

## HYDROXYPROLINE DATING: EXPERIMENTS ON THE <sup>14</sup>C ANALYSIS OF CONTAMINATED AND LOW-COLLAGEN BONES

Anat Marom<sup>1,2</sup> • James S O McCullagh<sup>3</sup> • Thomas F G Higham<sup>1</sup> • Robert E M Hedges<sup>1</sup>

**ABSTRACT.** Dating of the amino acid hydroxyproline from bone collagen has been shown to produce accurate and reliable radiocarbon dates. This article presents further application of the method demonstrating it can be used to obtain dates for both low-collagen and contaminated bones, extending the capability of <sup>14</sup>C dating archaeological bone from conventional limits imposed by alternative pretreatment methods. The method therefore has the potential for significantly benefiting the accelerator mass spectrometry (AMS) dating community in the <sup>14</sup>C dating of archaeological bone.

### INTRODUCTION

Dating archaeological bones that have low collagen content or are contaminated from the burial environment or museum conservation work can sometimes result in inaccurate results. This problem is especially acute for old bones, close to the radiocarbon limit, and can compromise the ability of <sup>14</sup>C specialists to contribute to the reconstruction of reliable archaeological chronologies. It is estimated, for example, that up to 70% of Paleolithic <sup>14</sup>C dates on bones are likely to be underestimates of the real age, blurring the picture of modern human dispersals and Neanderthal extinction (Higham 2011). In order to find ways to minimize these uncertainties and reduce contamination issues, a method for isolating and <sup>14</sup>C dating the collagenous amino acid hydroxyproline was developed (Abelson and Hoering 1961; Ho et al. 1969; Hare 1980; Stafford et al. 1982, 1991; Gillespie et al. 1984, 1986; Stafford et al. 1988, 1991; van Klinken and Mook 1990; van Klinken et al. 1994; van Klinken and Hedges 1995; McCullagh et al. 2010; Marom 2012; Marom et al. 2012). Hydroxyproline (Hyp) constitutes about 10% of bone collagen by mass but is not found in significant amounts elsewhere in nature, and can therefore be used as a bone-specific biomarker. The Hyp dating approach is fundamentally different from conventional pretreatment methods as it focuses on isolating and dating a specific single molecule, rather than the removal of extraneous and potentially contaminating molecules from intact collagen. Here, we demonstrate a further practical benefit of the approach validating the method's applicability to archaeological samples.

The Hyp dating method that has been developed uses a mixed-mode (i.e. ion-exchange combined with hydrophobic chemistry), semipreparative HPLC methodology. By isolating and AMS dating Hyp from a range of known-age bones, the average background carbon added from the procedure has been estimated and shown to be minimal:  $3.3 \pm 1.4 \mu\text{g}$  for each 500–1000  $\mu\text{g}$  graphite, of which  $1.5 \pm 0.3 \mu\text{g}$  is of modern age, a suitable level for <sup>14</sup>C dating (Marom et al. 2012). The method has proven to be a powerful tool that can help resolve archaeological questions. Recently, for instance, it was used to provide the earliest direct ages for the presence of anatomically modern humans on the Russian Plain and resolved a long-standing chronometric problem in the dating of the key Sungir Mid-Upper Paleolithic burials (Marom et al. 2012).

It is generally assumed (using the conventional bulk dating protocol) that humic acids should be removed by an alkali wash step combined with gelatinization. However, van Klinken and Hedges (1995) showed that only dating the HPLC-purified collagen-specific tri-peptides (“the tri-peptide”

<sup>1</sup>Research Laboratory for Archaeology and the History of Art, University of Oxford, Oxford OX1 3QY, United Kingdom.

<sup>2</sup>Current address: Kimmel Centre for Archaeological Science, Weizmann Institute of Sciences, POB 26, Rehovot 76100, Israel. Corresponding author. Email: anat.marom-rottem@weizmann.ac.il.

<sup>3</sup>Chemistry Research Laboratory, University of Oxford, Oxford OX1 3TA, United Kingdom.

method) or the CO<sub>2</sub> from carboxylic groups of proteinaceous molecules (the “ninhydrin method”) were capable of completely removing humic acids that they had deliberately added to the collagen. Conventional <sup>14</sup>C chemistries failed to do this (van Klinken and Hedges 1995). To test the Hyp method’s contamination removal efficiency, a known-age bone was artificially contaminated with a known-age contaminant. The results presented below demonstrate the successful removal of contamination and the superiority of the Hyp method over conventional acid-base-acid (ABA) pretreatment in this respect. In addition, experimental work is presented on bone that was artificially “weathered” in an accelerated fashion by exposure to heat. Hyp survival is evaluated in order to assess its availability for bones that are poorly preserved in collagen (see Supplemental file available with the online version of this article). Finally, problematic archaeological bones are tested. These include a bone with too little surviving collagen to be datable by the bulk collagen method, a bone that produced different dates using different pretreatment methods, implying it is contaminated, and 2 bones of the same individual, which produced very different dates, again, implying they are severely contaminated. The results reported here add to our understanding of the advantages and limits of the Hyp dating method and help in providing a framework for its implementation in <sup>14</sup>C dating laboratories.

## **METHODS**

We used several bone samples in our experiments. An ancient bone background standard (~60–70 ka BP) from Alaska was used in the contamination experiment (Brock et al. 2010a) (the “Lemon Mine” bone). A modern elephant bone was used as a reference material for the diagenesis experiment. A horse 2nd/3rd phalange obtained from the Kostenki 14 site was used to check the applicability of the Hyp dating method to low-collagen bones. This bone was never exposed to any conservation material; its nitrogen percentage was 0.3%, as opposed to 3.5–4.5% found in modern bone (Stafford et al. 1988). La Ferrassie 1 is a Neanderthal skeleton found in the Dordogne, France. Its distal left tibia and distal right tibia were both dated using the Hyp method in order to resolve the discrepancy between both dates obtained by conventional bulk <sup>14</sup>C methods. A horse astragalus from Flixton, UK, suspected of being contaminated from the burial environment, was also dated using the Hyp method.

The Hyp dating method has been described thoroughly elsewhere (McCullagh et al. 2010; Nalawade-Chavan et al., these proceedings), and will only be presented here briefly. Bone collagen was extracted after demineralization and gelatinization of cleaned and crushed bone. The resulting collagen was hydrolyzed and filtered using a 0.2- $\mu$ m PTFE filter (Chromacol, Fisher Scientific UK Ltd., Loughborough, UK) to remove precipitates. The amino acid mixture was separated into individual amino acids using a mixed-mode HPLC separation method incorporating weak cation exchange and reversed-phase components combined in the same stationary phase (McCullagh et al. 2010). The collected Hyp fraction was dried and combusted, followed by CO<sub>2</sub> graphitization and measurement of its <sup>14</sup>C content by AMS (Brock et al. 2010a).

### **Contamination Protocol**

Tea was used to artificially contaminate a <sup>14</sup>C-depleted bone. Tea contains polyphenolic compounds, which are the origin of humic and fulvic acids found in soils; all bind to proteins such as collagen using a mechanism similar to that found in leather tanning (e.g. using oak bark). The bone was crushed and decalcified, and then immersed in 15 mL of hot tea, vibrating for 1 hr. The sample was then rinsed repeatedly, until the water was not stained anymore. It was thereafter repeatedly rinsed with alkali, until the wash water was colorless. It was then assumed that any contaminants left in the

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sample were bound to the collagen. After re-acidification, a part of the sample was subjected to ultrafiltration (Brock et al. 2010a). The samples were freeze-dried and combusted on an EA (Carlo Erba NA 2000) coupled to a gas source isotope ratio mass spectrometer (IRMS) (Sercon 20/20, Cheshire, UK) for  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , and C:N measurement.

### RESULTS

#### Contamination Experiment

We used tea to contaminate the Lemon Mine background bone, so as to mimic the contamination of bone in the burial environment (for details on the contamination protocol and bone used see Methods section). The success of contamination was verified by altering the C:N ratio from 3.2 (normal for intact collagen) to 3.4 (see Table 1), and  $^{14}\text{C}$  dating. The tea-contaminated bone treated with ABA and ultrafiltration yielded a  $^{14}\text{C}$  date of  $22,170 \pm 140$  BP, indicating that ~6% of the carbon dated was modern carbon contaminant (Table 1). The contaminated bone was then hydrolyzed and filtered (see Methods). The  $^{14}\text{C}$  date of the remaining collagen was  $33,200 \pm 600$  BP, indicating that the majority of the contamination was removed. HPLC was then used to separate the Hyp fraction, and AMS dating yielded a  $^{14}\text{C}$  age of  $>44,100$  BP, a value indistinguishable from the untreated Lemon Mine Hyp date and indicating complete contamination removal (see Table 1).

Table 1 The C:N ratios and  $^{14}\text{C}$  dates of tea-contaminated Lemon Mine collagen, after subjecting it to different treatments; LM = Lemon Mine, ABA = acid-base-acid (ABA) protocol; UF = ultrafiltration; and AF is the ORAU laboratory code for ABA+UF.

	Treatment	C:N	Graphite size (mg)	$^{14}\text{C}$ date BP ( $\pm 1\sigma$ error)	% modern ( $F^{14}\text{C} \times 100$ )
LM	ABA UF (AF)	3.2	2.1	$>49,000^a$	~0%
LM Hyp	HPLC-separated fraction	5.1	1.0	$>44,200$	0.20%
LM+Tea	ABA	3.4	—	—	—
LM+Tea	ABA UF (AF)	3.4	2.4	$22,170 \pm 140$	6.33%
LM+Tea	Hydrolyzed and filtered	3.2	0.9	$33,200 \pm 600$	1.63%
LM+Tea Hyp	HPLC-separated fraction	5.0	0.8	$>44,100$	0.16%

<sup>a</sup>Note that the bulk date for the Lemon Mine (one characteristic date out of hundreds) is older than the Hyp ones. “AF” samples’  $^{14}\text{C}$  age is corrected for pretreatment contamination, while other samples are not. Additionally, the bulk samples were bigger, and therefore subjected to bigger combustion correction. While these corrections could be justifiable, they could also be an over-correction, yielding slightly older dates.

#### Archaeological Bones

The Kostenki-Borschevo complex of sites, on the banks of the river Don near Voronezh in Russia, are key Paleolithic sites dating to Marine Oxygen Isotope stages 3–2. In several of the sites, a visible tephra layer is present, identified as the Campanian Ignimbrite (CI) and dated by  $^{40}\text{Ar}/^{39}\text{Ar}$  to  $39,280 \pm 110$  cal yr BP (De Vivo et al. 2001). The CI layer serves as a major chronostratigraphic marker for the Middle to Upper Paleolithic transition, providing a temporal marker that is not based on  $^{14}\text{C}$  dates (Hoffecker et al. 2008).

At the ORAU, bones are prescreened for collagen content by measuring their nitrogen percentage. Bones with  $<0.76\%$  N content are usually rejected for collagen extraction (although this is now often set at a lower cutoff of 0.5%) and are assumed to have too little collagen to obtain reliable results (Brock et al. 2010b). Because the Kostenki horse bone was expected to have low collagen content as indicated by its low nitrogen values (see Methods), the whole bone (140 g) was used to

extract the remaining collagen. Some 1.2 g of collagen was extracted and 30 mg of this was hydrolyzed and single amino acids separated on a preparative HPLC system using a method suitable for the isolation of Hyp. The Hyp fraction produced a  $^{14}\text{C}$  date of  $35,700 \pm 900$  BP.

Many dates have been obtained for samples from Kostenki, mostly by  $^{14}\text{C}$ . However, bone  $^{14}\text{C}$  dates from this site are considered unreliable, as they often exhibit a large range of ages for samples from the same layer. These inconsistencies are probably the result of younger carbon contamination originating from humic acids (Haesaerts et al. 2004).  $^{14}\text{C}$  dates on charcoals and OSL dates from sediment underlying the CI tephra at Kostenki 12 and Kostenki 14 yielded ages  $>40,000$  BP<sub>OSL</sub> (Hoffecker et al. 2008). The horse bone was found in cultural layer VIb, below the CI tephra level, and above the horizon of hearths.  $^{14}\text{C}$  ages for the cultural level VIb, using the IntCal09 calibration curve (Reimer et al. 2009), range between 42.1 and 38.7 ka cal BP (at 95.4% probability). The  $^{14}\text{C}$  ages for the “Horizon of hearths” range between 42.8 and 39.6 ka cal BP (at 95.4% probability), based on the dates published by Hoffecker et al. (2008). The horse date is therefore expected to be not older than 42.8 and not younger than 39.3 ka cal BP. The Hyp fraction of the Kostenki 14 horse yielded a calibrated age of 42.3–38.9 ka cal BP (95.4% probability), which is within the expected range. A model was made using OxCal v 4.2 (Bronk Ramsey 2009), assuming that the horse bone precedes the date of charcoal from the level of volcanic ash (LVA), taken as an approximation for the pre-eruption date (Douka et al. 2010). The modeled calibrated date was narrowed to 42.0–39.4 ka cal BP, which is closer to the expected value (see Figure 1). The Hyp date obtained from the Kostenki 14 horse bone suggests strongly that reliable  $^{14}\text{C}$  dates can be obtained from low-collagen bones using the Hyp dating method.

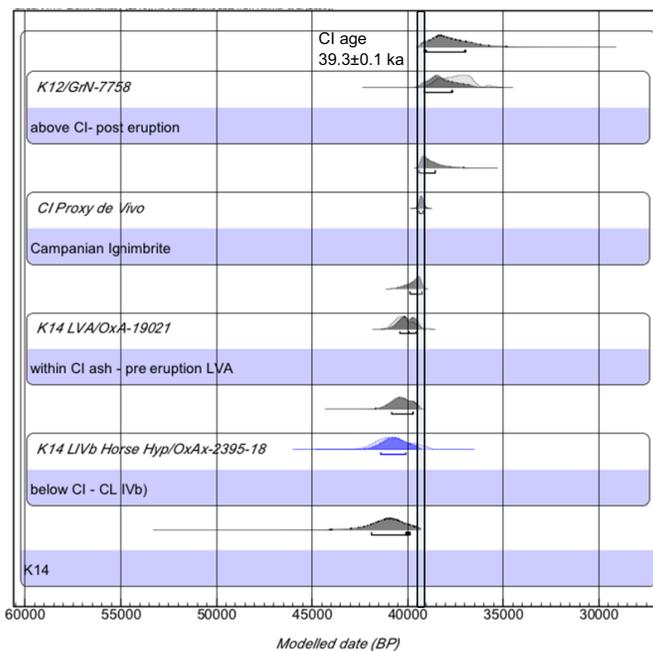


Figure 1 Bayesian model built with OxCal v 4.1.7, using IntCal09 (Bronk Ramsey 2009; Reimer et al. 2009) to calibrate the Kostenki dates; 68.2% probability range error is marked on the likelihood distribution of the calibrated date range. The CI eruption date (line) is given here as the  $^{40}\text{Ar}/^{39}\text{Ar}$  age of De Vivo et al. (2001), at  $39.28 \pm 110$  cal BP. The K14 horse Hyp predates the CI eruption.

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We also used hydroxyproline dating to try to determine the age of the La Ferrassie 1 Neanderthal skeleton. Previously, this had proved impossible using conventional techniques due to an unresolved contamination issue in the bone. La Ferrassie is a Middle-Upper Paleolithic site in the Dordogne, France, in which the burials of 2 Neanderthal adults and 5 children and neonates were found in the early 20th century. The Upper Paleolithic material found in La Ferrassie is important in understanding the Upper Paleolithic sequence in France. One of the most important individuals found at the site is La Ferrassie 1, the skeleton of an adult male. His skull, the largest and most complete Neanderthal skull ever found, has many of the typical Neanderthal traits such as the low, sloping forehead and large nasal opening.

The distal left tibia and distal right tibia of La Ferrassie 1 were sampled and dated at the ORAU previously. The surface of the bones seemed to have been treated with preservation material that the sampling process attempted to avoid, by sampling the interior of the bone. The results obtained with ultrafiltration were very different from each other, with the left tibia yielding a date of 32,750 ± 450 BP, and the right, 11,540 ± 55 BP (Table 2). These results, together with the high C:N values, suggested that both bones were contaminated, the right tibia to a greater extent than the left.

Some 30.7 mg of the contaminated collagen of the left tibia and 19.8 mg of the right tibia, both previously extracted, together with the corresponding low molecular weight collagen that passed through the ultrafilter, were hydrolyzed and separated on the HPLC, and the Hyp fraction was dated. The Hyp dates were both older from the bulk dates. However, the discrepancy between them was not resolved, with the left tibia now dating to 35,700 ± 1500 BP and the right tibia to 12,910 ± 90 BP (Table 2), a difference of ~20 ka. We conclude that the Hyp dating method was unable to eliminate all of the contaminant in this case, which is probably of collagenous origin.

Table 2 C:N values and dates of La Ferrassie left and right tibia.

	PNumber	Treatment	C:N	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	<sup>14</sup> C date BP (±1σ error)
Left tibia	22,719.0 AF* 01	Ultrafiltration	3.4	-18.9	11.4	32,750 ± 450
	22,719.1 NRC 01	Hyp separation	4.9	-22.8	13.4	35,700 ± 1500
Right tibia	22,720.0 AF* 01	Ultrafiltration	3.5	-19.3	10.7	11,540 ± 55
	22,720.2 NRC 01	Hyp separation	4.9	-22.9	12.7	12,910 ± 90

A possible candidate is collagen-based glue, commonly used by curators in the past, customarily obtained from boiling collagen-containing animal parts such as hides and bones. Based on their age shift from the left tibia Hyp date (assuming this is the correct age), the presence of 23% collagen contamination in the right tibia and 1% in the left can be assumed, and the stable isotopes values of the contaminant can be calculated. The contaminant's  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  are -20.9‰ and 8.4‰, respectively, both depleted relatively to the  $\delta^{13}\text{C}$  of -18.9‰ and  $\delta^{15}\text{N}$  of 11.4‰ of the Neanderthal sample, and in accord with the contaminant being from collagen of a C<sub>3</sub>-eating herbivore (e.g. glue made of horse collagen).

At the Flixton site in the UK, a number of horse dates show inconsistency and are suspected of being contaminated by carbon derived from the burial environment. The paleolake Flixton was an ancient lake in the Vale of Pickering, in North Yorkshire, on the western shore of which lay Star Carr. Star Carr is one of the most important Mesolithic sites in England, occupied intermittently in ~9500–8000 BC (Dark et al. 2006). At the time, this was a reed swamp-fringed lake, inhabited by Mesolithic hunter-gatherers. Its importance lies in its unusually good preservation of organic materials and exceptionally rare finds from the site include barbed points, inferred stag headdresses, and

a boat paddle, as well as the earliest evidence of carpentry in Europe (Clark 1954; Conneller et al. 2012). Thirty-eight mostly incomplete horse bones and 4 teeth were found at Flixton 2, an island in the middle of Lake Flixton, in a layer of fine detritus mud (layer H). The pollen record and stratigraphic analysis suggest that the horse bones date to the Upper Paleolithic.

A bulk sample of the mud associated with the horse bones, taken from around a horse astragalus (find No. 2713), yielded a  $^{14}\text{C}$  date of  $9850 \pm 80$  BP (CAR-1016) (Cloutman 1988). Another bulk mud sample, from the top of that layer, produced a  $^{14}\text{C}$  date of  $9270 \pm 210$  BP (Q-66) (Clark 1954). The reliability of these dates is questionable, however, having been produced from the organic material within the sediment, and liable to humic contamination. Based on the pollen record, it is thought that around 9.7 ka BP the climate in this area became warm, leading to the spread of the forest (Dark et al. 2006). As a result, horses are believed to have become locally extinct. It is therefore assumed that horses from this site could not be younger than 9.7 ka BP. A number of the horse bones had been  $^{14}\text{C}$  dated over the years, some of them several times, with dates ranging from 10,150 to 9160 BP, with C:N ratios ranging between 3.3 and 3.5 (Table 3). Interestingly, the older dates are those treated with an ion-exchange column as part of the pretreatment (with the exception of sample OxA-6329, which gave the date  $9160 \pm 80$  BP, see Table 3). Ion exchange was routinely conducted in the past at the ORAU (Hedges and Law 1989). It was phased out and replaced by ultrafiltration due to column bleed, which affected low-yield collagen samples and rendered them younger than their true age. These results suggest that ultrafiltration is less efficient in decontaminating these types of samples than the ion-exchange protocol.

Table 3  $^{14}\text{C}$  dates and C:N ratios from different samples from Flixton 2. AF = ultrafiltration; AI = ion exchange; \* designates solvent wash. In bold, the Hyp sample from this study. All the dates except the last two (Schadla-Hall and Lane, in press) were dated in Oxford. NM = not measured. NP = not published.

Sample	Lab code	Treatment	C:N	$\delta^{13}\text{C}$	$^{14}\text{C}$ age BP ( $\pm 1\sigma$ error)
XB23 left astragalus	X-2395-14	Hyp	5.0	-24.6	<b>10,155 <math>\pm</math> 55</b>
	OxA-6328	AI*	3.3	-20.2	10,150 $\pm$ 90
	OxA-20322	AF	3.4	-21.3	9626 $\pm$ 39
	OxA-20356	AF*	3.5	-21.2	9640 $\pm$ 40
	OxA-21175	AF*	3.5	-20.5	9290 $\pm$ 45
1st phalange	OxA-20695	AF*	3.3	-20.5	9920 $\pm$ 45
	OxA-6318	AI	NM	-20.8	10,090 $\pm$ 90
Bone	OxA-6329	AI*	3.5	-20.3	9160 $\pm$ 80
1st phalange	OxA-20696	AF	3.3	-21.0	9975 $\pm$ 45
	OxA-6319	AI	NM	-20.8	10,150 $\pm$ 80
Soil around bone	CAR-1016		NP	NP	9850 $\pm$ 80
Mud from top of an organic mud layer	Q66		NP	NP	9270 $\pm$ 210

The collagen from the horse left astragalus from Flixton 2 that produced the seemingly too-modern date of  $9290 \pm 45$  (OxA-21,175) was further separated using the Hyp dating method. The hydrolyzed collagen (30 mg) was injected into the HPLC. The Hyp fraction yielded a date of  $10,155 \pm 55$  BP (12,390–11,260 cal BP using the IntCal09 calibration curve, at 95.4% probability; Table 3 and Figure 2). This result matches much more closely the archaeological and paleoenvironmental age estimates, along with the ion-exchanged dated bones from the site (Figure 2). It is concluded that the Flixton horse bone is contaminated (probably with humic acids), and that the contamination was

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removed by dating the Hyp fraction, and mostly removed by ion exchange, but not by the normal pretreatment procedures. There are interesting parallels with the dating of the Meisenheim elk skeleton in Germany (Fiedel et al., these proceedings). Here, the only method applied that yielded an age consistent with the age of the elk, which was found beneath the Laacher See tephra, was the Hyp dating method. The site, like Flixton, is humic-rich, and almost certainly the cause of the more modern dates obtained using other routine methods.

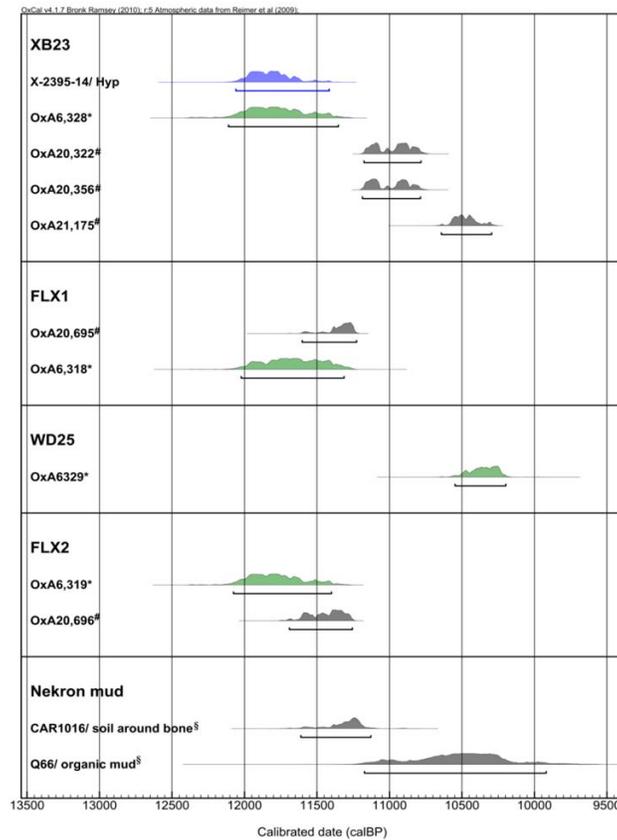


Figure 2 Calibrated <sup>14</sup>C dates for Flixton 2 horse bones, produced with OxCal v 4.1.7 and IntCal09 calibration curve (Bronk Ramsey 2009; Reimer et al. 2009). The 68.2% error is marked on the likelihood distribution of the calibrated date range. \* denotes samples pretreated with an ion exchange method (AI) and their likelihood distributions are colored in green; # denotes samples ultrafiltered prior to AMS dating (AF); § denotes bulk date. Dates are as in Table 3.

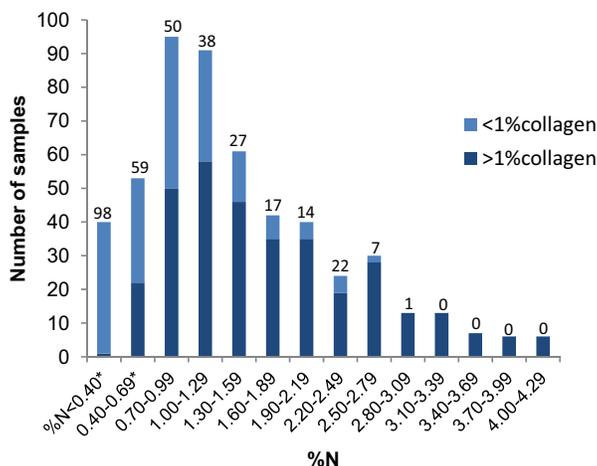
## DISCUSSION

The data presented in this paper suggest strongly that the Hyp dating method is particularly advantageous for accurately dating bones contaminated with high-molecular-weight and/or cross-linked carbon contaminants, and those that yield questionable <sup>14</sup>C dates or are considered undatable. In most cases, however, existing pretreatment methods appear to be sufficient for obtaining reliable results. In some cases, we have shown that the problem of bones from contexts producing unexpectedly variable dates can be resolved through testing using Hyp methods. One problem in <sup>14</sup>C dating

of bone is that contamination is often hard to identify through routine analytical methods. The carbon:nitrogen atomic ratio (C:N) can provide an indication that contamination is present but it is not sufficiently precise to definitely rule out its presence. Intact collagen will have a C:N ratio of 3.21 (Ambrose 1990). Bones are considered “datable” when their collagen C:N ratio is 2.9–3.6 (DeNiro 1985; Ambrose 1990), or 3.1–3.5 in Oxford (van Klinken and Hedges 1995). High values (i.e. >3.5) may indicate diagenetic alteration has taken place, causing de-amination and/or contamination by exogenous carbon-containing compounds. Since the C:N ratio is less sensitive to contamination than the <sup>14</sup>C measurement, there is no guarantee that even bones with “normal” C:N (e.g. 3.1–3.3, given the measurement error) are completely contaminant-free (van Klinken 1999). As we have shown here, the Hyp method has great potential for yielding reliable dates for those potentially contaminated either from the burial environment or by conservation material, unless of course they have been contaminated by collagen-derived consolidants.

Bones with very low collagen content are also considered problematic for dating. Some bones that yield trace amounts of collagen have indeed lost most of their collagen. It is expected, however, that some of the very low-collagen bones may still contain endogenous collagen, probably partially degraded. By its very nature, the ultrafiltration step will reduce yields significantly as a result of the fact that everything smaller than 30 kDa (about a third of the collagen chain) will pass through the filter and will not be dated. For older bones (e.g. Paleolithic), in which the collagen quality has deteriorated, a greater proportion of collagen is lost during ultrafiltration (Brock et al. 2010b). The artificial weathering experiment presented in this paper shows that Hyp may be retained in the soluble fraction of low-collagen bones. For bones in which the acid-insoluble fraction of the collagen is insufficient to yield a reliable date using the standard method, the Hyp dating method may enable dating by extracting Hyp from both the organic and the mineral fractions.

Figure 3 The number and percentage of bone samples that yielded <1% and >1% wt collagen for 0.3% ranges of whole bone %N content for a data set of 521 bones from a wide range of contexts and ages. \* The data set is biased against the lower %N bones, as most of these samples are failed and thus not analyzed. In addition, the screening for %N is mostly done for bones that are either considered to be poorly preserved or are from archaeological sites known for poor or variable preservation, meaning that the pattern presented here may not apply to all sites.



At ORAU, bones from sites known to have variable or poor collagen preservation are prescreened in order to identify samples suitable for <sup>14</sup>C dating using conventional methods (samples that yield >1% wt collagen) by measuring the %N content of whole bones (Brock et al. 2007, 2010b; Marom 2012). Although when measuring the whole bone nitrogen content it is not only the nitrogen from collagen that is being measured but also nitrogen from NCPs and potentially nitrogen from depositional soil (e.g. microbial proteins, humic acids) or conservation material, it has been shown that %N > 0.7 is a good indicator for the survival of collagen, with ~70% prediction success (Brock et al. 2010b). Figure 3 shows the analysis of 521 bones from a wide range of contexts and ages: 82% of

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the bones (428) had whole bone %N content higher than the 0.7% threshold. Of those, 26% (112 samples, 21% of the whole data set) were “false positives,” i.e. yielded <1% collagen and were therefore failed. These represent either contaminated bones, or, most likely, bones with degraded collagen that passed through the ultrafilters (with up to 70% of the “collagen” being <30 kDa; Marom 2012). Those bones with high nitrogen content but low collagen yield will fail to be dated using the ultrafiltration method, but may potentially succeed using the Hyp method if the Hyp content is sufficient. Even bones with low %N, considered undatable by conventional bulk dating methods, may still yield reliable  $^{14}\text{C}$  dates using the Hyp dating method if enough material is available, as the Kostenki horse bone has shown. The set of circumstances under which the hydroxyproline dating method should be used, and the recommended workflow, are summarized in Figure 4.

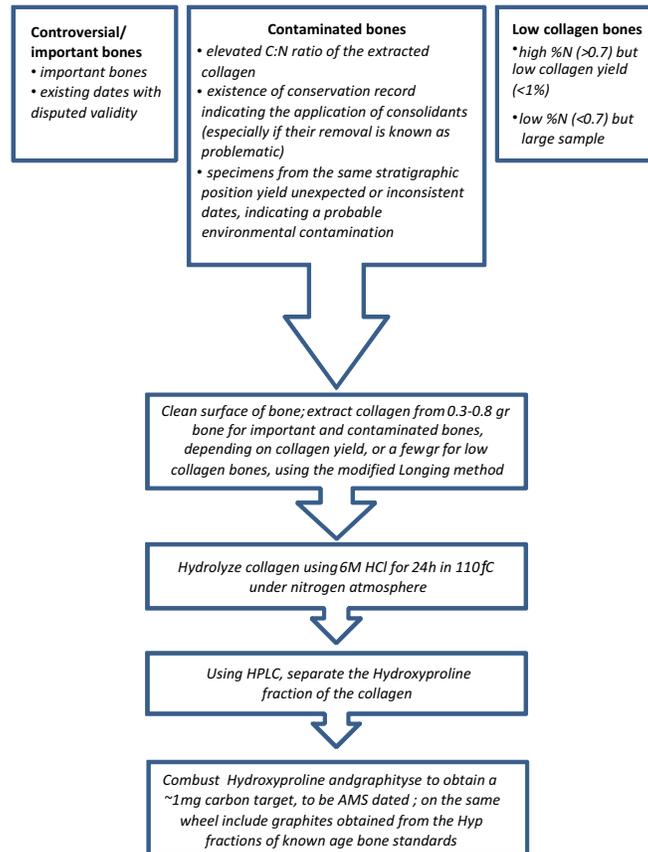


Figure 4 A suggested set of circumstances under which the hydroxyproline method should be employed, and the recommended workflow.

## CONCLUSIONS

The artificial contamination and degradation experiments presented in this paper serve as a “proof of concept” that the Hyp dating method has the potential to date accurately bones that were subjected to heat and contamination. These bones would not be dated or would yield erroneous results using standard pretreatment procedures. The successful application of the method, to problematic archaeological bones from the sites of Kostenki and Flixton, further demonstrates the application of

the method. The discrepancy between the date produced by the left and right tibia of the La Ferrassie 1 Neanderthal skeleton is probably a result of collagen-derived contamination, which the method is unable to remove of course, as some of the Hyp itself is from an exogenous source. Even when a bulk date can be obtained for a problematic bone, the Hyp date should provide a greater degree of certainty, being derived from a single compound that is more likely to be bone specific than all compounds dated as a result of conventional pretreatment approaches. It is therefore concluded that the Hyp dating method should be the method of choice for dating important, badly preserved, or contaminated bones, when sufficient sample material is available.

## ACKNOWLEDGMENTS

The authors would like to thank Dr C Conneller, University of Manchester, for her help and advice on the Flixton site. The  $^{14}\text{C}$  dates were financed by an Oxford Radiocarbon Accelerator Dating Service (ORADS) grant through the Natural Environment Research Council.

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