmental factors may cause differences among consumer bulk δ^{13}C and δ^{15}N values. The carbon and nitrogen isotope values

✉ Jeremy McCormack
mccormack@em.uni-frankfurt.de

1 Department of Human Evolution, Max Planck Institute for Evolutionary Anthropology, Leipzig, Germany

2 Institute of Geosciences, Goethe-University Frankfurt, Frankfurt am Main, Germany

3 Institute for Geosciences, Johannes Gutenberg-University, Mainz, Germany

4 Chaire de Paléoanthropologie, Collège de France, Paris, France

5 School of Anthropology and Conservation, University of Kent, Canterbury, UK

6 Department of Antiquities of Jordan, Amman, Jordan

of primary producers (food web baseline) can vary significantly, and these differences are passed along to higher trophic level consumers. Isotopic baseline variability can compromise (palaeo)ecological dietary interpretations when comparing animals from different times and/or regions, consumers that integrate multiple food webs, or when investigating highly mobile animals (Casey and Post 2011).

Including additional geochemical dietary proxies, independent from $\delta^{13}C$ and $\delta^{15}N$ values, can help verify traditional stable isotope results and provide otherwise inaccessible dietary information, ultimately leading to more robust (palaeo)ecological interpretations (e.g. McCormack et al. 2021). In the past decade, non-traditional metal isotopes such as calcium, magnesium, and zinc (Zn) from the skeletal mineral phase (bioapatite) have shown potential as (palaeo) dietary and trophic level proxies in the marine (Martin et al. 2015; Jaouen et al. 2016a) and terrestrial systems (Heuser et al. 2011; Martin et al. 2020; Jaouen et al., 2016b). Zinc $^{66}Zn/^{64}Zn$ ratios (expressed as the $\delta^{66}Zn$ value) show decreasing $\delta^{66}Zn$ values in carnivores' bioapatite relative to herbivores (Jaouen et al. 2016b; Bourgon et al. 2020). Muscles and most organs appear typically ^{66}Zn depleted compared to the diet of an animal and its bulk body $\delta^{66}Zn$ composition (Moynier et al. 2013; Balter et al. 2013; Mahan et al. 2018), resulting in lower $\delta^{66}Zn$ values as the trophic level increases. This trophic level effect has been demonstrated for tooth enamel/enameloid (Bourgon et al. 2020, 2021; McCormack et al. 2022), tooth dentine (McCormack et al. 2022), and bone (McCormack et al. 2021), with an offset of bone/dentine values of approximately + 0.2‰ compared to enamel/enameloid within an individual (Jaouen et al., 2016b; McCormack et al. 2022).

Combining independent dietary proxies (e.g. $\delta^{15}N$ with $\delta^{66}Zn$) can help avoid misinterpretation of physiological or environmental controls on the isotopic composition as dietary. In addition, bioapatite $\delta^{66}Zn$ values show potential for identifying specific feeding behaviours, such as the consumption of bone and omnivory (Jaouen et al., 2016b; Bourgon et al. 2020, 2021). At least for marine mammal bone, $\delta^{66}Zn$ values within a species appear more homogenous geographically than $\delta^{13}C$ and $\delta^{15}N$ values, indicating a potentially lower isotopic variability at the base of the marine food web (McCormack et al. 2021). The inclusion of zinc isotope analysis may thus allow for a more direct comparability of marine species trophic ecology (and consumers thereof) between spatially and temporally distinct locations than traditional $\delta^{13}C$ and $\delta^{15}N$ isotope analysis alone.

Here, we investigate a method to routinely combine collagen extraction used for bulk $\delta^{13}C$ and $\delta^{15}N$ analyses and ^{14}C dating with $\delta^{66}Zn$ analyses on the same sampled material, thereby avoiding resampling of precious archaeological skeletal material. Many laboratories routinely use diluted hydrochloric acid (HCl) to dissolve the mineral portion of the bone as the first step in the collagen extraction and purification process, which is then discarded (e.g. Longin 1971; Brock et al. 2010; Sealy et al. 2014; Talamo et al. 2021). Here, we demonstrate that the acidic solution can be used for subsequent $\delta^{66}Zn$ analysis following the bone mineral's dissolution. Using the acidic solution allows the combination of traditional $\delta^{13}C$ and $\delta^{15}N$ (and ^{14}C dating) with $\delta^{66}Zn$ analyses, allowing more comprehensive (palaeo)ecological and dietary reconstructions through a multi-proxy approach. One of the challenges lies in potential Zn contamination introduced to the mineral solution, as collagen extraction is usually not performed in metal-free cleanrooms with acid-precleaned equipment. Therefore, we systematically tested potential Zn contamination introduced to the HCl solution during a standard collagen extraction protocol (e.g. Fewlass et al. 2019; Talamo et al. 2021), as well as methods to reduce Zn contamination without substantially modifying the collagen extraction protocol or contaminating the collagen for subsequent analyses.

We then compared $\delta^{66}Zn$ values from bone fragments dissolved in two ways: (1) dissolution during collagen extraction carried out in a standard collagen extraction laboratory for ^{14}C dating and (2) dissolution within a metal-free Pico Trace clean laboratory (standard for Zn purification and analyses). Our bone samples come from two archaeological sites with drastically different levels of bone preservation. Bones from the Ain Difla rockshelter in Jordan are fragmentary and mostly unidentifiable (based on morphology), coming from a depositional context indicating the likelihood of very low levels of collagen preservation. In contrast, the environmental context of the cave of Ranis-Ilsenhöhle in Germany is likely to demonstrate exceptionally well-preserved collagen. Thus, bones from these sites should represent the two extreme conditions regarding collagen preservation in archaeology and are ideal for testing our method.

Material and methods

All isotopic analyses were carried out in the Department of Human Evolution at the Max Planck Institute for Evolutionary Anthropology (MPI-EVA), Leipzig, Germany.

Material

Ranis-Ilsenhöhle, Germany

Ranis-Ilsenhöhle is a cave located in Thuringia, Germany (50°39′45.3″N, 11°33′53.5″E), containing archaeological deposits spanning the Middle Palaeolithic to the recent Holocene (Hülle 1977). New excavations were carried out between 2016 and 2022 by the Department of Human Evolution at the MPI-EVA and the Landesamt für Denkmalpflege

und Archäologie Thüringen (Weimar, Germany). The bones from layers 7–9 included in this study were excavated in 2020, and all appeared well preserved. They were pretreated as part of a ^{14}C dating program to reconstruct the site's chronology and were analysed through collagen peptide mass fingerprinting, also known as zooarchaeology by mass spectrometry (ZooMS), to identify the taxon.

Ain Difla, Jordan

Ain Difla in Wadi Hasa, Jordan (30°54′13.14″N, 35°48′43.70″E), is a rock shelter containing Middle Palaeolithic and arguably Initial Upper Paleolithic deposits (Clark et al. 1997; Rezek 2020). The bones in this study (Supplementary Data 1) were excavated in the upper part of the archaeological sequence at the site during the excavation undertaken in 2019 by the Department of Human Evolution (MPI-EVA). This upper part (divided provisionally into layers 1–7 during the 2019 field season) corresponds to arbitrary levels 1–5 of Clark's excavations (Clark et al. 1997). Bone remains are rare at the site and those excavated were fragmented, bleached, and highly weathered, making them difficult to identify to species level based on their morphology. We selected bones for collagen extraction and ^{14}C dating to reconstruct the chronology of the corresponding deposits. We also sampled them for taxonomic identification through ZooMS.

Collagen extraction laboratory Zn contamination tests

As collagen extraction is usually not performed in metal-free cleanrooms, a series of blank tests, i.e. without actual sample material, were performed to identify any potential non-sample related Zn contamination sources when applying our collagen extraction protocol carried out in a standard laboratory. Blank tests were carried out in a laminar flow hood and performed using the same equipment and steps used for the demineralisation of archaeological samples following our typical collagen extraction protocol (Fewlass et al., 2019; Talamo et al. 2021). Briefly, demineralisation involves ~ 5 ml 0.5 M HCl added into borosilicate glass tubes containing the bone sample using a borosilicate glass beaker and subsequent removal of HCl with glass Pasteur pipettes when demineralisation is complete (see also "Collagen extraction bone dissolution"). For the blank tests, we assessed each step of the demineralisation protocol for potential Zn contamination and collected the ~ 5 ml 0.5 M HCl solution in precleaned perfluoroalkoxy alkane (PFA) vials (Table 1).

The same steps were then repeated with additional HCl precleaning of the glass beakers and tubes and personnel

Table 1 Zinc concentration ([Zn]) in collagen extraction blank tests. We used approximately 5 ml 0.5 M HCl for each blank test. All glassware was initially cleaned and heated at 500 °C for 8 h. For the HCl-precleaned glass equipment blanks, personnel also wore vinyl gloves. All procedural blank HCl solutions were collected in acid-precleaned PFA vials after the blank tests

Blank preparation	No HCl precleaning (nitrile gloves)	HCl precleaning (vinyl gloves)	HCl precleaning and suprapure HCl (vinyl gloves)
	[Zn] ng/ml	[Zn] ng/ml	[Zn] ng/ml
0.5 M HCl directly into PFA vial from glass beaker	5	6	1
0.5 M HCl directly into PFA vial from glass beaker	5	4	4
0.5 M HCl directly into PFA vial from glass beaker	6	4	1
0.5 M HCl into glass tube for 24 h; closed with lid	13	4	18
0.5 M HCl into glass tube for 24 h; closed with lid	110	4	1
0.5 M HCl into glass tube for 24 h; closed with lid	10	3	6
0.5 M HCl into glass tube for 24 h; open in flow hood	15	4	1
0.5 M HCl into glass tube for 24 h; open in flow hood	7	4	1
0.5 M HCl into glass tube for 24 h; open in flow hood	7	4	1
0.5 M HCl pipette between 2 glass tubes	9	19	9
0.5 M HCl pipette between 2 glass tubes	6	13	11
0.5 M HCl pipette between 2 glass tubes	5	13	9
0.5 M HCl into glass tube for 24 h; closed with aluminium foil	7	3	1
0.5 M HCl into glass tube for 24 h; closed with aluminium foil	16	4	1
0.5 M HCl into glass tube for 24 h; closed with aluminium foil	7	4	1
0.5 M HCl into glass tube for 1 month in fridge; closed with lid	7	4	2
0.5 M HCl into glass tube for 1 month in fridge; closed with lid	22	11	1
0.5 M HCl into glass tube for 1 month in fridge; closed with lid	14	5	1

wearing vinyl gloves rather than nitrile gloves, as commonly used in our laboratory. Such practice is a crucial consideration as vinyl gloves have been shown to have a lower Zn contamination potential than nitrile gloves (Jaouen et al. 2020). Hydrochloric acid glass cleaning steps involved filling beakers and tubes with 2 M high-purity HCl for 24 h and subsequently rinsing them with ultrapure water to remove any Zn. In a final procedural blank test, we repeated the same steps with HCl-precleaned glass beakers and tubes and wore vinyl gloves while using suprapure HCl instead of the laboratory-grade HCl, which is generally used for collagen extraction (Table 1).

To test the contamination potential of the glass Pasteur pipettes and a possible glass pipette cleaning protocol, we pipetted approximately 2 ml suprapure HCl from HCl-precleaned glass tubes into PFA vials, reusing the glass pipette three times. This procedure was repeated for three glass pipettes.

All blank solutions were subsequently taken to our clean laboratory, evaporated, and redissolved in 1 ml 3% nitric acid (HNO_3). Zinc concentrations were measured using a Thermo Fisher Neptune multicollector–inductively coupled plasma–mass spectrometer (MC-ICP-MS). Zn contamination was estimated by comparing the procedural blanks' Zn signal intensity (V) to a solution with a known Zn concentration (300 ng/ml).

Bone sampling and dissolution

Ten bone samples from Ain Difla and nine bone samples from Ranis were sampled using a diamond-tipped cutting wheel, ultrasonicated in ultrapure water (Milli-Q water, TOC < 5 ppb) between samples. Bone surfaces were mechanically abraded (cleaned) with a sandblaster to remove exogenous material. For each bone, between 350 and 680 mg of bone was sampled for the collagen extraction protocol, and ~30–50 mg was sampled for the clean laboratory dissolution protocol (Table 2). All ~30–50 mg bone samples were subsequently cleaned by ultrasonication in Milli-Q water for 5 min and dried in a drying chamber at 50 °C (standard practice for Zn isotope analyses). Samples for the collagen extraction protocol were weighed into glass tubes, which had been previously heated at 500 °C for 8 h to remove organics and cleaned in 2 M high-purity HCl for 24 h before rinsing with ultrapure water to remove Zn contaminants. Sampling and subsequent work were carried out using vinyl gloves. A cave bear long bone (R-EVA 2907) from Austria dating to > 50,000 BP was sampled and prepared with each batch for both dissolution protocols as it is used as a 'blank' bone during all ^{14}C pretreatments to monitor lab-based contaminants.

Collagen extraction bone dissolution

Collagen extraction was carried out using the protocol described in Fewlass et al. (2019) for stable isotopic analysis and ^{14}C dating. All glassware is routinely cleaned and heated at 500 °C for 8 h to remove organics. For the initial demineralisation stage, ~5 ml 0.5 M HCl was added to each sample tube using a glass beaker (additionally precleaned in 2 M HCl at room temperature). Samples were kept in the refrigerator (4 °C) during demineralisation, with daily checks for CO_2 effervescence and softness (using glass pipettes). HCl was renewed once or twice per week. To estimate the potential of Zn contamination, procedural blanks were prepared alongside the samples for the demineralisation stage of the collagen extraction. The dissolved mineral and blank solution (HCl) were collected in acid-cleaned PFA vials. The vials were previously weighed to calculate the volume of the mineral solution collected during the collagen extraction protocol. All PFA vials used in this study were precleaned in our clean laboratory in a series of steps, including 3 M HNO_3, ultrapure water, and 6 M HCl, each step heated to 80 °C for 8 h.

After demineralisation, the PFA vials containing the HCl solutions were transferred to a metal-free Pico Trace clean laboratory for preparation for Zn isotope analyses. Based on the final solution volume, corrected for the density of 1.01 g/ml 0.5 M HCl, and the initial sample weight, a 30 to 40 mg pretreatment equivalent (between 1 and 2 ml) of the solution was extracted and evaporated. The residue was then dissolved in 1 ml 1.5 M hydrobromic acid (HBr) and placed in an ultrasonic bath for 30 min for the ensuing column chromatography Zn purification.

Following the demineralisation stage, collagen extraction of samples proceeded without further modifications. Samples were treated with 0.1 M NaOH for 30 min to remove humic acid contamination and treated again with 0.5 M HCl (15 min) to remove atmospheric CO_2. Samples were gelatinised in weakly acidic water (HCl pH3) at 75 °C for 20 h, and then filtered to remove > 80 µm particles (Eeze-Filter™ from Elkay Laboratory Products (UK) Ltd.). Gelatin extracts were then ultrafiltered to concentrate the large molecular weight (> 30 kDa) fraction (Sartorius "VivaspinTurbo" ultrafilters with 30 kDa molecular weight cut-off), precleaned to remove carbon contaminants according to Bronk Ramsey et al. (2004). Extracts were freeze-dried (48 h) and weighed to determine the collagen yield, expressed as a percentage of the initial dry bone weight.

Clean laboratory bone dissolution

Bone samples were dissolved in a metal-free cleanroom in closed PFA vials with 1 ml 1 M HCl on a hotplate for 3 h at 120 °C and then evaporated at 120 °C. The residue was

Table 2 Zinc isotope values and concentrations for Ain Difla and Ranis bone samples prepared for collagen extraction (coll. extraction) and dissolved under metal-free cleanroom (clean lab.) conditions. Differences are given between $\delta^{66}Zn$ ($\Delta^{66}Zn$) and Zn concentration ([Zn]) of the same bone sampled dissolved using both protocols. Taxon identifications from Ranis bones were determined through ZooMS analysis

Sample	Taxon	Site	Sampled for coll. extraction	Sampled for clean lab	Collagen yield	Collagen extraction protocol		Clean laboratory protocol		Difference between dissolution protocols	
			(mg)	(mg)	(%)	$\delta^{66}Zn$ (‰ JMC Lyon)	[Zn] (µg/g)	$\delta^{66}Zn$ (‰ JMC Lyon)	[Zn] (µg/g)	$\Delta^{66}Zn$	[Zn] (µg/g)
3526	ungulate medium	Ain Difla	653.9	55.94	0.5	1.32	65	1.33	65	0.01	0
3527	mammal_unknown	Ain Difla	675.5	61.03	0.3	1.56	67	1.53	64	0.03	2
3528	mammal_unknown	Ain Difla	654.2	49.28	0.4	1.46	52	1.49	49	0.02	3
3529	mammal_unknown	Ain Difla	510.4	51.04	0.6	1.46	62	1.49	64	0.03	2
3530	mammal_unknown	Ain Difla	609	44.3	0.5	1.21	66	1.21	69	0.00	3
3531	mammal_unknown	Ain Difla	565.9	45.99	0.6	1.23	75	1.30	72	0.07	3
3532	mammal_unknown	Ain Difla	514.2	54.19	0.7	1.53	78	1.52	87	0.01	9
3533	mammal_unknown	Ain Difla	589	52.22	0.5	1.34	100	1.33	102	0.01	1
3534	mammal_unknown	Ain Difla	557.5	67.73	0.3	1.34	85	1.41	91	0.07	6
3535	mammal_unknown	Ain Difla	621.9	51.53	0.4	1.30	84	1.31	83	0.01	2
3606	*Rangifer*	Ranis	461.7	39.6	8.6	0.41	660	0.43	673	0.02	13
3607	*Rangifer*	Ranis	414.8	39.14	8.4	0.31	465	0.32	635	0.01	170
3608	Ursidae	Ranis	470.2	41.08	14.5	0.59	369	0.55	488	0.04	119
3609	Ursidae	Ranis	397.3	39.98	15.1	0.45	497	0.47	507	0.01	10
3610	*Rangifer*	Ranis	399	40.32	11.6	0.37	663	0.37	717	0.00	54
3611	*Rangifer*	Ranis	361.1	41.02	8.5	0.34	672	0.33	789	0.01	117
3612	*Rangifer*	Ranis	386.7	40.38	10.2	0.51	650	0.48	650	0.03	1
3613	Ursidae	Ranis	341.5	40.2	15.3	0.48	614	0.46	641	0.02	27
3614	Equidae	Ranis	413.3	40.47	12.8	0.55	534	0.59	732	0.04	198

then dissolved in 1 ml 1.5 M HBr and placed in an ultrasonic bath for 30 min.

Column chromatography and zinc isotope measurements

The column chromatography steps for quantitative recovery of sample Zn were the same for all samples regardless of the dissolution methods used. Each column chromatography batch included one clean laboratory chemistry blank and one reference standard (NIST SRM 1400). Zn purification was performed in two steps, following the modified ion exchange method adapted from Moynier et al. (2006), first described in Jaouen et al. (2006b). One millilitre of AG-1 × 8 resin (100–200 mesh) was placed in 10 ml hydrophobic interaction columns (Macro-Prep® Methyl HIC). Resin cleaning involved 5 ml 3% HNO_3 followed by 5 ml ultrapure water, repeated twice. The resin was then conditioned with 3 ml 1.5 M HBr. After loading the sample, 2 ml 1.5 M HBr was added for matrix residue elution, followed by Zn elution with 5 ml 3% HNO_3. Following the second column step, the solution was evaporated at 105 °C and the residue was redissolved in 1 ml 3% HNO_3.

Zn isotope ratios and Zn concentrations were measured using a Thermo Fisher Neptune MC-ICP-MS at the Max Planck Institute for Evolutionary Anthropology (Leipzig, Germany). Instrumental mass fractionation was corrected by Cu doping following the protocol of Maréchal et al. (1999) and Toutain et al. (2008). The Alfa Aesar zinc plasma standard solution was used for standard bracketing. All $\delta^{66}Zn$ values are expressed relative to the JMC-Lyon isotope standard. Zn concentrations were estimated following a protocol adapted from one used for Sr by Copeland et al. (2008) by applying a regression equation based on the Zn signal intensity (V) of three solutions with known Zn concentrations (150, 300, and 600 ng/ml). The $\delta^{66}Zn$ measurement uncertainty was estimated from standard replicate analyses and was better than ± 0.04 ‰ (2 SD). All samples were measured at least twice with a mean sample reproducibility of ± 0.01‰ (2 SD). NIST SRM 1400 was analysed alongside the samples with $\delta^{66}Zn$ values, as reported elsewhere (Jaouen et al., 2016b, 2020; Bourgon et al. 2020; McCormack et al. 2021). SRM reference material and samples show a normal Zn mass-dependent isotope fractionation, i.e. the absence of isobaric interferences, whereby the $\delta^{66}Zn$ versus $\delta^{67}Zn$ and $\delta^{66}Zn$ versus $\delta^{68}Zn$ values fall onto lines with slopes close to the theoretic mass fractionation values of 1.5 and 2, respectively.

Carbon and nitrogen isotope analysis

Where sufficient collagen was extracted (e.g. Ranis bone samples), ~ 0.5 mg collagen was weighed into a tin cup and analysed with a Thermo Finnigan Flash Elemental Analyzer (EA) coupled to a Thermo Delta Plus XP isotope ratio mass spectrometer to obtain elemental (C%, N%, C:N) and stable isotopic data ($\delta^{13}C$, $\delta^{15}N$). Carbon and nitrogen stable isotope values were two-point scale normalised to the VPDB (Vienna PeeDee Belemnite) and AIR (atmospheric N_2) scale respectively, using IAEA-CH-6 (sucrose, $\delta^{13}C = -10.449 \pm 0.033$ ‰), IAEA-CH-7 (polyethylene, $\delta^{13}C = -32.151 \pm 0.050$ ‰), IAEA-N-1 (ammonium sulphate, $\delta^{15}N = 0.43 \pm 0.2$ ‰), and IAEA-N-2 (ammonium sulphate, $\delta^{15}N = 20.41 \pm 0.2$ ‰). An in-house methionine standard was used as a quality control, which gave average values of $\delta^{13}C = -28.05 \pm 0.05$ ‰ (1 SD) and $\delta^{15}N = -6.41 \pm 0.05$ ‰ (1 SD), as well as an in-house collagen standard which gave values of $\delta^{13}C = -19.82 \pm 0.3$ ‰ (1 SD) and $\delta^{15}N = 4.89 \pm 0.1$ ‰ (1 SD). Samples were measured in duplicate, and variation between samples fell within ± 0.2 ‰ instrumental error.

Zooarchaeology by mass spectrometry (ZooMS)

Zooarchaeology by mass spectrometry (collagen type I peptide mass fingerprinting) analyses followed protocols detailed elsewhere (Buckley et al. 2009; Van Doorn et al. 2011; Welker et al. 2016). In short, nine bone specimens from Ranis-Ilsenhöhle were minimally destructively sampled using a diamond-tipped cutting wheel. Soluble collagen from the bone sample was extracted using a semi-destructive soluble collagen extraction technique, through incubation in 100 µl of 50 mM ammonium bicarbonate (NH_3CO_3, AmBic) buffer at 65 °C for 1 h. Subsequently, in order to improve and verify the AmBic taxonomic identity, all bone samples were demineralised in 120 µl of 0.6 M HCl at 4 °C for 21 h. After being rinsed thrice with AmBic, bone samples were incubated for 1 h in 100 µl of AmBic solution at 65 °C. Then, 50 µl of the resulting supernatant was digested with trypsin (0.5 µg/µL, Promega) overnight at 37 °C, acidified using 1 µl of 10% trifluoroacetic acid (TFA) and cleaned on C18 ZipTips (Thermo Scientific). Eluted peptides were subsequently spotted in triplicate on a MALDI Bruker plate with the addition of α-cyano-4-hydroxycinnamic acid (CHCA, Sigma). MALDI-TOF MS analysis was conducted at the IZI Fraunhofer in Leipzig, and spectra were identified through peptide marker mass identification in comparison to a database containing peptide markers masses for all medium to large-sized mammalian genera in existence in Europe during the Pleistocene (Welker et al. 2016).

To assess any potential contamination by non-endogenous peptides, we performed a set of blank extractions alongside the rest of the samples to exclude any potential protein contamination introduced during laboratory extraction. The MALDI-TOF MS spectra show no collagenous peptides,

demonstrating that the taxonomic identification does not derive from laboratory contamination.

All Ain Difla bone samples were analysed through ZooMS, following protocols described above, but had insufficient collagen preserved to provide identifiable spectra, thus preventing any taxonomic assignment.

Results

The Zn blank tests performed in the collagen extraction laboratory showed Zn concentrations between 5 and 110 ng/ml using glass equipment (500 °C, 8 h) that was not precleaned with HCl (Table 1). Blank tests performed with HCl-precleaned glass equipment and handled with vinyl instead of nitrile gloves led to Zn concentrations between 3 and 19 ng/ml for the blanks. Blanks produced with HCl-precleaned glass equipment, using vinyl gloves and suprapure HCl, had the lowest Zn contaminations, with Zn concentrations between < 1 and 18 ng/ml (Table 1). Importantly, glass pipettes were not additionally cleaned with HCl to remove Zn for either of these blank tests (only heated at 500 °C for 8 h to remove organics). Our results indicate that the glass pipettes can induce a minor source of Zn contamination with Zn concentrations between 5 and 19 ng/ml (Table 1). However, a subsequent test, which involved pipetting approximately 2 ml suprapure HCl from HCl-precleaned glass tubes into PFA vials, reusing the glass pipette thrice, did not document any noteworthy Zn contamination (< 0.5 ng/ml, Supplementary Table 1).

Blanks collected alongside the pretreatment of the bone samples using HCl-precleaned glass equipment and personnel wearing vinyl gloves had Zn concentrations between 1 and 8 ng/ml for the Ain Difla samples and 31 ng/ml for the Ranis samples. In comparison, the final mineral solution collected after Zn purification from the collagen extraction protocol yielded Zn concentrations between 1565 and 3112 ng/ml for Ain Difla and 8760 to 19,884 ng/ml for Ranis bones. The highest Zn concentration within the blanks of each collagen extraction procedure represents less than 1% of the amount of Zn present in the final dissolved bone mineral solution. No noteworthy amount of Zn was added during the subsequent column chemistry, as all clean laboratory chemistry blanks had Zn concentrations < 1 ng/ml.

The unsystematic difference in δ^{66}Zn values between a sample prepared following the collagen extraction protocol and the same sample dissolved under cleanroom conditions (Δ^{66}Zn) is on average 0.02‰ and thereby within measurement uncertainty (±0.04 ‰, 2 SD). Only two samples from Ain Difla have Δ^{66}Zn values exceeding 0.04‰ with values of 0.07‰ (Fig. 1, Table 2). The average difference in bone Zn concentration between both dissolution protocols is 2.2 µg/g for the Ain Difla and 55.6 µg/g for Ranis samples (Table 2). The cave bear bone (R-EVA 2907) that was prepared with both the Ranis and Ain Difla samples for both dissolution protocols ($n = 4$) has a mean δ^{66}Zn value

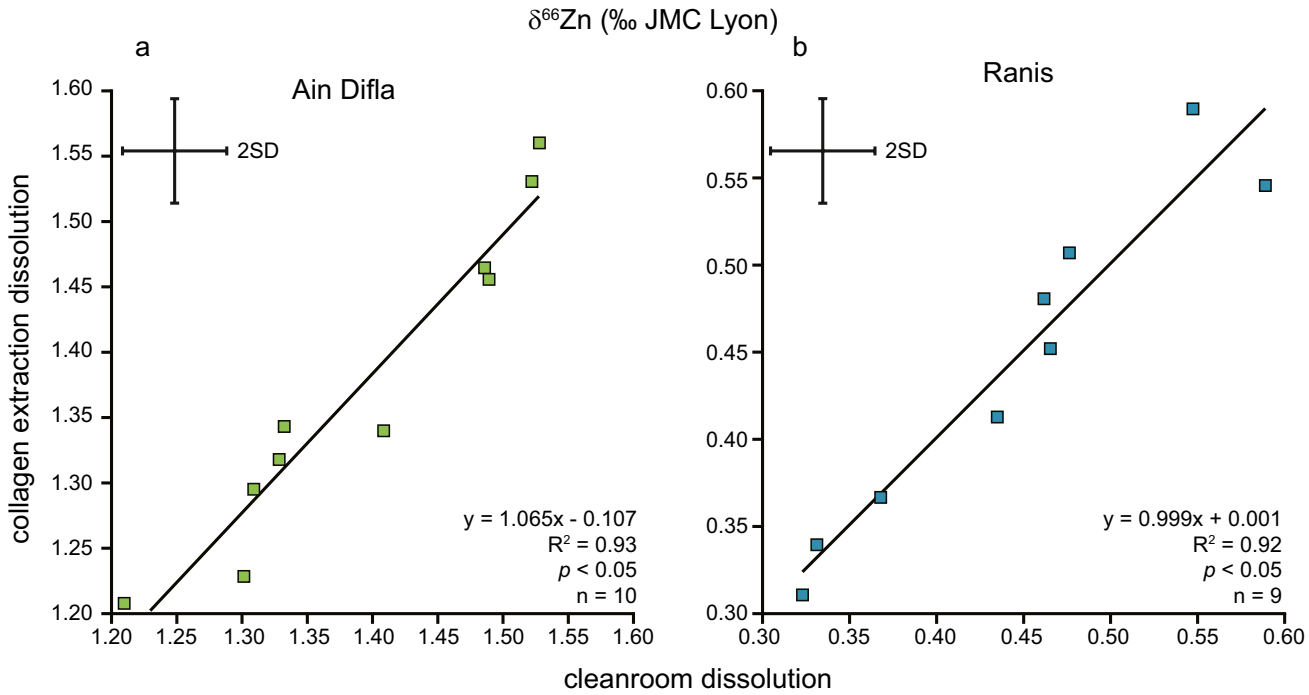


Fig. 1 Zinc isotope values from bone samples from Ain Difla (**a**) and Ranis (**b**) subjected to dissolution via the collagen extraction protocol (y-axis) and within a metal-free cleanroom (x-axis). Error bars represent maximum measurement uncertainty

of 1.38 ± 0.05‰ with a Zn concentration of 79.4 ± 3.6 µg/g (Supplementary Table 2). For each bone, the two samples taken for collagen extraction and cleanroom dissolution have homogenous Zn isotope values and concentrations, even though cleaning of the samples by ultrasonication in Milli-Q water was only carried out for samples taken for cleanroom dissolution. Given the reproducibility of the Zn values between bone samples, we conclude that no Zn contamination was introduced from the diamond-tipped cutting wheel during sampling.

The δ^{66}Zn values of bones from Ranis range between + 0.31 and + 0.59‰ with Zn concentrations between 369 and 789 µg/g. The bone fragments from Ain Difla have significantly higher δ^{66}Zn values between + 1.21 and + 1.56 ‰ and lower Zn concentrations between 49 and 102 µg/g. We do not observe a significant correlation between δ^{66}Zn values and Zn concentrations, expressed as 1/[Zn], for Ain Difla ($R^2 = 0.08$, $p = 0.42$) or Ranis ($R^2 = 0.20$, $p = 0.23$), when the average of both dissolution methods is used to compare δ^{66}Zn values with Zn concentrations (Fig. 2).

Generally, stable isotope and ^{14}C dating laboratories require a minimum collagen yield of ~1% to proceed with further analysis (van Klinken 1999). The Ain Difla bone collagen preservation was too low for further analyses (isotopic, ^{14}C or ZooMS). All Ranis samples had collagen yields, elemental (wt% C, wt% N, $C:N_{atomic}$) compositions, and isotope ranges characteristic of collagen that is uncontaminated and unaltered by the burial environment (Supplementary Data 1; DeNiro 1985; Ambrose 1990; van Klinken 1999). Carbon isotope values were between − 21.6 and − 18.1‰ and nitrogen values between + 1.7 and + 6‰ (Supplementary Data 1). The spectra obtained from Ranis-Ilsenhöhle bone specimens through ZooMS analysis have been taxonomically identified as *Rangifer tarandus* (5 bone specimens), Ursidae (3 bone specimens), and Equidae (1 bone specimen) (Supplementary Data 1). Peptide marker series can be similar for some closely related species, which is the case for Ursidae and Equidae. Ursidae includes species from the genera *Ursus*, most likely *U. spelaeus* or *U. arctos*, and Equidae includes species from the genera *Equus*, most likely *E. ferus*.

Discussion

We observe no systematic difference in δ^{66}Zn values, whether the bone samples were dissolved during collagen extraction without clean laboratory conditions or dissolved within a metal-free cleanroom (Fig. 1). The minimal variability in δ^{66}Zn values and Zn concentrations between both dissolution methods in some samples is likely related to analytical uncertainty and/or intra-bone Zn heterogeneity linked to the differences in sample sizes used for both protocols. Significant Zn contamination to the dissolved mineral solution during collagen extraction can be excluded. Indeed, Zn contamination, monitored with procedural blanks during the collagen extraction, demonstrates that a potential Zn contamination during our collagen extraction was likely less than 1% of the Zn present in the final dissolved mineral solution. However, Zn contamination remains a potential issue. It needs to be closely monitored through procedural blanks collected along with the dissolved mineral phase

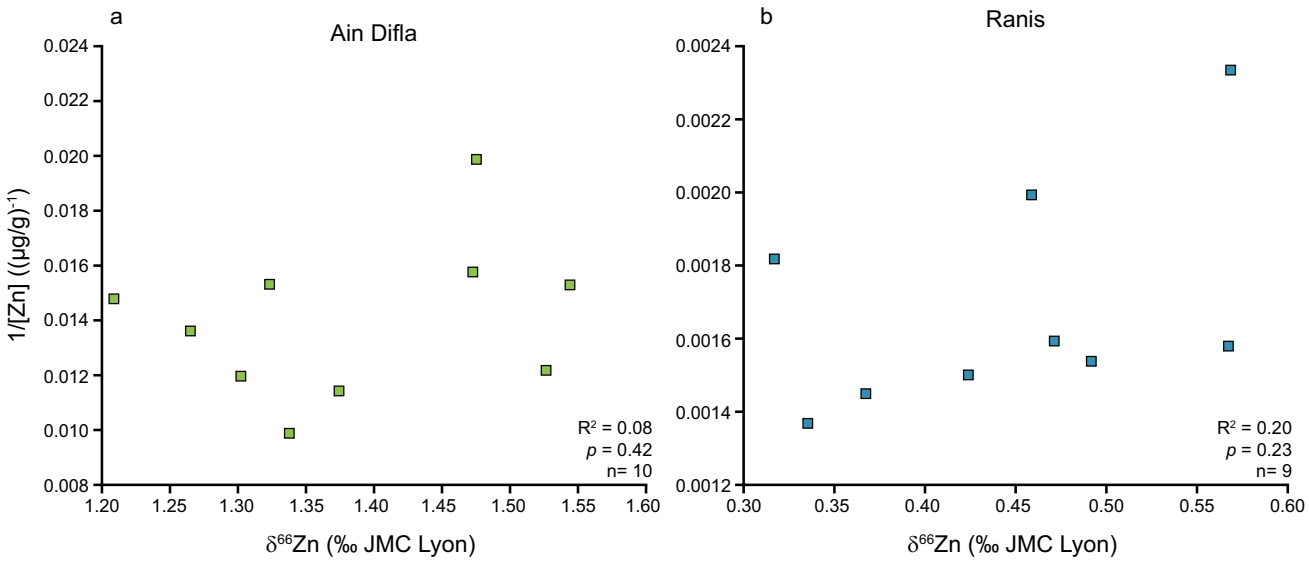


Fig. 2 Zinc isotope values from bone samples from Ain Difla (**a**) and Ranis (**b**) plotted against Zn concentration expressed as 1/Zn concentration (1/[Zn]). We observe no statistically significant relationship between bone δ^{66}Zn values and Zn concentration. Mean values of both dissolution protocols were used here

during collagen extraction and ideally in a series of blank tests before sample analysis for each laboratory adopting the protocol.

Hydrochloric acid precleaning of all glass equipment can eliminate a potential Zn contamination source, reducing total Zn contamination (Table 1). Zinc contamination introduced by atmospheric deposition was not observed in our collagen laboratory, as tubes left unclosed in the flow hood for 24 h did not yield a higher Zn contamination than closed tubes (Table 1). In contrast, tubes closed with a plastic lid indicate a higher Zn contamination than observed for blanks with no lid or blanks closed with aluminium foil. Still, even though plastic lids may act as a potential Zn contamination source, we did not observe them having any impact on our δ^{66}Zn values from bones samples, despite using plastic lids during collagen extraction.

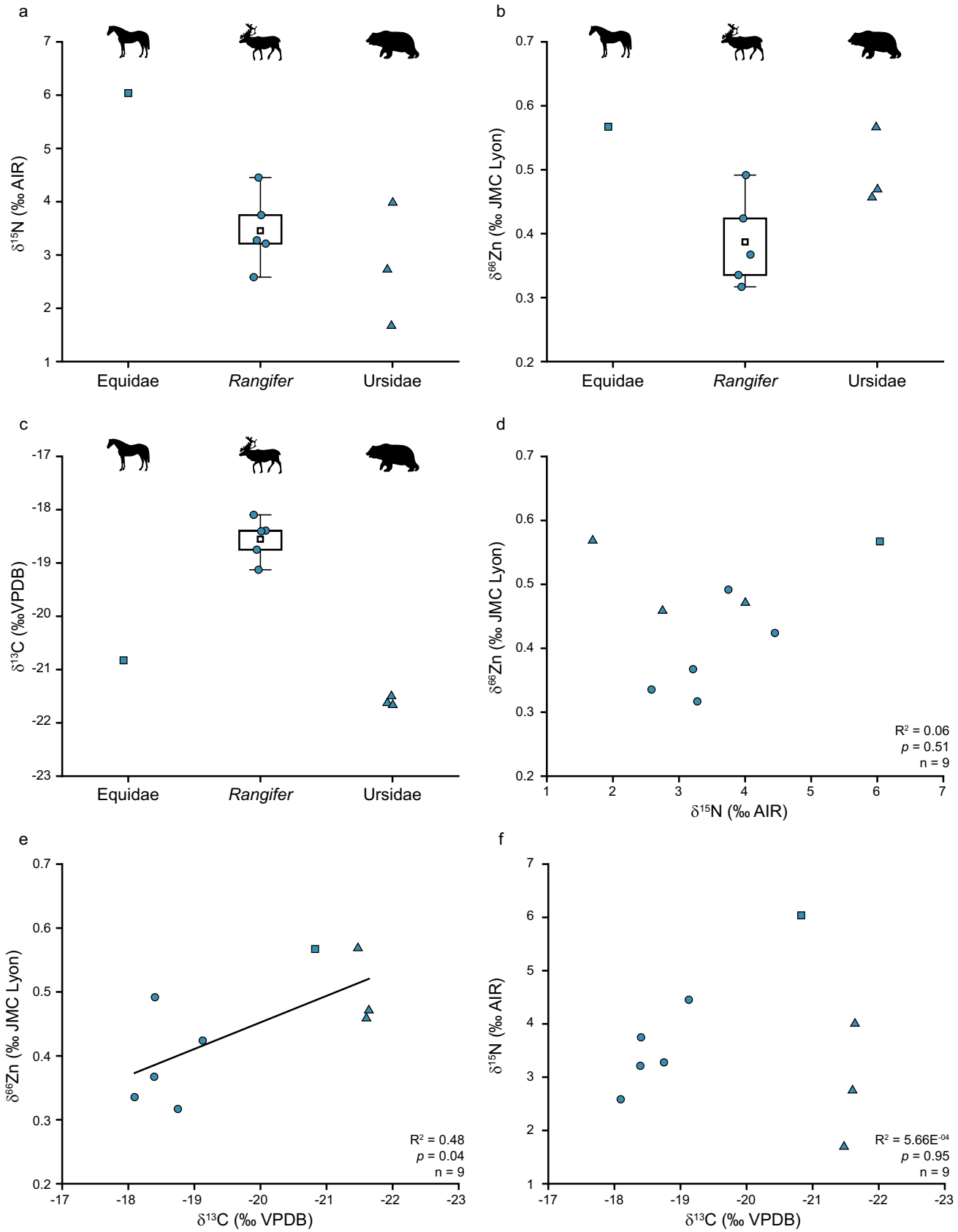
Our results demonstrate that δ^{66}Zn bone mineral analyses can be coupled to collagen extraction procedures with only minor adjustments to minimise Zn contamination. This combination of protocols is possible due to the relatively high concentration of Zn in bioapatite, permitting Zn contamination to be higher than under cleanroom conditions without necessarily affecting the final sample δ^{66}Zn value. Zinc contamination might pose a greater issue when using smaller sample sizes for collagen extraction. Still, our results show that sampling only 10 mg of bone for collagen extraction would still result in mean Zn concentrations for the dissolved mineral solution in 1 ml 3% HNO_3, after column chromatography Zn purification, of approximately 727 and 6303 ng/ml for Ain Difla and Ranis bones, respectively. When applying all steps to minimise Zn contamination presented here, including the use of vinyl gloves during all preparation steps, HCl precleaning of all glass equipment used and the use of high purity HCl for bioapatite demineralisation, Zn contamination can be kept to negligible levels (< 10 ng/ml) even for small sample sizes (Table 1).

The collagen laboratory demineralisation protocol applied here involves bone mineral dissolution taking place over days to weeks at low temperatures (Fewlass et al., 2019; Talamo et al. 2021) but some laboratories use faster demineralisation protocols involving powdered samples. If the necessary steps are taken to avoid Zn contamination during demineralisation, our protocol could be easily adapted to these methods. Indeed, this type of demineralisation is very similar to the demineralisation protocols commonly applied for Zn analysis on both bone and enamel samples (Jaouen et al. 2016a, b; Bourgon et al. 2020; McCormack et al. 2021). However, special care should be taken to avoid Zn contamination during bone or dentine powder grinding.

Coupling δ^{66}Zn with collagen extraction protocols provides an additional nitrogen-independent dietary and trophic level indicator, enabling more robust palaeoecological interpretations by (in)validation of results, as a baseline control, and by providing additional dietary/physiological information, depending on the sample material. The homogeneity of same sample δ^{66}Zn values independent of the here applied demineralisation method within both Ranis and Ain Difla bones implies that our protocol is applicable across the range of collagen preservation in archaeological skeletal remains. Although it remains to be tested, there is also the potential of using aliquots of the dissolved bioapatite as demineralised herein to also analyse other dietary tracers likely less impacted by contamination issues, such as calcium isotopes, together with δ^{66}Zn. Another benefit of this method is that collecting the dissolved mineral solution for further δ^{66}Zn analyses is not limited to laboratories with the necessary infrastructure to perform clean laboratory Zn purification and δ^{66}Zn MC-ICP-MS analyses. When the dissolved mineral solution is collected in precleaned closed PFA vials, it can be safely stored and transported to other laboratories for further processing and analyses if necessary.

Stable isotope values of both inorganic and organic components of vertebrate skeletal remains are less likely to be influenced by diagenetic alteration in more recent skeletal material compared to the Palaeolithic material examined herein; i.e. the likelihood of alteration increases with age, but the level of preservation will also strongly depend on the burial environment (van Klinken 1999; Collins et al. 2002; Clementz 2012). However, collecting the dissolved mineral solution for δ^{66}Zn analyses may provide a dietary proxy even when collagen extraction fails to provide bulk δ^{13}C and δ^{15}N data due to poor organic preservation. For example, the bone fragments from Ain Difla are not sufficiently preserved to allow taxonomic identification or collagen extraction for additional analyses, yet the δ^{66}Zn bone values and Zn concentrations are within the range of modern herbivore bones from Kenya (Jaouen et al., 2016b). Nevertheless, care must be taken when interpreting δ^{66}Zn values in the absence of collagen. The loss of collagen can potentially impact bone mineral δ^{66}Zn values as the increase in porosity following the loss of the organic matrix can lead to diagenetic modification via bioapatite recrystallisation, contamination, or secondary mineral precipitation (Hedges 2002; Trueman et al. 2004). A significant modification of original bone mineral elemental values might result in a correlation between the elemental concentration, expressed as 1/concentration and the isotopic composition of the element in question (Reynard and Balter 2014). The lack of a correlation between δ^{66}Zn and Zn concentration for bones from both Ain Difla and Ranis suggests that soil Zn addition and/or diagenetic bone Zn exchange is unlikely to have completely modified the samples' δ^{66}Zn values (Fig. 2).

There are apparent differences in the Zn concentration and isotope composition between Ain Difla and Ranis, which could be related to differences in diagenetic modification between the bones of these sites and/or environmental


a
δ15N (‰ AIR)
Equidae
Rangifer
Ursidae
b
δ66Zn (‰ JMC Lyon)
Equidae
Rangifer
Ursidae
c
δ13C (‰VPDB)
Equidae
Rangifer
Ursidae
d
δ66Zn (‰ JMC Lyon)
R² = 0.06
p = 0.51
n = 9
δ15N (‰ AIR)
e
δ66Zn (‰ JMC Lyon)
R² = 0.48
p = 0.04
n = 9
δ13C (‰ VPDB)
f
δ15N (‰ AIR)
R² = 5.66E-04
p = 0.95
n = 9
δ13C (‰ VPDB)

◂**Fig. 3** Nitrogen, zinc, and carbon isotope values from bone samples from Ranis. **a**, **b**, and **c** respectively show $\delta^{15}N$, $\delta^{66}Zn$, and $\delta^{13}C$ bone values arranged according to their taxonomy (x-axis). **d** shows $\delta^{66}Zn$ plotted against $\delta^{15}N$; **e** shows $\delta^{66}Zn$ plotted against $\delta^{13}C$; **f** shows $\delta^{15}N$ plotted against $\delta^{13}C$. Note, that the only statistically relevant relationship for **d**–**f** is given between $\delta^{66}Zn$ and $\delta^{13}C$ (i.e. $p < 0.05$). The boxes in **a**–**c** (for $n > 5$) represent the 25th–75th percentiles, with the mean as a smaller box and whiskers showing the 10th–90th percentiles. Squares represent Equidae, circles *Rangifer* and triangles Ursidae

differences in local Zn baselines. With our current dataset, we cannot unambiguously exclude either possibility. Zinc concentration in bones can vary depending on the dietary Zn uptake, as demonstrated by the large variability in recent and archaeological bone and dentine Zn concentrations observed in, e.g., marine Arctic remains and terrestrial remains from Kenya and Idaho (24 to 1025 µg/g, Kohn et al. 2013; Jaouen et al. 2016a, b; McCormack et al. 2021). It is noteworthy that we observe a correlation between bone $\delta^{66}Zn$ values and bulk collagen $\delta^{13}C$ values at Ranis, suggesting a dietary influence on both proxies and further indicating preservation of bone $\delta^{66}Zn$ values (Fig. 3e). Still, without clearly identifiable carnivore remains analysed in conjunction with herbivore remains and an observed trophic offset between them, we cannot exclude a possible diagenetic alteration of the bones $\delta^{66}Zn$ values for either site. We thus recommend careful evaluation of potential Zn diagenetic alteration (e.g. Kohn and Cerling 2002) when using older fossil bone or dentine material for palaeoecological investigations.

Conclusions

We demonstrate that bone collagen extraction protocols for ^{14}C dating and $\delta^{13}C$ and $\delta^{15}N$ analyses can be routinely coupled with $\delta^{66}Zn$ analyses of the dissolved mineral solution without the necessity of resampling precious archaeological or palaeontological skeletal material. Zinc concentration in bioapatite is typically high, allowing the collection of the dissolved mineral solution from collagen extraction protocols for further $\delta^{66}Zn$ analyses with only minor and easily implemented adjustments to established protocols to minimise Zn contamination. We observe no difference in bone $\delta^{66}Zn$ values between bones dissolved following our collagen extraction protocol, with further collagen processing, in a standard collagen extraction laboratory and the same material dissolved in a metal-free clean laboratory. Instead of discarding the dissolved mineral solution after collagen extraction, the solution can be retained and used for $\delta^{66}Zn$ analysis to gain an additional dietary and trophic level indicator that will allow more robust (palaeo)ecological interpretations. Additionally, collecting the demineralisation solution may provide $\delta^{66}Zn$ as a dietary proxy even if collagen preservation is insufficient for traditional $\delta^{13}C$ and $\delta^{15}N$ analyses. This approach can also be implemented in dating/isotopic laboratories without cleanroom and MC-ICP-MS facilities, as the solution can be safely stored and transported to laboratories with the necessary setup for further Zn purification and isotope analysis.

Obtaining necessary samples from archaeological material for interpretations of the palaeoenvironment, chronology, and genetic makeup is understandably associated with certain unease due to the destructive nature of many sampling methods. Accordingly, any method that allows the utilisation of a by-product that is usually and regularly disposed of from ongoing analyses (in our case, the mineral solution of bioapatite) for additional or supplementary knowledge should be especially adopted. Apart from the obvious benefits for conservation of archaeological specimens undergoing sampling, such a method of salvaging samples and products of their analysis increases the value of existing archaeological collections and, at the same time, lowers the quantity of new archaeological material necessary to excavate. Equally important, such methods could prove invaluable for archaeological sciences to operate as a scientific practice (Kosso 2011; Smith 2017) because they supplement and add to insights gained by other analyses (in this case, traditional dietary $\delta^{13}C$ and $\delta^{15}N$ analyses), which can then be used for (in)validation and falsification-control of results. For these reasons, we think that such an approach and the development of such methods should warrant a particular research emphasis in archaeological sciences in general.

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Data availability All data are available in the main text or the supplementary materials.

Declarations

Conflict of interest The authors declare no competing interests.

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