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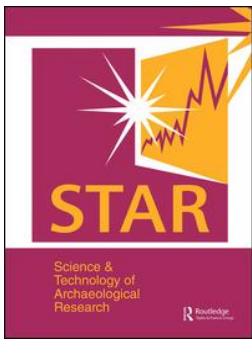
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Measuring the impact of parchment production on skin collagen stable isotope ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) values

Sean Doherty ^{a,b}, Michelle M. Alexander ^b, Jiří Vnouček ^{b,c}, Jason Newton ^d and Matthew J. Collins ^{e,f}

^aDepartment of Archaeology, University of Exeter, Exeter, UK; ^bBioArCh, Department of Archaeology, University of York, York, UK; ^cRoyal Danish Library, Copenhagen, Denmark; ^dNERC Life Sciences Mass Spectrometry Facility, Scottish Universities Environmental Research Centre, East Kilbride, UK; ^eMcDonald Institute for Archaeological Research, University of Cambridge, Cambridge, UK; ^fSection for Evolutionary Genomics, GLOBE Institute, University of Copenhagen, Copenhagen, Denmark

ABSTRACT

Parchment is one of the most abundant resources in archives across the world and is a unique time-sensitive material through which centuries of livestock economies, trade and craft can be explored. We examine the impact of structural and chemical modifications during production on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in the skin, particularly the removal of cutaneous keratins and lipids and the conversion of amide functional groups into carboxyl groups via alkaline hydrolysis. Through the manufacture of 51 parchment skins (sheep, goat, calf and pig) using both historic and modern manufacturing techniques, we found production resulted in a small enrichment in ^{13}C (average +0.12‰) and ^{15}N (+0.26‰). Our results pave the way for the isotopic analysis of parchment in paleodietary and paleoenvironmental studies for the historic period and establish the acceptable C:N ratios in deamidated collagenous tissues.

KEYWORDS

Stable isotope analysis; parchment; skin

1. Introduction

Palaeodietary and zooarchaeological stable isotope analysis has traditionally focussed on the analysis of bone collagen and dentine due to their preservation and ubiquity in the archaeological record. While abundant, these materials are constrained by their archaeological phasing or radiocarbon date, which at best assigns the material to a single century. Historic parchment, in contrast, is both numerous and typically dated to the year of use and, as with unprocessed skin (White and Schwarcz 1994; Iacumin et al. 1996; Iacumin et al. 1998; Finucane 2007; Corr et al. 2009; Basha et al. 2016; Lamb 2015), offers the possibility of a time-sensitive analysis of dietary and husbandry trends from the weeks and months prior to the animals' death.

While the isotope analysis of parchment has been conducted (Campana et al. 2010; Pollard and Brock 2011), the impact of production on measured values in the skin has not been explored. These previous studies have observed $\delta^{15}\text{N}$ values in modern and historic parchment far higher than those expected from terrestrial herbivores, with values >10‰ suggesting isotopic fractionation as a result of the structural and chemical modifications, the skin undergoes during processing, particularly amide sidechain hydrolysis during liming (Campana et al. 2010; Pol-

lard and Brock 2011). To address this, we present the isotopic analysis of 51 paired skin and parchment samples from a range of species to assess the impact of production on collagen isotope values and elemental composition, paving the way for the future analysis of the historical documents using this technique.

2. Parchment production

Parchment production transforms wet, perishable animal skin into a dry, durable sheet, suitable for writing purposes. Despite numerous manufacturing “recipes”, the basic principles have remained largely unchanged since the eighth century (Ryder 2009; Reed 1973; Haines 1990) (Figure 1). Once removed from the animal, the skin is limed, exposing it to a highly alkaline solution, typically calcium hydroxide ($\text{Ca}(\text{OH})_2$), sodium sulfide (Na_2S) or sodium hydroxide (NaOH). This process results in, (a) breaking the disulfide bonds in the keratinous hair and epidermis, facilitating their removal (Bienkiewicz 1983; Covington 2009); (b) hydrolysis of triglyceride esters (saponification), removing around 50% of cutaneous lipids (Koppenhoefer 1938; Koppenhoefer 1939), improving the whiteness of resulting parchment and the absorption of inks; and (c) hydrolysis of some amide groups attached to aspartic and glutamic acid

CONTACT Sean Doherty  sean@palaeome.org

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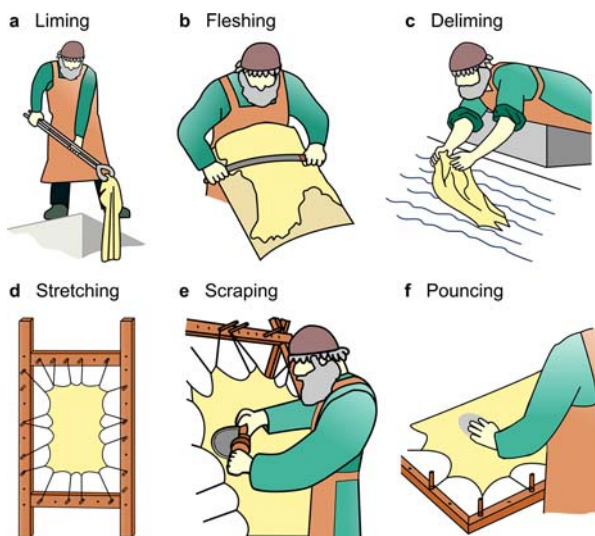


Figure 1. The parchment making process.

residues (deamidation), lowering the collagen's isoelectric point and “opening up” the collagen fibre network, enabling greater penetration of the solution and the removal of non-collagenous proteins, purifying the collagen substrate (Covington 2009; Menderes et al. 1999).

The skin is then fleshed with a double-handled knife, removing both the epidermis and adhering adipose tissue, leaving the collagen-rich dermis layer (Figure 2). Following this, the skin is delimed, typically in water, to lower the pH prior to tensioning. The skin is then stretched under great pressure, where it is shaved with a *lunellum* (half-moon blade) to smooth the surface and remove any remaining epidermal or adipose tissue. The skin is finally allowed to dry under tension, after which the surface may be pounced with a mildly abrasive tool (often a pumice stone) to produce a uniform surface.

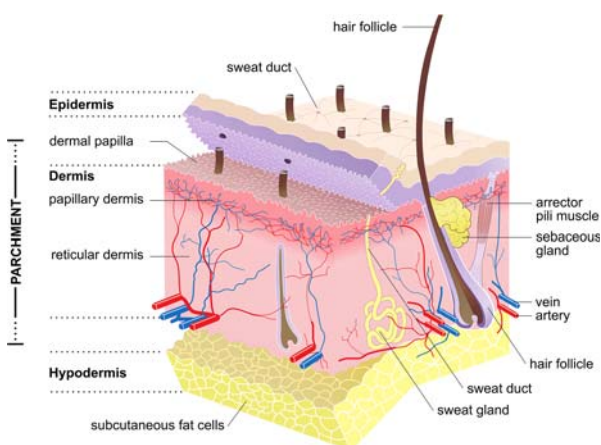


Figure 2. Structure of mammalian skin and the layers typically present in parchment (Source: Sean Doherty, Wikimedia Commons).

3. Material and methods

3.1. Experimental parchment production

Fresh skins were obtained from 42 sheep (*Ovis aries*) (E. Yorks. UK; Leics. UK; Notts. UK; Prague, CZ; Kansas, US), 2 goats (*Capra aegagrus hircus*) (Prague, CZ), 2 calves (*Bos taurus*) (Vienna, AU) and 5 pigs (*Sus scrofa domestica*) (Notts. UK). The animals were flayed shortly after death and their skins salted and stored at 5°C prior to analysis.

To maximise this study's comparability with both mediaeval and post-mediaeval parchment, two production methods were used. Method 1 followed traditional pre-nineteenth century European techniques as detailed in historic recipes (Ryder 2009; Reed 1973; Saxl 1954), with the skins unhaired in a straight lime solution, and delimed in water alone. Method 2 used industrial techniques through the addition of chemical depilatory agents during unhairing and acids during deliming (Covington 2009).

3.1.1. Method 1 (Historical method)

Each skin was washed and rehydrated in water at 8°C (pH 7.5) for 48 h. The water was replenished every 8 h and adhering foreign material was removed. The unsplit skins were submerged in a 3.5% calcium hydroxide solution (pH 13.5) in 220 L HDPE drums at room temperature for 6–18 days and agitated three times per day to ensure an even exposure across the skin. The ability to remove the fibre was appraised daily, and deemed sufficiently limed when it could be removed at the root by hand with ease (Covington 2009). Once unhaired, the skins were fleshed on the beam, with the epidermis and subcutaneous tissue removed with a double-handled knife. The skins were subsequently returned to the lime for a further 12 h, allowing for a uniform penetration of the solution, which may have been inhibited by hair or fat. The skin was then placed on the beam and mechanically squeezed with the knife to force out as much liquid as possible (“scudding”). To neutralise the alkalinity, each skin was washed vigorously for 30 min in running water and allowed to soak for 48 h, with the water replaced every 8 h. Each skin was then tightly stretched with ropes on a wooden frame, and allowed to dry under tension. While still wet and then again once dry, each side was shaved with a sharp knife to remove further layers of the dermis and produce a clean even surface.

3.1.2. Method 2 (Modern method)

Each skin was washed and rehydrated as in Method 1. The unsplit skins were treated in a 0.1% sodium hydrosulfide (NaHS) and 3% calcium hydroxide solution in large rotating tanning drums at room temperature for 30 min and agitated throughout. After

this, an additional 3% sodium hydrosulfide and 0.3% sodium hydroxide were then added. The drums were agitated intermittently for 1 h and then left to stand for 18 h, during which time the hair had completely dissolved. The skins were then fleshed on the beam and neutralised in water and 0.75% formic acid (HCOOH) for 6 h. The skins were then stretched, shaved and left to dry under tension as in Method 1.

3.2. Sample preparation and analysis

Due to the limited isotopic analysis of parchment, there is currently no standard method of sample preparation. Parchment is a complex material in which the chemistry and integrity of the collagen is altered during manufacture (and potentially during conservational treatments), and can degrade via oxidation, hydrolysis and biological attack (Hedges et al. 1989; Badea et al. 2012; Brock 2013). Surface contaminants, including inks, glues and chalk are common, as well as areas of localised gelatinisation and deterioration (Brock 2013). It is often essential that only a small physical sample is taken to minimise the aesthetic change to the manuscript. As such, careful sampling and pre-treatment is required to minimise and remove sources of contamination and ensure accurate analysis.

There is currently no widely accepted method for checking the integrity of collagen from skin or parchment (Lamb 2015); however, C:N ratios (an integrity standard for bone collagen and dentine (Ambrose 1990; van Klinken 1999)) have been widely used. Modern collagen (Col1) has an atypically low C:N ratio of 3.11 due to the abundance of glycine (C:N 2:1) (Table 1). Ratios higher than this in ancient collagen may reflect the loss of nitrogen during deamidation and the conversion of amide functional groups of asparagine (Asn) and glutamine (Gln) to carboxyl groups, and the loss of nitrogen through the citrullination of arginine (Arg). Based upon the atomic elemental composition of collagen, in the unlikely circumstance of all asparagine amino acids being converted to aspartic acid (Asp), we calculate a C:N ratio of 3.14, and if the complete conversion of glutamine to

glutamic acid (Glu) occurs, a C:N ratio of 3.19. We calculate a C:N ratio of 3.25 with the complete citrullination of arginine to citrulline (Cit), although there is nominal conversation during liming (Bowes and Kenten 1948). Therefore, if all amide groups (Asn, Gln) and guanidino groups (Arg) undergo hydrolysis (i.e. deamidated to carboxylic acids, and citrullinated to an intermediate metabolic amino acid), collagen would have a C:N ratio of 3.36. Ratios higher than this may indicate the presence of other proteins and lipids (van Klinken 1999; Schoeninger and DeNiro 1984; Kiljunen et al. 2006), such as elastin which has a C:N ratio of 5.8.

Brock (2013) examined the impact of different pre-treatment protocols on C:N ratios in parchment for carbon-14 dating. The highest C:N ratios were observed in untreated parchment and the lowest in those treated with strong acids or alkalis, the former highlighting the necessity for some sample preparation. The most consistently acceptable ratios (3.3) were produced from samples that had undergone lipid extraction followed by collagen extraction (demineralisation, gelatinisation, filtration and freeze-drying). This is consistent with the results from other analyses of parchment and mummified skin, where those that have not undergone collagen extraction have been shown to produce high C:N ratios, some in excess of 4.0 (Iacumin et al. 1996; Iacumin et al. 1998; Basha et al. 2016), while those that have undergone lipid and collagen extraction average around 3.3 (Finucane 2007; Pollard and Brock 2011; Brock 2013; Kiljunen et al. 2006).

Endogenous and exogenous lipids are often present in parchment in significant quantities (Ghioni et al. 2005; Strlič et al. 2009; Možir et al. 2014) and must be removed due to the different isotopic compositions of collagen and lipids (Liden, Takahashi, and Nelson 1995). Lipids have more negative $\delta^{13}\text{C}$ values than other biochemical compounds due to kinetic isotope effects that occur during lipid synthesis (DeNiro and Epstein 1977; Logan et al. 2008). Variability in tissue lipid content can, therefore, alter bulk $\delta^{13}\text{C}$ values, although, as they

Table 1. Atomic elemental composition of bovine type I collagen (Col1a1, P02453 and Col1a2, P02465).

Atoms	H	C	N	O	S	C:N ratio
Col1a1	6302	4055	1300	1324	7	
Col1a1	6302	4055	1300	1324	7	
Col1a2	6295	4016	1299	1282	4	
Proline hydroxylation ^b		12,126		286		
Total ^a	18,899	12,150	3899	4216	18	3.11
Percent composition	48.23%	31.01%	9.95%	10.76%	0.05%	
Effect of deamidation						
If all Asn → Asp		12,150	3866			3.14
If all Gln → Glu		12,150	3809			3.19
If all Arg → Cit		12,150	3740			3.25
If all deamidated		12,150	3617			3.36

^aIncludes additional C, H and O from glycosylation, based upon frequency reported in bovine bone collagen (Terajima et al. 2014).

^bIncludes additional O from hydroxylation of prolines assuming average of 94 hydroxyprolines per 1000 residues (based upon bulk hydroxylation levels in bovine skin, Burjanadze 1982; Menashi et al. 1976).

are composed mainly of carbon they have little impact on $\delta^{15}\text{N}$ values (Logan et al. 2008). In this study, lipids were removed from both skin and parchment samples with DCM/MeOH, a solvent mixture commonly used in tissues where triglycerides dominate (Ferraz et al. 2004; Colonese et al. 2015; Guiry et al. 2016). Due to the potential for residual calcium carbonate/hydroxide to remain in the skin from liming, a brief demineralisation step was included to remove this. Following the results of Brock (Brock 2013), samples were then gelatinised, filtered and freeze-dried to purify the collagen substrate for analysis. The process for each sample type is outlined in full below.

3.2.1. Unprocessed skin

Samples were taken from the belly region after soaking, but prior to liming. Adhering hair and fat deposits were removed with a scalpel to leave a dermis-rich sample, which was freeze-dried for 48 h, and ground to a coarse powder using a ball mill (Retsch MM400). In line with published analyses of modern and archaeological skins (Finucane 2007; Browning et al. 2014; Bergamo, Botta, and Copertino 2016) samples underwent lipid and collagen extraction prior to analysis. Samples were defatted via solvent extraction, DCM/MeOH (2:1 v/v), by ultrasonication for 1 hr, with the supernatant removed and solvent mixture replaced every 15 min. The samples were briefly demineralised in 0.6 M HCl at 4°C for 1 h, rinsed with distilled water and gelatinised in 0.001 M pH 3 HCl at 80°C for 48 h. The supernatant containing the collagen was filtered (60–90 μm Ezee-Filter™, Elkay Laboratories, UK), frozen and freeze-dried.

3.2.2. Parchment

Samples were cut from the parchment adjacent to the location sampled for the fresh skin. The lipids were solvent extracted following the same procedure used for skin. Samples were subsequently demineralised in 0.6 M HCl at 4°C for 6 h to remove residual calcium carbonate/hydroxide, rinsed with distilled water, and gelatinised in 0.001 M pH 3 HCl at 80°C for 48 h. The supernatant was then filtered, frozen, and freeze-dried.

3.3. Stable isotope analysis

Prepared collagen (0.9–1.1 mg) was weighed out in duplicate in 5×3.5 mm tin capsules (Elemental Microanalysis, Okehampton, UK) for carbon and nitrogen isotope analysis. Samples prepared under Method 1 were analysed at the Natural Environment Research Council Life Sciences Mass Spectrometry Facility (NERC LSMSF) in East Kilbride, where isotope ratio determinations were carried out on a ThermoElectron DeltaPlusXP (Thermo Fisher Scientific,

Bremen, Germany) with an Elementar Pyrocube elemental analyser (Elementar, Langensfeld, Germany). Stable carbon and nitrogen isotopic compositions were calibrated relative to VPDB and AIR using USG40 (glutamic acid) and ratios reported permil (‰). Measurement uncertainty was monitored using three in-house standards with well characterised isotopic compositions: ^{13}C -enriched alanine ($\delta^{13}\text{C}$: $-8.36 \pm 0.13\text{‰}$, $\delta^{15}\text{N}$: $2.08 \pm 0.06\text{‰}$), ^{15}N -enriched glycine ($\delta^{13}\text{C}$: $-38.58 \pm 0.09\text{‰}$, $\delta^{15}\text{N}$: $23.54 \pm 0.08\text{‰}$) and gelatine (Sigma-Aldrich, US. $\delta^{13}\text{C}$: $-20.31 \pm 0.18\text{‰}$, $\delta^{15}\text{N}$: $5.54 \pm 0.08\text{‰}$). Following the calculations outlined in Szpak, Metcalfe, and MacDonald (2017), the total analytical uncertainty was estimated to be $\pm 0.18\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 0.20\text{‰}$ for $\delta^{15}\text{N}$.

Samples manufactured following Method 2 were analysed at the University of York where determinations were carried out on a Sercon GLS analyser coupled to a Sercon 20–22 Mass Spectrometer (Sercon, Crewe, UK). Stable carbon and nitrogen isotopic compositions were calibrated relative to the VPDB and AIR using IAEA-600 (caffeine) and IAEA-N2 (ammonium sulphate), with ratios reported permil (‰). Measurement uncertainty was monitored using standards of cane sugar (IA-R006 – Iso-Analytical Ltd, UK. $\delta^{13}\text{C}$: $-11.64 \pm 0.03\text{‰}$) and fish gelatine (Sigma-Aldrich, US. $\delta^{13}\text{C}$: $-15.36 \pm 0.05\text{‰}$, $\delta^{15}\text{N}$: $15.2 \pm 0.05\text{‰}$). The total analytical uncertainty was estimated to be $\pm 0.19\text{‰}$ for $\delta^{13}\text{C}$ and $\pm 0.11\text{‰}$ for $\delta^{15}\text{N}$. Slight inter-lab variations in isotopic values are expected, although as the pairwise analysis was conducted at a single location, it is likely insignificant to interpretation (Pestle, Crowley, and Weirauch 2014). Reproducibility between samples was better than $\pm 0.2\text{‰}$ (1σ) for both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in skin, and parchment.

Statistical analysis was carried out using the IBM SPSS Statistic 22.0 software package (IBM 2013). Shapiro–Wilks tests for normality showed that the distribution of $\delta^{15}\text{N}$ values in skin and parchment were normal, but that $\delta^{13}\text{C}$ values were not ($p < 0.01$). As such, the statistical significance of differences between skin and parchment values was determined using a Wilcoxon signed-rank test for paired samples. In order to assess the difference and account for analytical uncertainty, for each paired skin and parchment sample, the isotopic data were normalised such that $\bar{x}_{\text{skin}} = 0$, and all replicates (skin and parchment) were reported as the difference Δ relative to this value. The difference between these two groups was determined using an Independent Samples *t*-test. The difference between $\Delta_{(\text{parchment-skin})}$ values produced following Method 1 and Method 2 was analysed using a Mann–Whitney U test.

4. Results

Isotopic and elemental composition results are reported in Table 2 and presented in Figure 3 (Method

Table 2. $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ isotope and elemental composition of skin and parchment samples.

Sample	Species	Method	Skin						Parchment					
			Yield (%)	$\delta^{13}\text{C}$ (‰)	%C	$\delta^{15}\text{N}$ (‰)	%N	C:N	Yield (%)	$\delta^{13}\text{C}$ (‰)	%C	$\delta^{15}\text{N}$ (‰)	%N	C:N
SH01	S	1	79.5	-25.4	42.4	9.7	16.8	2.9	57.6	-25.0	44.7	9.9	15.4	3.4
SH02	S	1	75.7	-25.8	45.5	9.2	16.0	3.3	57.6	-25.4	42.6	9.5	15.2	3.3
SH03	S	1	80.9	-26.0	42.5	10.0	16.2	3.1	55.6	-25.7	41.6	10.1	15.0	3.2
SH04	S	1	79.1	-25.8	43.8	8.4	16.1	3.2	64.6	-25.5	43.9	8.5	15.8	3.2
SH05	S	1	69.5	-25.7	41.9	8.4	16.0	3.1	54.6	-25.3	42.9	8.6	15.5	3.2
SH06	S	1	77.2	-26.0	43.2	8.9	16.2	3.1	71.5	-25.6	43.9	9.2	15.6	3.3
SH07	S	1	80.8	-25.8	42.9	9.0	16.1	3.1	63.9	-25.6	43.1	9.0	15.4	3.3
SH08	S	1	73.8	-26.0	42.9	9.7	15.9	3.1	60.0	-25.4	44.9	9.9	16.1	3.3
SH09	S	1	70.0	-24.7	42.5	8.4	16.1	3.1	41.8	-24.7	44.2	8.7	15.9	3.2
SH10	S	1	76.8	-25.1	43.6	8.7	15.9	3.2	44.2	-25.1	43.6	8.8	15.6	3.3
SH11	S	1	60.4	-24.9	43.5	8.1	16.0	3.2	61.7	-24.4	42.9	8.5	15.4	3.3
SH12	S	1	74.3	-26.0	43.7	9.3	15.2	3.3	68.2	-25.5	44.2	9.4	15.8	3.3
SH13	S	1	60.2	-23.3	43.2	7.6	16.1	3.1	55.1	-23.4	43.4	7.9	15.4	3.3
SH14	S	1	74.4	-25.7	43.4	9.3	15.7	3.2	69.1	-25.6	43.7	10.0	15.6	3.3
SH15	S	1	61.9	-24.6	43.8	8.3	15.6	3.3	62.1	-24.8	43.2	9.0	15.5	3.3
SH16	S	1	67.5	-25.4	44.2	8.0	15.9	3.2	54.8	-25.3	44.4	8.5	16.0	3.2
SH17	S	1	60.9	-24.9	43.8	8.5	16.1	3.2	53.7	-24.7	43.5	8.8	15.7	3.2
SH18	S	1	82.3	-25.3	43.3	9.2	15.6	3.2	77.9	-25.9	43.5	9.6	15.7	3.2
SH19	S	1	69.9	-26.0	42.7	8.6	15.5	3.2	63.5	-25.8	43.2	8.8	15.7	3.2
SH20	S	1	75.6	-25.1	44.7	8.6	15.9	3.3	71.7	-24.9	43.6	9.1	15.6	3.3
SH21	S	1	76.9	-24.8	43.3	8.5	15.5	3.3	65.4	-25.3	42.9	8.9	15.6	3.2
SH22	S	1	81.4	-25.7	44.1	8.3	15.3	3.4	71.7	-25.1	44.5	8.5	15.9	3.3
SH23	S	1	77.6	-25.8	42.9	8.4	15.1	3.3	66.6	-26.0	43.5	8.6	15.6	3.3
SH24	S	1	64.2	-25.7	44.9	8.2	15.9	3.3	51.7	-25.4	43.3	8.7	15.3	3.3
SH25	S	1	70.3	-23.8	43.3	6.1	15.7	3.2	72.3	-23.8	43.6	6.5	15.1	3.4
SH26	S	1	84.8	-25.7	44.0	5.1	15.4	3.3	76.4	-25.4	44.1	5.7	15.9	3.2
SH27	S	1	81.9	-25.3	43.4	7.3	15.1	3.4	68.3	-25.3	43.6	7.6	15.7	3.3
SH28	S	1	64.4	-24.7	44.7	8.0	15.6	3.3	61.8	-24.6	43.9	8.2	15.4	3.3
SH29	S	1	63.8	-25.6	43.9	9.7	15.3	3.3	51.3	-26.0	42.9	9.8	15.1	3.3
SH30	S	1	55.6	-25.5	43.6	9.5	15.6	3.3	57.1	-25.4	44.0	9.4	16.0	3.2
SH31	S	1	56.4	-25.4	41.4	10.1	15.4	3.1	59.4	-25.9	42.9	10.3	15.4	3.2
SH32	S	1	71.1	-25.6	44.3	10.0	15.8	3.3	66.5	-25.5	43.8	10.3	15.9	3.2
SH33	S	1	69.7	-25.8	43.8	10.0	15.5	3.3	63.7	-26.1	42.2	10.2	15.3	3.2
SH34	S	1	50.0	-26.0	43.5	10.5	15.6	3.2	56.1	-25.8	43.0	10.8	15.6	3.2
SH35	S	1	69.5	-25.4	43.8	7.4	15.5	3.3	63.9	-25.5	43.5	7.8	15.0	3.4
SH36	S	1	46.7	-25.9	43.6	7.2	15.7	3.2	54.4	-25.9	43.3	7.6	15.8	3.2
SH37	S	1	53.5	-26.4	43.1	9.1	15.3	3.3	52.2	-25.8	42.9	9.4	15.6	3.2
SH38	S	1	66.7	-25.8	46.1	9.6	15.7	3.4	70.5	-25.9	43.3	9.7	15.7	3.2
SH39	S	2	74.2	-24.9	43.6	6.9	15.5	3.3	63.1	-24.1	43.3	6.8	15.1	3.3
SH40	S	2	68.6	-21.9	43.6	6.6	16.0	3.2	67.0	-21.7	42.3	7.1	15.4	3.2
SH41	S	2	71.7	-20.7	43.2	12.3	15.8	3.2	67.3	-20.6	43.7	12.6	16.1	3.2
SH42	S	2	70.6	-24.2	43.7	6.3	15.6	3.3	67.4	-24.4	43.0	6.6	15.5	3.2
GT01	G	1	84.8	-23.8	44.2	8.1	15.4	3.3	75.2	-23.8	42.6	8.3	15.5	3.2
GT02	G	1	78.9	-23.5	44.4	6.7	15.8	3.3	65.7	-23.5	43.2	6.9	15.6	3.2
PG01	P	1	67.3	-23.3	43.6	4.0	15.9	3.2	56.3	-23.4	42.9	4.1	15.5	3.2
PG02	P	1	64.7	-23.3	43.9	4.3	15.9	3.2	57.2	-22.9	42.9	4.1	15.5	3.2
PG03	P	1	58.4	-23.0	42.7	5.6	15.7	3.2	57.9	-22.7	42.5	5.7	15.5	3.2
PG04	P	1	61.6	-23.0	43.7	5.8	15.8	3.2	63.0	-22.5	43.6	6.0	15.8	3.2
PG05	P	1	63.8	-23.6	43.4	4.5	15.8	3.2	67.8	-23.3	43.5	4.5	15.7	3.2
CF01	C	1	77.4	-24.5	44.6	3.9	15.6	3.3	72.8	-24.5	43.7	4.0	15.9	3.2
CF02	C	1	78.2	-24.5	42.9	4.7	15.3	3.3	71.0	-24.5	43.2	5.2	15.7	3.2

Note: Species, S = sheep, G = goat, P = pig, C = calf. Method, 1 = Historic, 2 = Modern.

1) and Figure 4 (Method 2). Results of statistical tests are presented in Tables 3 and 4.

4.1. Collagen quality indicators in skin and parchment

Average collagen yields of 70% and 63% were obtained from skin and parchment, respectively, consistent with collagen constituting around 90% of the protein fraction in skin (Wenstrup, Murad, and Pinnell 1991). These are comparable with those reported by Brock (Brock 2013) from historic parchment and far greater than the 2–4% threshold applied to bone to identify problematic samples (van Klinken 1999; DeNiro and Weiner 1988). While acknowledging the

varying sample sizes between species, average collagen yields in skin were highest in goats (82%), then calves (78%) and sheep (70%) and lowest in pigs (63%); a trend that matches the decreasing density of the dermal fibre network and increasing proportion of cutaneous lipids in these species (Reed 1973; Covington 2009).

Collagen yields were on average 7% lower in parchment than in skin. This is surprising as the removal of non-structural proteins during liming is thought to result in parchment being around 95% collagen (Kennedy and Wess 2008). Skin is heterogeneous and minor differences are to be expected between various locations, but this reduction may indicate a degree of collagen loss and damage associated with sample

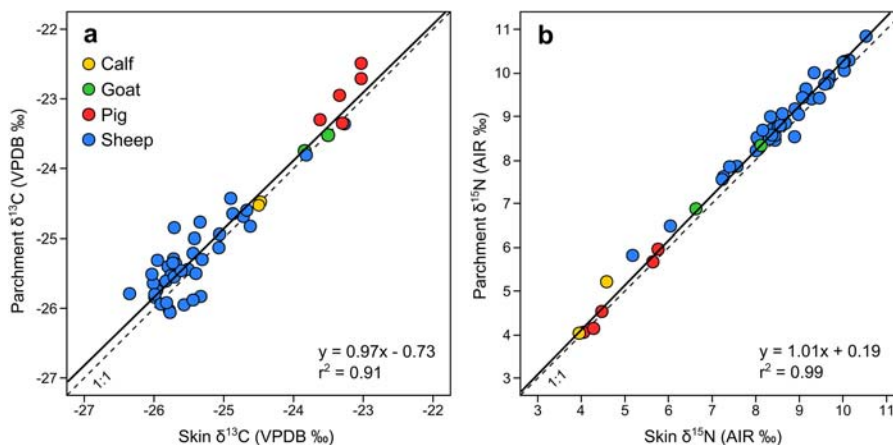


Figure 3. Comparison of stable isotope values from skin and parchment produced using Method 1, (a) $\delta^{13}\text{C}$, (b) $\delta^{15}\text{N}$. Solid line = Linear trend line; Dashed line = 1:1

preparation. The greatest differences were seen in a stillborn lamb (SH09, 22% lower) and a 2-day old lamb (SH10, 32% lower) which may have been more susceptible to damage due to the higher proportion of finer type III collagen fibres in foetal skin (Epstein and Munderloh 1978). Brock (2013) similarly observed that collagen extraction resulted in the lowest yields of any pretreatment method (47–71%), although produced consistently good C:N ratios.

In skin, %C ranged from 41.4 to 46.1, and 41.6 to 44.9 in parchment, and %N ranged from 15.1 to 16.8 in skin and 15.6 to 16.1 in parchment, consistent with those reported in modern type I collagen dominated tissues (Ambrose 1990; DeNiro 1985). Samples produced C:N ratios ranging from 2.9 to 3.5, with an average of 3.2 (2.9–3.4) in skin, and 3.2 (3.2–3.4) in parchment, with 16 (31%) skins showing higher ratios after processing, 12 (24%) lower, and 23 (45%) showing no difference. Of those that showed an increased ratio, it was often accompanied by an enrichment in ^{15}N . A number of samples presented C:N ratios greater than 3.2, a ratio indicative of where all asparagine and glutamine amino acids have deamidated. Due

to the improbability of this scenario, it may point to the presence of additional proteins (particularly elastin) or residual lipids.

4.2. Impact of production

The processing of skin to parchment resulted in a mean enrichment of 0.26‰ in ^{15}N across both production methods (standard deviation of 0.19, and standard error of 0.03), which was statistically significant ($P \leq 0.001$) (Table 3). Of the four species processed following Method 1, calfskin displayed the greatest enrichment (mean: +0.30‰), followed by sheepskin (mean: +0.29‰) and goatskin (mean: +0.20‰) with a negligible difference in pigskins (mean: +0.04‰). Of these, only the difference in sheepskin was statistically significant ($P \leq 0.001$). There is no significant difference in the $\Delta^{15}\text{N}_{(\text{parchment-skin})}$ offset between sheepskin processed following Method 1 or 2 ($P = 0.98$) (Table 4).

The impact of production on carbon values was more variable, although resulted in a mean enrichment of 0.12‰ in ^{13}C across both production methods (standard deviation of 0.31, and standard error of

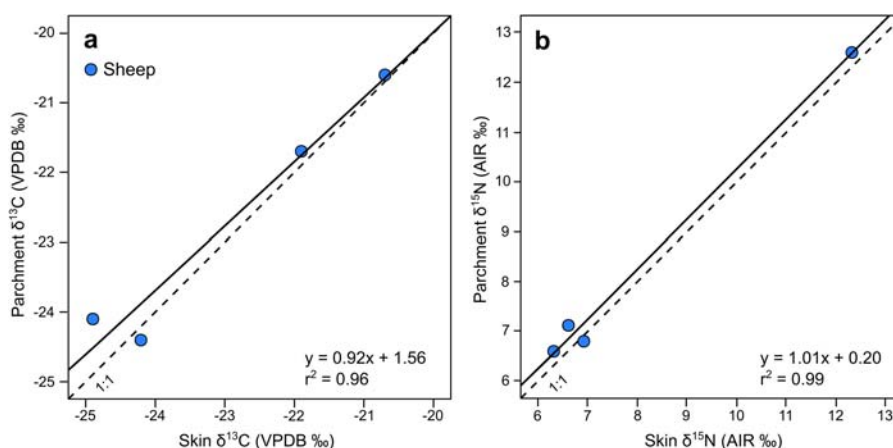


Figure 4. Comparison of stable isotope values from skin and parchment produced using Method 2, (a) $\delta^{13}\text{C}$, (b) $\delta^{15}\text{N}$. Solid line = Linear trend line; Dashed line = 1:1

Table 3. Significant differences in isotope values between skin and parchment (Wilcoxon signed-rank test for paired samples).

$\delta^{13}\text{C}$									
Species	Method	<i>n</i>	$\delta_{(\text{skin})}$	SD	$\delta_{(\text{parchment})}$	SD	$\Delta_{(\text{parchment-skin})}$	Z	<i>P</i>
All	1 and 2	51	-24.9	1.2	-24.8	1.2	+0.12	-2.793	0.005*
Sheep	1	38	-25.4	0.6	-25.3	0.6	+0.12	-2.072	0.038*
Sheep	2	4	-22.9	2.0	-22.7	1.9	+0.22	-0.921	0.36
Goat	1	2	-23.7	0.2	-23.6	0.2	-0.04	-1.000	0.32
Pig	1	5	-23.2	0.3	-23.0	0.4	+0.28	-1.761	0.08
Calf	1	2	-24.5	0	-24.5	0	-0.03	-0.447	0.66
$\delta^{15}\text{N}$									
All	1 and 2	51	8.0	1.9	8.3	1.9	+0.26	-5.825	<0.001*
Sheep	1	38	8.7	1.1	8.9	1.0	+0.29	-5.268	<0.001*
Sheep	2	4	8.0	2.9	8.3	2.9	+0.26	-1.473	0.14
Goat	1	2	7.4	1.0	7.6	1.0	+0.20	-1.414	0.16
Pig	1	5	4.8	0.8	4.9	0.9	+0.04	-0.557	0.58
Calf	1	2	4.3	0.6	4.6	0.9	+0.30	-1.342	0.18

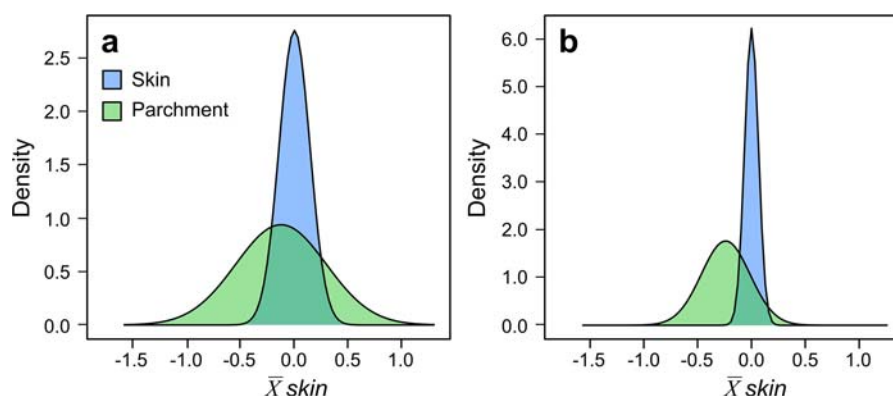
*Statistically significant differences ($P < 0.05$).

0.04), which was statistically significant ($P = 0.005$). Within the species manufactured following Method 1, pig (mean: +0.28‰) and sheepskin (mean: +0.12‰) showed a mean enrichment with processing, while goat (mean: -0.04‰) and calfskin (mean: -0.03‰) showed a negligible mean difference. As with nitrogen values, only the difference in sheepskin is statistically significant ($P = 0.038$). There is no significant difference in the $\Delta^{13}\text{C}_{(\text{parchment-skin})}$ offset between sheepskin processed following Method 1 or 2 ($P = 0.76$).

The mean difference between skin and parchment carbon values is smaller than the estimated analytical uncertainty, while the difference in nitrogen values is only slightly greater. To incorporate this uncertainty within statistical testing, replicates were normalised to the mean value of the corresponding skin (Figure 5) and the difference between these groups analysed

Table 4. Significant differences in parchment to skin offset in sheep between Method 1 (Historic) and Method 2 (Modern) (Mann-Whitney *U* for two independent samples).

	Method 1 (<i>n</i> = 38)	Method 2 (<i>n</i> = 4)	<i>U</i>	Z	<i>P</i>
$\Delta^{13}\text{C}_{(\text{parchment-skin})}$	+0.12	+0.22	69.0	-0.301	0.76
$\Delta^{15}\text{N}_{(\text{parchment-skin})}$	+0.29	+0.26	75.0	-0.044	0.98

**Figure 5.** Distribution of (a) $\delta^{13}\text{C}$ and (b) $\delta^{15}\text{N}$ values in skin and parchment replicates normalised to the mean value of skin.

through an Independent Samples t-test. Despite the small mean differences, skin and parchment carbon ($t = 2.84$, $df = 124.18$, $P = 0.005$) and nitrogen values ($t = 10.34$, $df = 117.19$, $P \leq 0.001$) differ significantly from each other. As with the paired samples, this difference is only significant within sheepskins (carbon: $t = 3.73$, $df = 101.01$, $P \leq 0.001$; nitrogen: $t = 9.15$, $df = 88.27$, $P \leq 0.001$).

5. Discussion

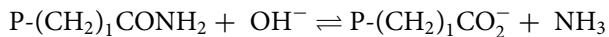
The observed variation between skin and parchment $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, as well as C:N ratios is likely the result of a range of factors resulting from changes made to the structure and chemistry of the skin during parchment production.

5.1. Impact of amide side chain hydrolysis

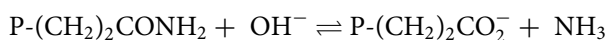
Forty-five of the 51 skins (88%) showed a small, but consistent ^{15}N -enrichment after processing, 24 (49%) of which were greater than analytical error (0.2‰). This is surprising, given that the most probable mechanism for elevated $\delta^{15}\text{N}$ values in parchment is the loss of the side chain-N. Asn side chain-N is significantly enriched in ^{15}N relative to peptide-N (average $\Delta_{\text{side-peptide}} = +11\text{‰}$) although is less significant for Gln

(average $\Delta_{\text{side-peptide}} = +3\%$) (Sacks and Brenna 2005) reflecting its central role in N metabolism. Thirty-one skins (61%) showed a ^{13}C -enrichment, of which nineteen (37%) were greater than experimental error. These results are suggestive of the kinetic isotope effect associated with peptide bond hydrolysis, preferentially eliminating the isotopically lighter nitrogenous compounds.

During liming, the amide functional groups of asparagine (Asp) and glutamine (Gln) are converted into carboxyl groups through alkaline hydrolysis, producing, respectively, aspartic acid:



and glutamic acid:



liberating carbon dioxide and ammonia. This deamidation reaction forms part of the controlled damage collagen undergoes during processing, and is essential in lowering the iso-electric point of collagen, swelling the skin and aiding the removal of non-collagenous proteins (Covington 2009). The rate of hydrolysis increases with prolonged liming as the rigidity of the collagen backbone decreases, making the partially deamidated collagen susceptible to further damage (van Duin and Collins 1998; Collins, Waite, and van Duin 1999). In less than 24 h of liming, around 50% of all available side chains are hydrolysed (Menderes et al. 1999). Cleavage of the carbon–nitrogen bond during hydrolysis has been shown to favour peptide bonds containing the lighter isotopes ^{12}C and ^{14}N , leading to a retention and enrichment of the heavier isotopes (Macko et al. 1986; Macko, Fogel-Estep, and Hare 1987; Bada, Schoeninger, and Schimmelmann 1989; Silber, Engel, and Macko 1992; McClelland and Montoya 2002; Chikaraishi et al. 2007; Chikaraishi et al. 2009; Miura and Goto 2012). Bada, Schoeninger, and Schimmelmann (1989), for example, observed a $\sim 7\%$ ^{15}N -enrichment on bovine collagen after 30% hydrolysis. This enrichment has been observed in deamidation associated with the transfer of amino acids from diet to consumer (Chikaraishi et al. 2007; Chikaraishi et al. 2009; Miura and Goto 2012; Hare et al. 1991; Popp et al. 2007) and in the archaeological degradation of proteins (Dent, Forbes, and Stuart 2004; von Holstein 2014). Sheepskins processed using Method 2 were only in lime for 24 h, but the parchment to skin offset is not significantly different from sheepskins processed using Method 1 which had been in lime for at least 6 days (Table 4). Further ^{15}N -enrichment may occur during the hydrolysis of arginine residues which has shown a preferential bias for releasing the lighter isotope during the urea cycle (Ambrose 2002), although during liming $<3\%$

of arginine residues are likely to be converted (Bowes and Kenten 1948; Jones 2004).

5.2. Removal of keratinous hair and epidermis

During production, the keratinous hair, wool and epidermis layer of the skin are removed chemically and mechanically. This has the potential to influence the isotopic value of the resulting parchment due to the isotopic disparity between keratin and collagen (Tieszen and Fagre 1993; O'Connell et al. 2012; von Holstein et al. 2013). Hair and wool fibres are made predominantly from keratin, which relative to collagen contains less glycine and proline and higher levels of cystine and tyrosine (Robbins 2012). This high cystine content results in an abundance of disulfide bonds, producing a “hard” keratin, as in nails and horn. The epidermis is composed predominantly of “soft” epithelial keratins, which have lower cystine and higher methionine and glycine content than hair keratin (Bieńkiewicz 1983; Fuchs 1983). Keratin constitutes $<2\%$ of the total composition of the skin (excluding the hair/wool), and in principle is entirely removed during production due to the different behaviour of collagen and keratin during liming (Bieńkiewicz 1983). At high pH, values the disulfide bonds undergo hydrolysis, dissolving the prekeratinised base of the hair, so that it is held by friction alone, and also weakens the epidermis (Covington 2009). This chemical attack is followed by the mechanical removal of the hair and epidermis during dehairing and shaving, with the resulting parchment made predominantly from the collagen-rich dermal/corium layer. The opening-up of the collagen fibres during liming, further results in the removal of non-collagenous components of the skin (such as non-structural proteins and lipids), increasing the relative proportion of collagen in parchment.

The isotopic relationship between collagen and keratin has been examined in a number of studies (O'Connell et al. 2012; von Holstein et al. 2013; DeNiro and Epstein 1978; O'Connell et al. 2001; Codron et al. 2012). Both are typically enriched over diet, with collagen enriched over keratin in both $\delta^{13}\text{C}$ (0–4‰) and $\delta^{15}\text{N}$ (0–2‰). In sheep, von Holstein et al. (2013) noted that collagen was enriched in ^{13}C over keratin by 2–2.7‰, but $\delta^{15}\text{N}$ differences were within experimental error. Isotopic variation between the two tissues is largely due to differences in the routing and composition of amino acids (von Holstein et al. 2013; O'Connell et al. 2001). Previous analysis has, however, been conducted on “hard” keratins, and due to the higher glycine content of epithelial keratins, it is likely that this isotopic variation between “soft” keratin and collagen is less pronounced. All visible signs of keratinous tissue were removed from the skin sample prior to analysis, but

it is possible that further removal during parchment production contributed to the observed enrichment.

5.3. Removal of lipids and non-collagenous proteins

The saponification of lipids during liming reduces the amount of ^{13}C -depleted lipids, potentially causing a ^{13}C -enrichment of parchment $\delta^{13}\text{C}$ values. Lipids are isotopically lighter in $\delta^{13}\text{C}$ than the protein component of animal tissue, and in ecological studies are typically removed through lipid extraction prior to analysis due to their impact on bulk isotope ratios (Guiry et al. 2016; Medeiros et al. 2015; Elliott, Roth, and Crook 2017). During liming, fatty acid esters undergo hydrolysis and are leached out into the lime solution, with as much as 50% of the total lipid content of skin removed (Koppenhoefer 1938; Koppenhoefer 1939). Lipids are likely to be lost during further delimiting and shaving; however, sheepskin parchment can still retain high levels of lipids (Ghioni et al. 2005). Pollard and Brock (Pollard and Brock 2011) noted an enrichment in ^{13}C in defatted parchment relative to that which had not undergone lipid extraction, although in this analysis lipids were extracted from both the fresh skin and parchment prior to analysis, reducing the influence they may have. Non-collagenous proteins are also removed during processing, reducing the amount of basic amino acids (arginine, lysine, and histidine), resulting in a proportional increase in glycine, which is enriched in ^{13}C relative to other amino acids (McMahon et al. 2015). Therefore, purification of the collagen substrate during processing may too influence the resulting bulk isotope values.

5.4. C:N ratios

The hydrolysis of asparagine and glutamine residues during liming and the subsequent loss of nitrogen is the likely cause of elevated C:N ratios seen in 16 skins after processing. Of those with ratio >3.2 , deamidation is likely the most important factor as the complete conversion of Gln and Asn is likely to result in a ratio of 3.22, although may be due in part to the presence of carbon-rich keratin or lipids. These results caution the interpretation of results from skin with values >4 (Iacumin et al. 1996; Iacumin et al. 1998; Badea et al. 2012) and may indicate the presence of resins, waxes or oils applied during mummification (Cockitt, Lamb, and Metcalfe 2020).

6. Conclusion

The structural and chemical modifications made to skin during parchment production typically results in a small enrichment in both ^{13}C and ^{15}N , although

below the commonly cited 1‰ level of variation likely to impact interpretation (Sealy et al. 2014). These results confirm Campana et al. (2010) and Pollard and Brock (2011) hypothesis that production alters the isotopic value of the skin, although is unlikely to be the cause of the high $\delta^{15}\text{N}$ values observed in both of these studies. Measuring the amino acid composition of paired samples could clarify the factors driving this mean enrichment, although it is likely a combination of factors, including the deamidation preferentially removing the lighter ^{12}C and ^{14}N isotopes, saponification removing ^{13}C -depleted lipids, and removal of the relatively depleted keratin component during liming and shaving.

This study confirms the potential for parchment to provide valuable time-sensitive insights for paleodietary (baselines) and paleoenvironmental studies for the historic period. With no statistically significant difference in the offset from skin to parchment manufactured using historic or modern techniques, parchment from the eighth to twenty-first century offers an exceptional resource for isotopically exploring historic animal/land management, craft and trade.

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Disclosure statement

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Notes on contributors

Sean Doherty is a Postdoctoral Research Fellow at the Department of Archaeology, University of Exeter. He specialises in examining human-animal-environment interactions through the synthesis of biomolecular, zooarchaeological and historical research.

Michelle Alexander is a Senior Lecturer in Bioarchaeology at the University of York. She specialises in the application of bioarchaeological techniques to understand major socio-

cultural/economic transitions and human-animal dynamics in the historical period.

Jiří Vnouček is a conservator at the Royal Library, Copenhagen, and previously the Head of Conservation at The National Library in Prague, Czech Republic. He holds a PhD from the University of York in the visual assessment of parchment, which drew upon his 20 years' experience in conservation and parchment production.

Jason Newton is head of NERC Life Sciences Mass Spectrometry Facility (LSMSF) in East Kilbride. He specialises in the ecological and environmental applications of stable isotope analysis, particular how stable isotopes may be used to elucidate food webs.

Matthew Collins is the Niels Bohr Professor of Palaeoproteomics at the Globe Institute's Section for Evogenomics at the University of Copenhagen, and the McDonald Professor of Palaeoproteomics at the McDonald Institute for Archaeological Research of the University of Cambridge. He specialises in the persistence of proteins in ancient samples, using modelling to explore the racemization of amino acids and thermal history to predict the survival of DNA and other molecules.

ORCID

Sean Doherty  <http://orcid.org/0000-0002-5503-2734>

Michelle M. Alexander  <http://orcid.org/0000-0001-8000-3639>

Jiří Vnouček  <http://orcid.org/0000-0002-1576-974X>

Jason Newton  <http://orcid.org/0000-0001-7594-3693>

Matthew J. Collins  <http://orcid.org/0000-0003-4226-5501>

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