Refining human palaeodietary reconstruction using amino acid $\delta^{15}N$ values of plants, animals and humans

Amy K. Styring [a,*], Rebecca A. Fraser [b], Rose-Marie Arbogast [c], Paul Halstead [d], Valasia Isaakidou [b], Jessica A. Pearson [e], Marguerita Schäfer [f], Sevasti Triantaphyllou [g], Soultana Maria Valamoti [g], Michael Wallace [c], Amy Bogaard [b], Richard P. Evershed [a]

[a] Organic Geochemistry Unit, Biogeochemistry Research Centre, School of Chemistry, University of Bristol, Bristol, UK
[b] School of Archaeology, 36 Beaumont Street, University of Oxford, Oxford, UK
[c] CNRS/UMR 7044 Maison Interuniversitaire des Sciences de l'Homme-Alsace, Strasbourg, France
[d] Department of Archaeology, University of Sheffield, Sheffield, UK
[e] Department of Archaeology, Classics and Egyptology, University of Liverpool, Liverpool, UK
[f] Institut für Prähistorische und Naturwissenschaftliche Archäologie, University of Basel, Basel, Switzerland
[g] School of History and Archaeology, Aristotle University of Thessaloniki, Thessaloniki, Greece

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ABSTRACT

An established method of estimating the trophic level of an organism is through stable isotope analysis of its tissues and those of its diet. This method has been used in archaeology to reconstruct past human diet from the stable nitrogen isotope ($\delta^{15}N$) values of human and herbivore bone collagen. However, this approach, using the ^{15}N-enrichment of human bone collagen $\delta^{15}N$ values over associated herbivore bone collagen $\delta^{15}N$ values to predict the relative importance of animal protein, relies on the assumptions that: (i) the $\delta^{15}N$ values of plants consumed by humans and herbivores are identical, and (ii) the ^{15}N-enrichment between diet and consumer is consistent. Bone collagen amino acid $\delta^{15}N$ values have the potential to tackle these uncertainties, as they constrain the factors influencing bone collagen $\delta^{15}N$ values. In this study, the $\delta^{15}N$ values of glutamic acid and phenylalanine in human and herbivore bone collagen isolates from Neolithic sites in Germany, Greece and Turkey were determined by gas chromatography-combustion-isotope ratio mass spectrometry. The fraction of animal protein in total dietary protein consumed by the humans was estimated by: (i) comparing bulk human and herbivore collagen $\delta^{15}N$ values, (ii) comparing bulk human and herbivore collagen and ancient charred cereal grain $\delta^{15}N$ values, (iii) comparing human bone collagen $\delta^{15}N_{Glutamic\ acid}$ and $\delta^{15}N_{Phenylalanine}$ values, and (iv) comparing $\delta^{15}N_{Glutamic\ acid}$ values of human and herbivore bone collagen and estimated $\delta^{15}N_{Glutamic\ acid}$ values of ancient charred cereal grains. Where determined cereal grain $\delta^{15}N$ values are higher than estimated herbivore forage values, estimates of animal protein consumption are significantly lower, emphasising the importance of the plant nitrogen contribution to human bone collagen. This study also highlights the need for further investigation into: (i) the $\Delta^{15}N_{Consumer-Diet}$ values of glutamic acid and phenylalanine in terrestrial ecosystems, and (ii) $\Delta^{15}N_{Glutamic\ acid-Phenylalanine}$ values of common plant foods in order to improve the accuracy and more widespread applicability of amino acid-based methods for palaeodietary reconstruction.

1. Introduction

Stable isotope analysis is routinely used to estimate the trophic position of an organism within a food web, based on the premise that the isotopic composition of a consumer's tissues originates from its diet, but is offset by trophic enrichment factors that are governed by underlying metabolic processes associated with nutrient assimilation and tissue biosynthesis. In archaeology, the

* Corresponding author. Present address: School of Archaeology, 36 Beaumont Street, University of Oxford, Oxford, UK. Tel.: +44 0 1865 288014.
E-mail address: amy.styring@arch.ox.ac.uk (A.K. Styring).

extent to which the $\delta^{15}N$ of human bone collagen lies above the $\delta^{15}N$ values of herbivore bone collagen from the same archaeological site is an established method of estimating human trophic position, or rather the proportion of animal protein in the human diet (Hedges and Reynard, 2007; Koch, 2007; Lee-Thorp, 2008; Minagawa and Wada, 1984; Schoeninger et al., 1983). Interpretations, however, can be confounded by uncertainty regarding the ^{15}N trophic enrichment factor between diet and consumer tissues ($\Delta^{15}N_{Consumer-Diet}$); studies have found this to vary between 2 and 6‰ (DeNiro and Epstein, 1981; Hare et al., 1991; Minagawa and Wada, 1984; O'Connell et al., 2012; Schoeninger and DeNiro, 1984). Since bulk bone collagen $\delta^{15}N$ values integrate the $\delta^{15}N$ values of their constituent amino acids, representing the net effect of dietary protein sources and metabolic cycling within the body, such variation in consumer-diet offsets is not easy to study using bulk collagen $\delta^{15}N$ values alone.

Determination of individual amino acid $\delta^{15}N$ values in bone collagen has the potential to shed more light on the underlying metabolic pathways responsible for the ^{15}N-enrichment of consumer tissues over diet and to allow further elucidation of the often complex biochemical processes contributing to the bulk $\delta^{15}N$ value (Hare et al., 1991; Naito et al., 2010a,b; Styring et al., 2010). Equations have been developed to estimate the trophic level of humans and fauna in aquatic, C3-plant-based and C4-plant-based ecosystems based on the fact that the $\delta^{15}N$ values of the amino acids glutamic acid (Glu) and phenylalanine (Phe) increase to different extents with trophic level (8 and 0.4‰ respectively; Chikaraishi et al., 2011, 2010, 2009, 2007; Steffan et al., 2013). The benefit of this approach is that the $\delta^{15}N$ values of Glu ($\delta^{15}N_{Glu}$) and Phe ($\delta^{15}N_{Phe}$) provide an internal trophic level indicator, precluding the need to rely upon the bone collagen $\delta^{15}N$ values of preserved fauna, whose tissues may not in fact have contributed to the human diet. The very small ^{15}N-enrichment of $\delta^{15}N_{Phe}$ with trophic level (Δ_{Phe}) suggests that Phe has undergone very little metabolism within the body and therefore bone collagen $\delta^{15}N_{Phe}$ values broadly reflect those of the diet, whereas the large ^{15}N-enrichment in $\delta^{15}N_{Glu}$ with trophic level (Δ_{Glu}) suggests that bone collagen $\delta^{15}N_{Glu}$ values result from subsequent N metabolism within the consumer.

The limitation of both of these methods is that they rely on the assumption that the $\delta^{15}N$ values of plants consumed by humans and the fauna they consume are identical. $\delta^{15}N$ value determinations of modern plants suggest that this assumption is implausible, since plants vary widely in their $\delta^{15}N$ values (e.g. Craine et al., 2009). One of the proposed reasons for high $\delta^{15}N$ values of human bone collagen, particularly during the European Neolithic, is consumption of manured crops by humans, since manuring increases the $\delta^{15}N$ values of plants (Bogaard et al., 2007; Dürrwächter et al., 2006). Studies have shown that manuring of crops can increase cereal grain and chaff $\delta^{15}N$ values by as much as 10‰ and that $\delta^{15}N$ values are correlated to the amount of manure applied (Bogaard et al., 2007; Fraser et al., 2011; Kanstrup et al., 2011). The effect of this increase on crop $\delta^{15}N$ values is to raise the bone collagen $\delta^{15}N$ value of humans eating manured crops above that of herbivores eating unmanured plants, leading to overestimation of the importance of animal protein in the diet (Fig. 1).

In the past, the stable isotope values of plants consumed by humans on an archaeological site have rarely been determined directly from those preserved, due to concerns about contamination and the robustness of plant isotope values after years of burial. Carbonisation is the most common process by which plant material can survive in archaeological contexts and various studies have examined the changes in the $\delta^{15}N$ values of cereal grains with charring (Bogaard et al., 2007; Fiorentino et al., 2012; Fraser et al., 2013a; Kanstrup et al., 2012). Within the charring conditions
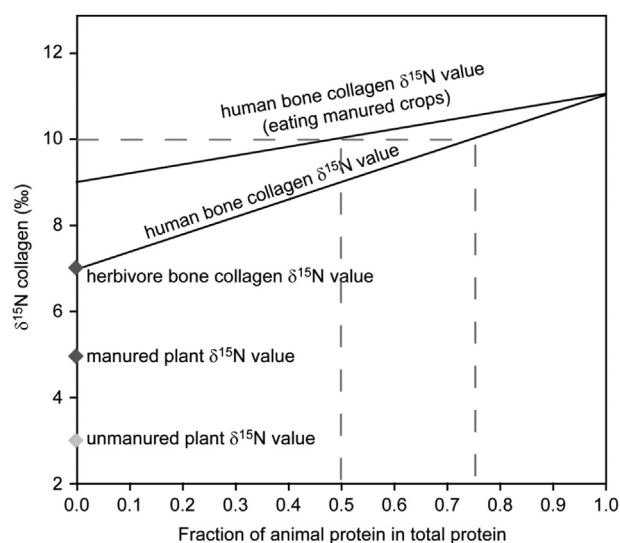

Fig. 1. Variation of human bone collagen $\delta^{15}N$ value with fraction of animal protein in the diet, assuming a $\Delta^{15}N_{Collagen-Diet}$ value of 4‰, for humans eating animals and unmanured or manured plants. In this case, manuring increases the $\delta^{15}N$ value of plant by 2‰. Following this model, a human bone collagen value of 10‰ could be due either to: (i) consumption of unmanured plants and 75% animal protein, or (ii) consumption of manured plants and 50% animal protein.

conducive to producing undistorted cereal grains similar to those found on archaeological sites (less than 250 °C for more than 6 h; Charles et al., in prep), the $\delta^{15}N$ values of charred einkorn grains were found to increase by around 1‰ (Fraser et al., 2013a). This was ascribed to the preferential loss of ^{14}N-containing volatiles, during the thermal conversion of starch and protein in the grains to high molecular weight melanoidins (Styring et al., 2013), which are relatively resistant to degradation (Almendros and Dorado, 1999). Biochemical investigation of ancient charred grains from two archaeological sites in Europe found them to contain similar proportions of N as their modern charred counterparts and acid-—base—acid pre-treatment, which is commonly used to remove contaminants in radiocarbon dating (Goh, 1991), resulted in little change in their %N or $\delta^{15}N$ values (Fraser et al., 2013a; Styring et al., 2013). Whilst further work is needed to establish the effect of a wider range of burial conditions and durations on the biochemical composition and $\delta^{15}N$ values of a wider range of cereal grain taxa, the charred grains preserved on archaeological sites have the potential to provide baseline $\delta^{15}N$ values of plants to include in palaeodietary predictions.

The amino acid $\delta^{15}N$ values of these cereal grains could also improve trophic level estimates by refining the amino acid trophic level equations. Although amino acids in archaeobotanical charred cereal grains do not survive in sufficient quantities for isotopic analysis (they contain 0.4% of the total hydrolysable amino acid content of fresh cereal grains; Styring et al., 2013), it may be possible to estimate their values based upon differences between bulk $\delta^{15}N$ values and amino acid $\delta^{15}N$ values determined in modern plants (Chikaraishi et al., 2010, 2011; Styring et al., 2014). Such calculations are discussed in more detail in Sections 2.5.3 and 2.5.4.

In this study, the bulk and amino acid $\delta^{15}N$ values of human and faunal bone collagen and the bulk $\delta^{15}N$ values of ancient charred cereal grains and pulse seeds from the archaeological sites of Vaihingen an der Enz, Germany, Makriyalos, Greece and Çatalhöyük, Turkey were determined by elemental analysis-isotope ratio mass spectrometry (EA-IRMS) and gas chromatography-combustion-IRMS (GC-C-IRMS). We use the $\delta^{15}N$ values obtained to compare four different methods for estimating the proportion of animal

protein consumed by humans at each of the sites: (i) the 'Standard method' comparing human and faunal collagen $\delta^{15}N$ values; (ii) the 'Standard method plus plants', comparing human, faunal and ancient charred cereal grain $\delta^{15}N$ values; (iii) the 'Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values method', comparing the difference between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values in human bone collagen with the difference between $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values in modern plants, and (iv) the 'Bone collagen and plant $\delta^{15}N_{Glu}$ values method', comparing the $\delta^{15}N_{Glu}$ value of human and faunal bone collagen and the estimated $\delta^{15}N_{Glu}$ value of ancient charred cereal grains. Each of these methods is described in more detail in Section 2.5. The archaeological sites were chosen because they all date to the Neolithic, between c. 8000 and 4500 BC, and display evidence of both herding and cultivation, but are located in very different environmental zones. Further details of the archaeological contexts can be found in Supplementary Text 1. Inline Supplementary Fig. S1 shows the site locations. Discussion of the relative merits and limitations of each method for estimating the proportion of animal protein consumed by humans will help to refine future interpretations of human diet from bone collagen $\delta^{15}N$ values. In particular, discussion of current amino acid-based stable isotope methodologies is hoped to inform priorities for future work needed to ground truth this approach.

Inline Supplementary Fig. S1 can be found online at http://dx.doi.org/10.1016/j.jas.2014.11.009.

2. Materials and methods

2.1. Bone collagen extraction and analysis

Human bone was sampled from the compact mid-shaft of the femur (preferred), another long bone, or the rib. For faunal sampling, the distal humerus was generally sampled, taking sidedness into account to avoid sampling the same individual more than once. Collagen was extracted from approximately 1 g of compact bone using a modified Longin method (Longin, 1971): bone samples were crushed and immersed in 0.5 M HCl until demineralised and then washed three times in Milli Q water. Samples were then gelatinised by adding pH 3 water and heating to 70 °C for 48 h. The gelatinous solution was then filtered through an 80 μm Ezee-filter and transferred to clean test tubes and freeze-dried. Bone collagen samples of approximately 0.75 mg were weighed into tin capsules for determination of $\delta^{13}C$ and $\delta^{15}N$ values. Collagen yields ranged from 0.9 to 17.0% and the molar C:N ratios between 2.9 and 3.6, which is within the accepted range for well-preserved collagen (DeNiro, 1985).

2.2. Archaeobotanical sampling and analysis

Ancient charred cereal grain and pulse seed samples consisted of at least ten whole grains/seeds from the same stratigraphic unit, derived mostly from visible concentrations such as 'storage deposits'. The grains/seeds selected were virtually undistorted morphologically (displaying slight puffing only), resembling modern material charred experimentally at around 230 °C for a prolonged period (up to 24 h; Fraser et al., 2013a). Grains/seeds were examined at x7-45 magnification for visible surface contaminants, such as adhering sediment or plant roots; these were removed by gentle scraping. Grains/seeds were weighed and placed in glass test tubes in preparation for an acid-base-acid (ABA) pre-treatment, a procedure commonly applied to charcoal and charred plant remains prior to radiocarbon and stable isotope analysis (Goh, 1991) and considered appropriate for use on archaeobotanical remains (Fraser et al., 2013a). This three-step procedure consisted of: 1) treatment with 10 mL of 0.5 M hydrochloric acid (HCl) at 70 °C for

30–60 min, or until any effervescing ceased, and then rinsing in distilled water three times, 2) treatment with 10 mL of 0.1 M sodium hydroxide (NaOH) at 70 °C for 60 min, followed by rinsing in distilled water until the solution was clear and the pH neutral, using a minimum of three rinses, 3) treatment with 10 ml of 0.5 M HCl at 70 °C for 30–60 min, followed by three rinses in distilled water and final freeze drying. Grains/seeds were ground to fine homogeneous powder using a mortar and pestle and weighed into tin capsules ready for bulk $\delta^{13}C$ and $\delta^{15}N$ determinations.

The C:N molar ratios of modern grains charred at 230 °C for 24 h under a reducing atmosphere have been found to range from 17.9 to 33.4, with an average C:N molar ratio of 22.7 ($n = 17$; Fraser et al., 2013a). Of the ancient charred grain samples from Vaihingen, 13 out of 13 had C:N molar ratios within this range (18.0–28.6); 3 out of 3 grain samples from Makriyalos had C:N molar ratios within this range (25.3–26.1) and 6 out of 15 grain samples from Çatalhöyük had C:N molar ratios within a similar range (13.2–21.1). The grains from Çatalhöyük tended to have higher %N contents than those from the other sites, which could be due to post-depositional contamination, loss of non-N containing organics during burial or natural variation in %N caused by the environment in which they were grown. Work by Vaiglova et al. (in press) found no indication of post-depositional contamination in lentils or peas from Çatalhöyük, which suggests that the high %N is a factor of the environment in which they were grown. This needs to be investigated further by N isotope analysis of modern plants from around the site.

The C:N molar ratios of modern pulse seeds charred at 230 °C for 24 h under a reducing atmosphere were found to range from 8.8 to 13.1, with an average C:N molar ratio of 10.8 ($n = 15$; Fraser et al., 2013a). Of the ancient charred pulse seed samples from Vaihingen, 3 out of 3 had C:N molar ratios within a similar range (10.0–13.7); 0 out of 1 pulse seed samples from Makriyalos had C:N molar ratios within this range (8.0) and 4 out of 8 pulse seed samples from Çatalhöyük had C:N molar ratios within a similar range (8.7–11.1). The seed samples from Makriyalos and Çatalhöyük have higher %N contents than those determined in modern charred pulse seeds, which could also be a feature of the crop growing conditions and/or burial environment. Given that the cereal grains from Makriyalos did not have unusually high %N, the high %N in the pulse seeds is probably due to a difference in microscale growing conditions rather than a depositional or environmental effect that would have increased the %N of both cereal grains and pulse seeds. More modern and archaeological plant samples need to be analysed to resolve this. To account for the observed effects of experimental charring (at 230 °C for 24 h under a reducing atmosphere) on cereal grain and pulse seed $\delta^{15}N$ values, a generous offset of 1‰ is subtracted from the measured $\delta^{15}N$ values (cf. Fraser et al., 2013a).

2.3. Bulk $\delta^{13}C$ and $\delta^{15}N$ determinations

Bulk $^{12}C/^{13}C$ analysis was performed by sample combustion in a Costech 4010 on-line to a VG TripleTrap and Optima dual-inlet mass spectrometer. Isotope ratios were calculated relative to the VPDB reference by comparison with co-run laboratory standards (of plant material) calibrated against NBS-19 and NBS-22. Bulk $^{15}N/^{14}N$ analysis was performed by sample combustion on a ThermoFinnigan system comprising an elemental analyser linked under continuous flow with a Delta + XL mass spectrometer (ThermoFinnigan, Bremen). Isotope ratios were calculated as $\delta^{15}N$ versus atmospheric N_2 by comparison with standards calibrated against IAEA-N-1 and N-2. The relative analytical errors (1 standard deviation) for replicate analytical standards were ±0.2‰ for $\delta^{13}C$ and ±0.4‰ for $\delta^{15}N$. Replicate analyses of a bone collagen sample VAH35 measured in eleven separate mass spectrometry runs had a

standard deviation (1SD) of ±0.3‰ for δ^{13}C, ±0.2‰ for δ^{15}N and ±0.2 for the C:N molar ratio (mean C:N of 3.3). For plant material, the precision (1SD) among replicates of a well-homogenized modern uncharred barley sample was ±0.2 for %N and ±0.4‰ for δ^{15}N analysed in 29 separate runs and ±3.5 for %C and ±0.1‰ for δ^{13}C, analysed in 21 separate runs.

2.4. Amino acid δ^{15}N value determinations

2.4.1. Hydrolysis of bone collagen

Approximately 2 mg of collagen was hydrolysed in culture tubes (6 M HCl, 2 mL, 100 °C, 24 h). On cooling, the hydrolysates were evaporated under a stream of N_2 and redissolved in 2 mL 0.1 M HCl before being stored at −18 °C until required for analysis. Fractions of the protein hydrolysates (250 μL) were transferred to culture tubes and dried under N_2 before undergoing derivatisation. A known quantity of norleucine was added to each sample as an internal standard.

2.4.2. Preparation of amino acid derivatives (N-acetyl-i-propyl esters)

Amino acids were converted to their i-propyl esters by addition of 1 mL of a 4:1 v/v mixture of isopropanol and acetyl chloride (acetyl chloride added dropwise in an ice bath). Culture tubes were then sealed and heated at 100 °C for 1 h. Reagents were evaporated under a gentle stream of N_2 at room temperature. Dichloromethane (DCM) was added (2 × 0.5 mL) and evaporated in an ice bath to remove excess reagents. Amino acid i-propyl esters were then treated with 1 mL of a mixture of acetic anhydride, triethylamine and acetone (1:2:5, v/v/v; 10 min, 60 °C). Reagents were evaporated under a gentle stream of N_2 in an ice bath. The samples were dissolved in 2 mL ethyl acetate and 1 mL saturated sodium chloride solution was added. After phase separation, the organic phase was collected and the extraction repeated with an additional 1 mL of ethyl acetate. The combined organic phases were evaporated under N_2 in an ice bath and the residual water removed with successive 1 mL aliquots of DCM and evaporated under N_2 in an ice bath. The N-acetyl-i-propyl (NAIP) esters were dissolved in ethyl acetate and stored at −18 °C until required for analysis.

2.4.3. Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS)

Amino acids were identified by GC by comparison of their retention times with those of amino acid standards and their δ^{15}N values were determined by GC-C-IRMS. A ThermoFinnigan Delta^{Plus} XP system (Thermo Electron Corporation) was used to determine the δ^{15}N values of derivatised amino acids. The mass spectrometer (EI, 100 eV, three Faraday cup collectors m/z 28, 29 and 30) was interfaced to a Thermo Electron Trace 2000 gas chromatograph via a ThermoElectron gas chromatograph combustion III interface (CuO/NiO/Pt oxidation reactor maintained at 980 °C and reduction reactor of Cu wire maintained at 650 °C). Samples were introduced using a PTV injector held at 200 °C. Helium at a flow of 1.4 mL min^{-1} was used as the carrier gas and the mass spectrometer source pressure was maintained at 9×10^{-4} Pa. The separation of the amino acids was accomplished using a DB-35 capillary column (30 m × 0.32 mm internal diameter; 0.5 μm film thickness; Agilent Technologies, UK). The oven temperature of the GC was started at 40 °C and held for 5 min before heating at 15 °C min^{-1} to 120 °C, then 3 °C min^{-1} to 180 °C, then 1.5 °C min^{-1} to 210 °C and finally 5 °C min^{-1} to 270 °C and held for 1 min. A Nafion membrane removed water and a cryogenic trap was employed in order to remove CO_2 from the oxidised and reduced sample.

All δ^{15}N values are reported relative to reference N_2 of known nitrogen isotopic composition, previously calibrated against the AIR

international isotope standard, introduced directly into the ion source in four pulses at the beginning and end of each run. Each reported value is a mean of triplicate δ^{15}N determinations. An amino acid standard mixture, comprising amino acids whose δ^{15}N values were individually determined by EA-IRMS, was run every 3 runs in order to monitor instrument performance. The δ^{15}N values of the amino acids in the standard mixture were within 0.8% of their δ^{15}N values measured separately by EA-IRMS, with a precision of better than 0.8% (cf. Styring et al., 2012). Fig. 2 shows a GC-C-IRMS chromatogram displaying the separation of amino acids in archaeological human bone collagen.

2.5. Methods of estimating the contribution of animal protein to human diet

The following four methods were used to estimate the proportion of animal protein consumed by humans at each of the archaeological sites in this study. See Table 1 for a description of each of the terms used in the equations.

2.5.1. 'Standard method'

The 'Standard method' assumes that humans eating only plant protein will have the same bone collagen δ^{15}N value as the local herbivores, whereas humans eating only animal protein will have a bone collagen δ^{15}N value a trophic level higher than the local herbivores (Hedges and Reynard, 2007). This approach also assumes that the ^{15}N trophic enrichment factor between diet and consumer tissues ($\Delta^{15}N_{Consumer-Diet}$) is the same for herbivores and carnivores and for diets of differing protein content, both of which are assumptions that are still under debate (e.g. Hussey et al., 2014). The equation for estimating the proportion of animal protein consumed is:

$$f = \frac{\delta^{15}N\left[\text{hum}\right] - \delta^{15}N\left[\text{herb}\right]}{\Delta^{15}N_{Consumer-diet}} \qquad (1)$$

See Supplementary Text 2 for an explanation of how this equation was derived.
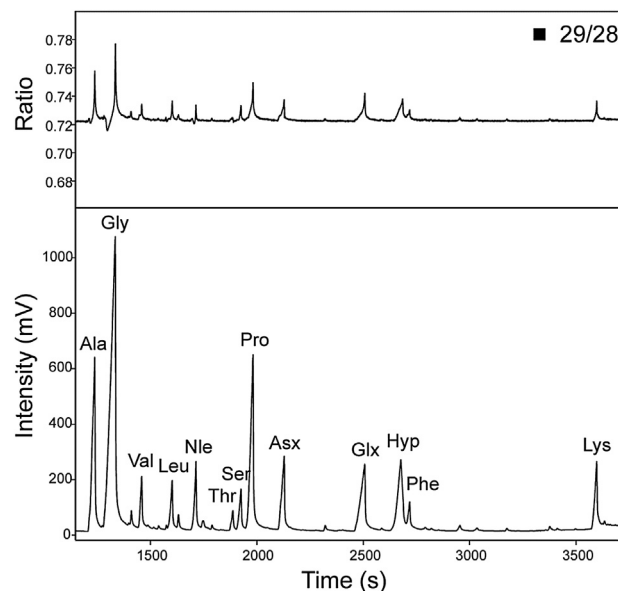

Fig. 2. A GC-C-IRMS chromatogram displaying the separation of amino acids in archaeological human bone collagen. Amino acids: Ala alanine, Gly glycine, Val valine, Leu leucine, Nle norleucine, Thr threonine, Ser serine, Pro proline, Asx aspartic acid, Glx glutamic acid, Hyp hydroxyproline, Phe phenylalanine, Lys lysine.

Table 1
Summary of the terms in equations used to estimate the proportion of animal protein in human diet.

Term	Description
$\delta^{15}N[gr]$	Mean of ancient charred cereal grain $\delta^{15}N$ values
$\delta^{15}N[herb]$	Mean of domestic herbivore collagen $\delta^{15}N$ values
$\delta^{15}N[hum]$	Mean of human collagen $\delta^{15}N$ values
$\delta^{15}N_{Glu}[gr]$	Estimated $\delta^{15}N_{Glu}$ value of ancient cereal grains on the site
$\delta^{15}N_{Glu}[herb]$	Mean of domestic herbivore collagen $\delta^{15}N_{Glu}$ values
$\delta^{15}N_{Glu}[hum]$	Mean of human collagen $\delta^{15}N_{Glu}$ values
$\delta^{15}N_{Phe}[hum]$	Mean of human collagen $\delta^{15}N_{Phe}$ values
$\Delta^{15}N_{Consumer\text{-}Diet}$	^{15}N trophic enrichment factor between consumer and diet $\delta^{15}N$, defined as 4‰ in this study (the mean of the 2–6‰ ^{15}N-enrichment determined in previous studies; Hedges and Reynard, 2007; O'Connell et al., 2012)
$\Delta^{15}N_{Glu\text{-}Phe}*$	Difference between the $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of plants at the base of the food chain
Δ_{Glu}	^{15}N trophic enrichment factor between consumer and diet collagen $\delta^{15}N_{Glu}$, defined as 8.0 ± 1.2‰ in this study (Chikaraishi et al., 2009)
Δ_{Phe}	^{15}N trophic enrichment factor between consumer and diet collagen $\delta^{15}N_{Phe}$, defined as 0.4 ± 0.5‰ in this study (Chikaraishi et al., 2009)
f	Proportion of animal protein in the human diet

Table 2
Summary of $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$ and $\Delta^{15}N_{Glu\text{-}Phe}*$ values determined for different plant types in previous studies.

Plant type	n	$\delta^{15}N_{Glu}$	$\delta^{15}N_{Phe}$	$\Delta^{15}N_{Glu\text{-}Phe}*$	Reference
C_3 tree and grass leaves	16	2.0 ± 4.9	10.4 ± 5.0	-8.4 ± 1.6	Chikaraishi et al., 2010
Barley and bread wheat grains from Rothamsted, UK	4	5.4 ± 4.2	12.3 ± 4.2	-6.9 ± 0.2	Styring et al., 2014
Barley and bread wheat grains from Bad Lauchstadt, Germany	4	3.0 ± 2.2	12.0 ± 1.9	-9.0 ± 0.3	Styring et al., 2014
Broad beans and peas from Bad Lauchstadt, Germany	4	0.9 ± 0.2	0.8 ± 1.1	0.1 ± 1.0	Styring et al., 2014
C_4 grass leaves	7	4.4 ± 6.1	4.0 ± 6.9	0.4 ± 1.7	Chikaraishi et al., 2010

2.5.2. 'Standard method plus plants'

The 'Standard method plus plants' assumes that humans eating only plant protein will have a bone collagen $\delta^{15}N$ value a trophic level higher than that of associated ancient charred grains and humans eating only animal protein will have a bone collagen $\delta^{15}N$ value a trophic level higher than that of local herbivores. Human bone collagen $\delta^{15}N$ values falling between the two indicate a mixed plant-animal diet of varying proportions. The equation is:

$$f = \frac{\left(\delta^{15}N[hum] - \Delta^{15}N_{Consumer-diet}\right) - \delta^{15}N\left[gr\right]}{\delta^{15}N[herb] - \delta^{15}N[gr]} \qquad (2)$$

See Supplementary Text 3 for an explanation of how this equation was derived.

2.5.3. 'Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values method'

The 'Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values method' was developed by Chikaraishi et al. (2009), who use this equation to calculate the trophic level of a consumer:

$$\text{Trophic level} = \frac{\left(\delta^{15}N_{Glu} - \delta^{15}N_{Phe} - \Delta^{15}N_{Glu-Phe}*\right)}{\left(\Delta_{Glu} - \Delta_{Phe}\right)} + 1$$

Since we are interested in the fraction of herbivore (animal) protein consumed in our study, it is necessary to subtract rather than add 1, so a human consuming 100% plant protein has a value of $f = 0$:

$$f = \frac{\left(\delta^{15}N_{Glu}\left[hum\right] - \delta^{15}N_{Phe}\left[hum\right] - \Delta^{15}N_{Glu-Phe}*\right)}{\left(\Delta_{Glu} - \Delta_{Phe}\right)} - 1 \qquad (3)$$

Table 2 summarises the difference in plant $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ ($\Delta^{15}N_{Glu-Phe}*$) values determined in previous studies. Chikaraishi et al. (2010) use a $\Delta^{15}N_{Glu-Phe}*$ value of -8.4‰ as the basis of their C_3 ecosystem trophic level equation, established from $\Delta^{15}N_{Glu-Phe}$ values determined in modern C_3 leaves ($n = 16$; Table 2). Styring et al. (2014) have determined the amino acid $\delta^{15}N$ values of manured and unmanured bread wheat (*Triticum aestivum*) and barley grains (*Hordeum vulgare*; $n = 8$) from the experimental farming sites of Rothamsted, UK and Bad Lauchstädt, Germany. The

$\Delta^{15}N_{Glu-Phe}$ values of these cereal grains were not affected by species or manure application, but differed significantly between locations; cereals grown at Rothamsted had a $\Delta^{15}N_{Glu-Phe}$ value of -6.9 ± 0.2‰, whereas those grown at Bad Lauchstädt had a $\Delta^{15}N_{Glu-Phe}$ value of -9.0 ± 0.3‰ ($t(6) = 10.606$, $p < 0.001$). The significant difference between the $\Delta^{15}N_{Glu-Phe}$ values of cereal grains from different sites cautions against using a standardised $\Delta^{15}N_{Glu-Phe}*$ value as the basis of a trophic level equation, without carrying out further $\Delta^{15}N_{Glu-Phe}$ value determinations in the relevant geographic region of the site, or without further assessment of variation in this value. We consider the effect of such variation in plant $\Delta^{15}N_{Glu-Phe}$ values on the estimate of animal protein consumption by humans using Equation (3) (see Section 3.3). Chikaraishi et al. (2010) and Styring et al. (2014) found that the $\Delta^{15}N_{Glu-Phe}$ values of C_4 terrestrial plants ($n = 7$) and pulses (broad beans and peas; $n = 4$) are much higher than that of C_3 terrestrial plants (Table 2), which would influence predictions of plant protein contribution to human diet if humans were eating significant quantities of C_4 plants and/or pulses. Although there is no evidence for significant C_4 plant consumption by humans at the sites in this study, since their $\delta^{13}C$ values are more negative than -18‰ (Section 3.1), there is a possibility that pulses played an important role in the Neolithic diet (cf. Bogaard, 2012, 2013; Valamoti, 2004).

The ^{15}N trophic enrichment factor between consumer and diet $\delta^{15}N$ values of Glu and Phe (Δ_{Glu} and Δ_{Phe}) from four controlled feeding experiments using green algae, zooplankton and fish were found to be 8.0 ± 1.2‰ and 0.4 ± 0.5‰, respectively (compiled by Chikaraishi et al., 2009). These Δ_{Glu} and Δ_{Phe} values have been used in estimates of trophic position in planktonic ecosystems (Hannides et al., 2009; McCarthy et al., 2007; McClelland et al., 2003; Schmidt et al., 2004), terrestrial ecosystems (Chikaraishi et al., 2011) and ancient human skeletal remains (Naito et al., 2010a,b). Recently, two studies have determined the $\Delta^{15}N_{Consumer-Diet}$ values of amino acids in seals fed on herring (Germain et al., 2013) and in four large carnivorous fish species (Hoen et al., 2014). They found that the difference between Δ_{Glu} and Δ_{Phe} values in these high trophic level consumers was much lower than that determined in lower trophic position marine organisms, implying that the difference between Δ_{Glu} and Δ_{Phe} values may be affected by the quantity and/or quality of protein in the diet (Hoen et al., 2014). Since there have been no feeding studies carried out on terrestrial mammals, we use the Δ_{Glu} and Δ_{Phe} values of 8‰ and 0.4‰, since they have been used in previous studies of human palaeodiet. In addition, the Δ_{Glu} and Δ_{Phe} values determined for seals in the Germain et al. (2013) study yield a difference between Δ_{Glu} and Δ_{Phe} values of 2.9‰ (2.9–0‰), which produces unrealistically high estimates (over 100% in most cases) of animal protein consumption by humans. It is clear that $\Delta^{15}N_{Consumer-Diet}$ amino acid values need to be determined for mammals

with similar digestive systems to humans (i.e. pigs) in order to resolve these uncertainties.

2.5.4. 'Bone collagen and cereal $\delta^{15}N_{Glu}$ values method'

The 'Bone collagen and plant $\delta^{15}N_{Glu}$ values method' takes into account the $\delta^{15}N_{Glu}$ values of human and faunal bone collagen and the estimated $\delta^{15}N_{Glu}$ value of ancient charred cereal grains from the same archaeological sites. This method focuses upon bone collagen and plant $\delta^{15}N_{Glu}$ values because the ^{15}N trophic enrichment factor between consumer and diet collagen $\delta^{15}N_{Glu}$ (Δ_{Glu}) is much larger than that of Phe (8.0‰ compared to 0.4‰), allowing greater distinction between the $\delta^{15}N_{Glu}$ values of humans eating only plant or only animal protein. The $\delta^{15}N_{Glu}$ value of ancient charred cereal grains can be estimated from comparison of their determined bulk $\delta^{15}N$ values and known differences between bulk $\delta^{15}N$ values and $\delta^{15}N_{Glu}$ values in modern cereal grains. Using the cereal grain amino acid $\delta^{15}N$ values determined by Styring et al. (2014), it is found that the $\delta^{15}N_{Glu}$ value relative to the bulk grain $\delta^{15}N$ value ($\Delta^{15}N_{Glu\text{-}Bulk}$ value) was not affected by site or manure application, but differed significantly between *T. aestivum* (-0.5 ± 0.5‰) and *H. vulgare* ($+1.4 \pm 0.5$‰; $t(6) = 5.548$, $p = 0.001$). Since the cereal grains from the archaeological sites in this study were predominantly glume wheats (*Triticum monococcum* and *Triticum dicoccum*), whose $\delta^{15}N_{Glu}$ values have not been determined in modern grains, the $\Delta^{15}N_{Glu\text{-}Bulk}$ value determined for *T. aestivum* (from the same genus as the glume wheats) was used to estimate the $\delta^{15}N_{Glu}$ value of ancient charred cereal grains. It would be advisable to determine $\Delta^{15}N_{Glu\text{-}Bulk}$ values for *T. monococcum* and *T. dicoccum* in future studies to test the reliability of this approach. Thus, the $\delta^{15}N_{Glu}$ value of the ancient charred cereal grains was estimated by subtracting 0.5‰ from the bulk $\delta^{15}N$ value of charred cereal grains from the site (also corrected for charring by subtracting 1‰ from the determined bulk $\delta^{15}N$ value). It is therefore assumed that humans eating 100% cereal protein will have a bone collagen $\delta^{15}N_{Glu}$ value 8.0‰ higher than that of the associated charred cereal grain $\delta^{15}N_{Glu}$ value and humans eating 100% animal protein will have a bone collagen $\delta^{15}N_{Glu}$ value 8.0‰ higher than that of the local herbivore bone collagen $\delta^{15}N_{Glu}$ value. The equation for this method is the same as Equation (2), but substitutes bulk collagen/grain $\delta^{15}N$ values with $\delta^{15}N_{Glu}$ values:

$$f = \frac{\left(\delta^{15}N_{Glu}[\text{hum}] - \Delta_{Glu}\right) - \delta^{15}N_{Glu}\left[\text{gr}\right]}{\left(\delta^{15}N_{Glu}[\text{herb}] - \delta^{15}N_{Glu}\left[\text{gr}\right]\right)} \qquad (4)$$

2.6. Statistical analysis

Independent *t*-tests were used to detect differences in crop stable isotope values between site, species and manuring regime for the modern field studies. A Kruskal Wallis test with post-hoc Bonferroni−Dunn test was used to detect differences in bulk collagen and amino acid $\delta^{15}N$ values between the herbivore species at each archaeological site due to non-normal distribution of the data.

3. Results

3.1. Bulk isotope analyses

3.1.1. Vaihingen an der Enz, Germany

Bulk $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen isolates from Vaihingen are plotted in Inline Supplementary Fig. S2. For the full $\delta^{13}C$ and $\delta^{15}N$ value dataset of bone collagen and archaeobotanical samples from the site, and for further discussion of land use and palaeodietary interpretation at Vaihingen using bulk collagen isotope values, see Fraser et al. (2013b). Mean $\delta^{15}N$ values are plotted in Fig. 3a and those used in the estimates of human diet are given in Table 3. Cereal grains (*T. monococcum* and *T. dicoccum*) exhibit mean $\delta^{13}C$ and $\delta^{15}N$ values of -24.1 ± 0.5‰ and 4.5 ± 0.5‰, respectively. The pulse seeds (*Pisum sativum* and *Lens culinaris*) have relatively high $\delta^{15}N$ values (2.8 ± 1.5‰) compared to atmospheric N_2. In modern studies, only pulses grown on artificial 'dung-soil' in Evvia, Greece were found to have such high $\delta^{15}N$ values (Fraser et al., 2011). The $\delta^{13}C$ and $\delta^{15}N$ values of the domestic herbivores are very similar to the average values determined at other Linearbandkeramik sites (cf. Dürrwächter et al., 2006; Oelze et al., 2011; Bickle and Whittle, 2013), and reflect a terrestrial herbivore diet in a temperate climate. Wild taxa constitute c.15% of the faunal remains (Schäfer, 2011) and the wild herbivores have similar $\delta^{15}N$ values to the domestic herbivores (average $\delta^{15}N$ value is 6.4 ± 0.4‰). Human bone collagen $\delta^{15}N$ values vary from 8.0 to 10.2‰, with a juvenile individual exhibiting a lower bone collagen $\delta^{15}N$ value of 5.8‰. This individual is discounted from the dietary calculations.

Inline Supplementary Fig. S2 can be found online at http://dx.doi.org/10.1016/j.jas.2014.11.009.

3.1.2. Makriyalos, Greece

Bulk $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen isolates from early Late Neolithic Phase I of Makriyalos (dating to 5500 to 5000 BC) are plotted in Inline Supplementary Fig. S3. The $\delta^{13}C$ and $\delta^{15}N$ values of 5 pigs determined by Triantaphyllou (2001) from late Late Neolithic Phase II of Makriyalos (c.4900 to 4500 BC) are also plotted, since pig bones account for a large proportion of the faunal assemblage (Pappa et al., 2004). Mean $\delta^{15}N$ values are plotted in Fig. 3b and those used in the estimates of human diet are given in Table 3. Cereal grains (*T. dicoccum*) exhibit mean $\delta^{13}C$ and $\delta^{15}N$ values of -24.4 ± 0.1‰ and 0.4 ± 0.3‰, respectively. More cereal grains and pulse seeds need to be analysed from this site to gain a better idea of the crop baseline $\delta^{15}N$ signature, but these initial isotope determinations provide a basis for further work. The large animal bone assemblage from Phase I is heavily dominated by domesticates (>99%), among which pigs, cattle and sheep are more strongly represented than goats and dogs. Relative proportions differ somewhat both between context types and according to method of quantification (Pappa et al., 2013, 2004). The cattle are not considered to contribute significantly to the human bone collagen isotope values, since their $\delta^{13}C$ values (-16.1 ± 2.0‰) are significantly higher than those of the humans (-20.4 ± 0.3‰; $t(16.68) = 8.70$, $p < 0.001$) and indicate a C_4 plant component to the cattle diet. The human bone collagen $\delta^{15}N$ values range from 5.3 to 9.3‰, with a mean of 7.6 ± 1.0‰. These values are very similar to those determined for the bone collagen isotope values of 18 human bone samples from the same Late Neolithic phase determined in a previous study (between 4.9 and 8.3‰; Triantaphyllou, 2001).

Inline Supplementary Fig. S3 can be found online at http://dx.doi.org/10.1016/j.jas.2014.11.009.

3.1.3. Çatalhöyük, Turkey

Bulk $\delta^{13}C$ and $\delta^{15}N$ values of bone collagen isolates from Çatalhöyük are plotted in Inline Supplementary Fig. S4. Bulk collagen isotope values were determined by Jessica Pearson and are discussed in more detail in Pearson (2013). The latter also compares human bone collagen $\delta^{15}N$ values from different areas, different buildings and across different levels of the site to determine potential differences in the importance of animal protein in the human diet. For the purposes of this study, we have taken the mean $\delta^{15}N$ value of all human bone collagen at the site. Mean $\delta^{15}N$ values are plotted in Fig. 3c and those used in estimates of human diet

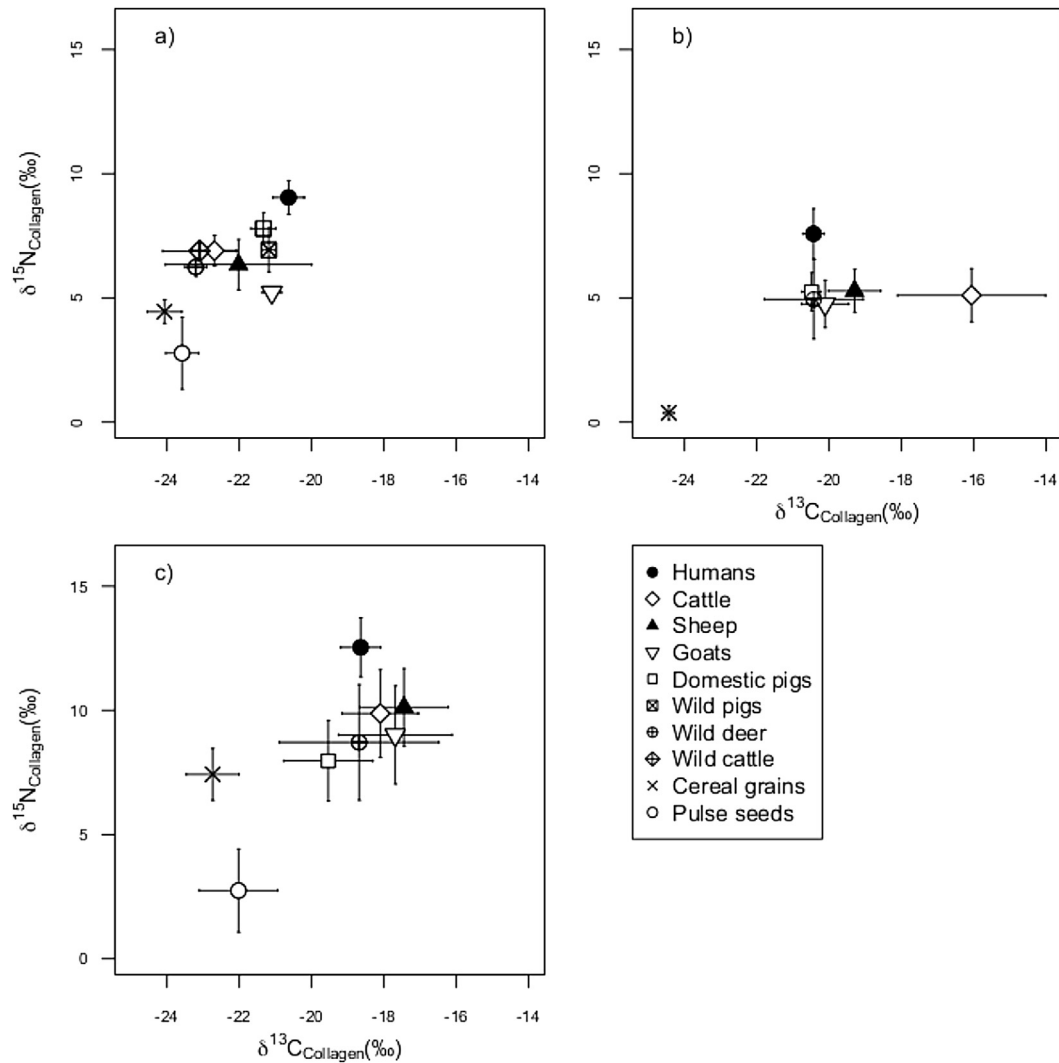**Fig. 3.** Mean $\delta^{13}C$ and $\delta^{15}N$ values (with ±1 SD) of human and faunal bone collagen and cereal grains and pulse seeds from: a) Vaihingen an der Enz, Germany, b) Makriyalos, Greek Macedonia, and c) Çatalhöyük, Turkey. Charred grain and pulse $\delta^{15}N$ values are adjusted for charring effect (cf. Fraser et al., 2013a,b).

are given in Table 3. The $\delta^{15}N$ values of cereals (*H. vulgare*, *T. dicoccum*, *Triticum durum/aestivum*, *T. monococcum*) and pulses (*P. sativum*) from the site are relatively high and it is unclear whether this is a result of manuring, or a factor of the environment. High plant $\delta^{15}N$ values can be caused by aridity (Hartman and Danin, 2010; Heaton, 1987), or waterlogged conditions, which result in denitrification (Finlay and Kendall, 2008). The range of isotope values for domestic sheep and goats from the site is very wide, suggesting that they fed across varied ecological zones (Pearson et al., 2007). Human bone collagen $\delta^{15}N$ values range from 9.2 to 15.1‰, which would seem to indicate varied sources of dietary protein.

Inline Supplementary Fig. S4 can be found online at http://dx. doi.org/10.1016/j.jas.2014.11.009.

3.2. Amino acid nitrogen isotope analyses

The bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values were determined for a subset of five humans, five domestic herbivores and five wild herbivores (three from Makriyalos) on each site. Individuals with bulk collagen $\delta^{13}C$ and $\delta^{15}N$ values closest to the mean for their species were selected from Vaihingen and Makriyalos in order to minimise variation. Since glutamine is deamidated to form

glutamic acid during hydrolysis of bone collagen, the $\delta^{15}N_{Glu}$ value determined by GC-C-IRMS represents both the nitrogen of glutamic acid and the amino-nitrogen of glutamine. The mean bone collagen $\delta^{15}N$, $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of each species from each site are given in Table 3. Estimated $\delta^{15}N_{Glu}$, $\delta^{15}N_{Phe}$ and $\Delta^{15}N_{Glu-Phe}$ values of the cereal grains are given in italics. Bone collagen $\delta^{15}N$, $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values for all of the individuals are given in Inline Supplementary Table S1.

Inline Supplementary Table S1 can be found online at http://dx. doi.org/10.1016/j.jas.2014.11.009.

3.2.1. Vaihingen an der Enz, Germany

There is no significant difference in the $\delta^{15}N_{Phe}$ value of humans, domestic cattle and wild deer from Vaihingen (10.7 ± 1.1‰; χ^2 (2) = 1.808, p = 0.405), which contrasts with the significant difference in their bulk $\delta^{15}N$ values (χ^2 (2) = 12.5, p = 0.002). This similarity in $\delta^{15}N_{Phe}$ values has also been observed for terrestrial mammals in other studies (e.g. Naito et al., 2010a,b; Styring et al., 2010) and indicates that the $\delta^{15}N_{Phe}$ values of the human, cattle and deer diets are very similar, since the $\delta^{15}N$ value of Phe increases by only 0.4‰ between diet and consumer. Phe undergoes very little metabolic routing in the body: it is not biosynthesised and is catabolised into tyrosine via a metabolic pathway that involves no

Table 3
Mean $\delta^{15}N$ values determined for bone collagen isolates and charred cereal grains and pulse seeds from the sites of Vaihingen, Makriyalos and Çatalhöyük.

Site	Species	n	Bulk $\delta^{15}N$ (‰)	$\delta^{15}N_{Glu}$ (‰)	$\delta^{15}N_{Phe}$ (‰)	$\Delta^{15}N_{Glu-Phe}$ (‰)
Vaihingen	All humans	47	9.1 ± 0.7	–	–	–
	Humans[a]	5	9.1 ± 0.1	12.2 ± 1.2	10.7 ± 1.6	1.5 ± 1.8
	All domestic herbivores	29	6.8 ± 0.8	–	–	–
	Domestic cattle[a]	5	6.8 ± 0.1	9.7 ± 0.8	10.8 ± 1.1	−1.1 ± 1.3
	All wild deer	9	6.2 ± 0.4	–	–	–
	Wild deer[a]	5	6.2 ± 0.1	8.7 ± 1.0	10.4 ± 0.9	
	Cereal grains	13	4.5 ± 0.5	4.0[b]	10.8[c]	−6.9, −9.0, −8.4[d]
	Pulse seeds	3	2.8 ± 1.5	–	–	–
Makriyalos	All humans	18	7.6 ± 1.0	–	–	–
	Humans[a]	5	7.7 ± 0.5	11.1 ± 1.4	9.8 ± 1.7	1.3 ± 1.5
	All domestic herbivores	54	5.1 ± 1.0	–	–	–
	Domestic sheep[a]	5	5.2 ± 0.1	7.8 ± 1.4	9.9 ± 1.7	−2.2 ± 1.5
	All wild deer	3	4.9 ± 0.6	–	–	–
	Wild deer[a]	3	4.9 ± 0.6	6.8 ± 1.6	8.6 ± 2.0	−1.8 ± 1.8
	Cereal grains	3	0.4 ± 0.3	−0.1[b]	9.9[c]	−6.9, −9.0, −8.4[d]
Çatalhöyük	All humans	67	12.5 ± 1.2	–	–	–
	Humans[a]	5	13.0 ± 1.0	16.7 ± 2.1	13.5 ± 2.2	3.3 ± 0.9
	All domestic herbivores	204	10.0 ± 1.7	–	–	–
	Domestic caprines[a]	5	10.0 ± 1.3	12.5 ± 2.1	13.3 ± 2.2	−0.8 ± 0.9
	All wild cattle	70	9.9 ± 1.8	–	–	–
	Wild cattle[a]	5	9.7 ± 1.4	12.7 ± 1.5	12.1 ± 1.6	0.6 ± 0.6
	Cereal grains	6	6.7 ± 0.9	6.2[b]	13.3[c]	−6.9, −9.0, −8.4[d]
	Pulse seeds	4	2.7 ± 1.7	–	–	–

[a] Individuals chosen for amino acid isotope analysis.
[b] Estimated by subtracting 0.5‰ from the average cereal grain $\delta^{15}N$ value.
[c] Estimated from the average domestic herbivore $\delta^{15}N_{Phe}$ value (see Section 3.2.1).
[d] $\Delta^{15}N_{Glu-Phe}$ values determined in modern *T. aestivum* and *H. vulgare* grains grown at (i) Rothamsted, UK, and (ii) Bad Lauchstädt, Germany (Styring et al., 2014) and (iii) in modern C$_3$ leaves (Chikaraishi et al., 2010).

breaking of a N bond (Salway, 1999) and therefore no isotopic fractionation would be expected. Unfortunately, the $\delta^{15}N_{Phe}$ values of human and faunal bone collagen and plant protein cannot be used to estimate the relative contributions of plant and animal protein to the diet because the differences in $\delta^{15}N_{Phe}$ values between fauna and plants are smaller than instrumental errors.

The human $\delta^{15}N_{Phe}$ value derives from both the plant and animal contributions to the diet, however, and since the determined human and domestic herbivore $\delta^{15}N_{Phe}$ values are very similar, it can be inferred that the $\delta^{15}N_{Phe}$ value of the plants eaten by the humans is also similar to that of the domestic herbivore $\delta^{15}N_{Phe}$ value, regardless of the relative contributions of animal and plant protein to the human diet. This similarity need not imply, however, that the bulk $\delta^{15}N$ values of plants eaten by humans and herbivores are also the same, because offsets between $\delta^{15}N_{Phe}$ and bulk $\delta^{15}N$ values vary among plant taxa and the $\delta^{15}N$ values of the 19 other amino acids in plant protein also contribute to the bulk plant $\delta^{15}N$ value (Styring et al., 2014).

Using the cereal grain amino acid $\delta^{15}N$ values determined by Styring et al. (2014), it is found that the $\delta^{15}N_{Phe}$ value relative to the bulk grain $\delta^{15}N$ value ($\Delta^{15}N_{Phe-Bulk}$ value) is 8.4 ± 1.5‰. Since this value differs slightly between site and species, we have decided to use an average $\Delta^{15}N_{Phe-Bulk}$ value in our calculations. Subtracting the $\Delta^{15}N_{Phe-Bulk}$ cereal grain value from the $\delta^{15}N_{Phe}$ value inferred for the plant portion of the human diet (Table 3) gives an estimate

of the bulk plant $\delta^{15}N$ value if humans were eating only cereal grains. For Vaihingen, the calculated cereal grain $\delta^{15}N$ value is 2.4‰, which is lower than the $\delta^{15}N$ values determined for the 13 cereal grains from Vaihingen (4.5 ± 0.5‰; Fig. 4a). This suggests that the plants consumed by humans from Vaihingen were not restricted to cereal grains with similar $\delta^{15}N$ values to those determined in this study, but may have included plants with lower $\delta^{15}N$ values such as pulses (Inline Supplementary Fig. S2).

The bone collagen $\delta^{15}N_{Glu}$ values are much more variable and differ even between individuals of the same species (within-species differences account for 21% of the variation). Nevertheless, the difference between the $\delta^{15}N_{Glu}$ and bone collagen $\delta^{15}N$ values ($\Delta^{15}N_{Glu-Bulk}$) between species is not significant (2.8 ± 0.8‰; χ^2 (2) = 0.740, p = 0.691). This is likely due to the central role that Glu plays both in the biosynthesis of other amino acids and in the excretion of waste N in the form of urea. The amino group of Glu is donated to keto acids to form other amino acids (Salway, 1999) and the first step in the formation of urea occurs during the deamination of Glu by Glu dehydrogenase (Sick et al., 1997). The $\delta^{15}N$ value of Glu is therefore much more sensitive to differences in metabolic function, which can vary between individuals and also tends to reflect the average $\delta^{15}N$ value of bone collagen amino acids.

3.2.2. Makriyalos, Greek Macedonia

There is no difference in the $\delta^{15}N_{Phe}$ value of humans, domestic sheep and wild deer from Makriyalos (9.8 ± 1.7‰; χ^2 (2) = 0.908, p = 0.635), which contrasts with the significant difference in bulk $\delta^{15}N$ values between humans and herbivores (χ^2 (2) = 8.580, p = 0.014). It can therefore be inferred that the $\delta^{15}N_{Phe}$ value of the plant portion of the human diet is similar to the $\delta^{15}N_{Phe}$ value of domestic herbivore bone collagen (Table 3), regardless of animal protein consumption. Using a $\Delta^{15}N_{Phe-Bulk}$ value of 8.4‰, the $\delta^{15}N$ value of plants consumed by humans at Makriyalos is estimated to be 1.5‰, which is slightly higher than the $\delta^{15}N$ values determined for the 3 cereal grains from Makriyalos (0.4 ± 0.3‰; Fig. 4b). This implies that the plants consumed by humans from Makriyalos were not restricted to cereal grains with similarly low $\delta^{15}N$ values to those determined in this study. Analysis of greater numbers of cereal grains and pulses from Makriyalos would reveal whether crops with these higher $\delta^{15}N$ values are preserved on the site.

Again, bone collagen $\delta^{15}N_{Glu}$ values are much more variable and differ between and within species (within-species differences account for 17% of the variation). In contrast to individuals at Vaihingen, there is a significant difference in the $\Delta^{15}N_{Glu-Bulk}$ values between species (χ^2 (2) = 8.949, p = 0.011). A post-hoc Bonferroni–Dunn test showed the significant difference to be between red deer and humans (p = 0.013), with $\Delta^{15}N_{Glu-Bulk}$ values of the red deer lower than the humans. This seems to indicate that the $\delta^{15}N_{Glu}$ value does not reflect the average of amino acid $\delta^{15}N$ values in red deer bone collagen at Makriyalos.

3.2.3. Çatalhöyük, Turkey

There is no significant difference in the $\delta^{15}N_{Phe}$ value of humans, domestic sheep/goats and wild/domestic cattle from Çatalhöyük (13.0 ± 1.6‰; χ^2 (2) = 2.660, p = 0.264), which contrasts with the significant difference in bulk $\delta^{15}N$ values (χ^2 (2) = 9.420, p = 0.009). It can therefore be inferred that the $\delta^{15}N_{Phe}$ value of the plant portion of the human diet is similar to that of the domestic herbivores (Table 3), regardless of animal protein consumption. Using a $\Delta^{15}N_{Phe-Bulk}$ value of 8.4‰, the $\delta^{15}N$ value of plants consumed by humans at Çatalhöyük is estimated to be 4.9‰, which is lower than the $\delta^{15}N$ values determined for the 6 cereal grain samples from the site (6.7 ± 0.9‰; Fig. 4c). This suggests that, like at Vaihingen, the plants consumed by humans from Çatalhöyük may have included
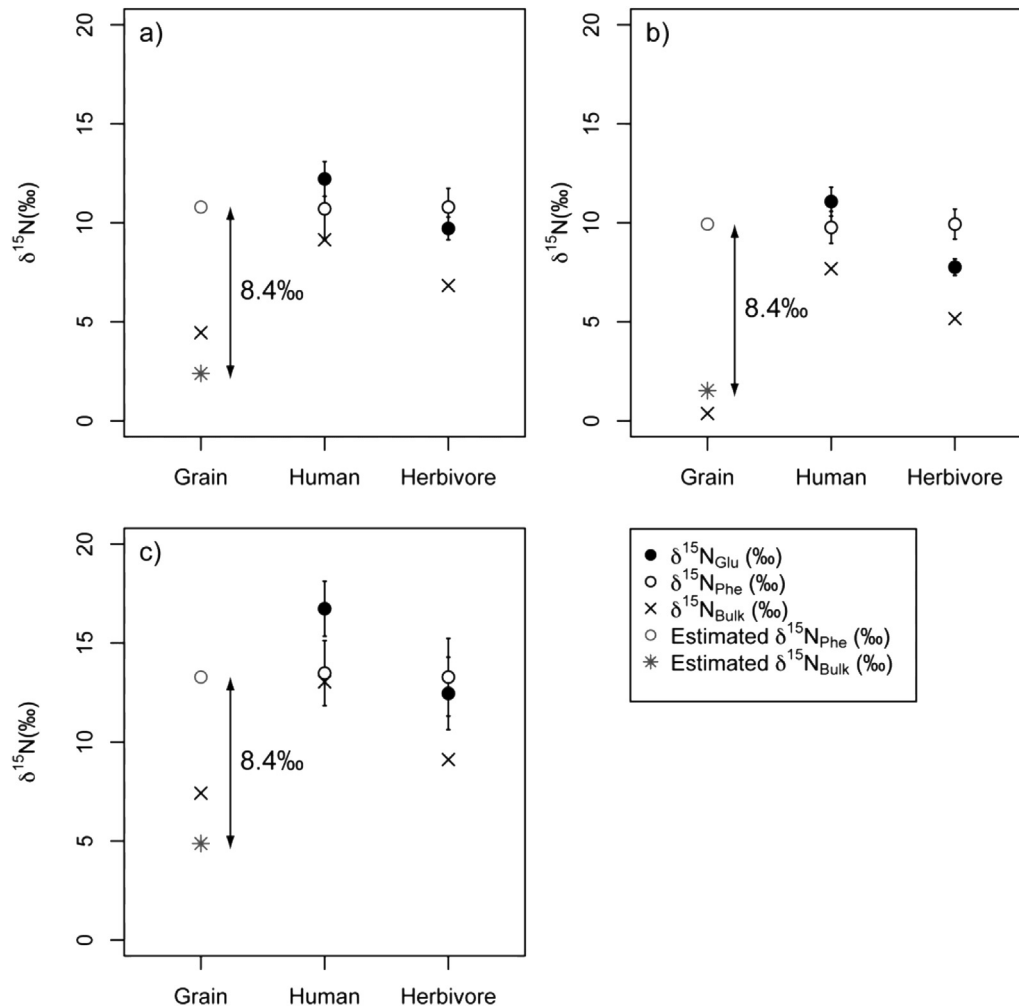
Fig. 4. Determined and estimated bulk plant, collagen and amino acid $\delta^{15}N$ values of plants, herbivores and humans from a) Vaihingen, b) Makriyalos, and c) Çatalhöyük. Estimated plant $\delta^{15}N_{Phe}$ values are inferred from human and herbivore bone collagen $\delta^{15}N_{Phe}$ values. Cereal grain $\delta^{15}N$ values were estimated by subtracting 8.4‰ from estimated plant $\delta^{15}N_{Phe}$ values ($\Delta^{15}N_{Phe-Bulk}$ values determined in modern cereal grains).

plants with lower $\delta^{15}N$ values such as pulses (Inline Supplementary Fig. S4).

Again, the bone collagen $\delta^{15}N_{Glu}$ values are much more variable and differ between and within species (within-species differences account for 32% of the variation). As observed at Vaihingen, the $\Delta^{15}N_{Glu-Bulk}$ values do not differ significantly between species ($3.3 \pm 0.7‰$; $\chi^2 (2) = 2.880$, $p = 0.237$) and the $\Delta^{15}N_{Glu-Bulk}$ value is similar to that of the individuals from Vaihingen.

3.3. Estimating the proportion of animal protein consumed by humans

Table 3 gives the average bulk $\delta^{15}N$ values, $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values used to estimate the proportion of animal protein consumed by the humans on each archaeological site, using each of the four methods described in Section 2.5. The estimates obtained from each of the methods are presented in Table 4. Estimates made using the 'Standard method' and the 'Standard model plus plants' were carried out using: (i) all human and domestic herbivore bulk bone collagen $\delta^{15}N$ values, and (ii) using only the bone collagen $\delta^{15}N$ values from the humans ($n = 5$) and domestic herbivores ($n = 5$) whose amino acid $\delta^{15}N$ values were determined in this study. The $\Delta^{15}N_{Consumer-Diet}$ value for bulk bone collagen is assumed to be 4‰.

3.3.1. Vaihingen an der Enz, Germany

Animal protein consumption among humans at Vaihingen is calculated to be 58% if the 'Standard method' includes the bulk collagen $\delta^{15}N$ values of all of the individuals. The proportion of animal protein consumption calculated using only the bulk collagen $\delta^{15}N$ values of the individuals chosen for amino acid analysis is the same as that calculated for all individuals, with a smaller range in the 95% confidence interval due to the smaller variation in bulk collagen $\delta^{15}N$ values.

The calculated proportion of animal protein consumed is much lower using the 'Standard method plus plants' (26%). This is because the cereal grain $\delta^{15}N$ values are relatively high compared to the estimated herbivore forage $\delta^{15}N$ values ($\delta^{15}N_{Herbivore}$ – 4‰ = 2.8‰ compared to 4.5‰). This indicates that the $\delta^{15}N$ values of plants consumed by humans and herbivores at Vaihingen are different and suggests that cereals consumed by humans were likely to have been manured (cf. Fraser et al., 2013b), accounting for the relatively high $\delta^{15}N$ values.

Using different $\Delta^{15}N_{Glu-Phe}*$ values in the 'Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values method' makes a considerable difference to the calculated animal protein consumption (between 11 and 38%), but regardless of the $\Delta^{15}N_{Glu-Phe}*$ value used, the calculated animal protein consumption is much lower than that calculated using only

Table 4
Estimated proportions of animal protein consumed by humans from Vaihingen, Makriyalos and Çatalhöyük, calculated using the four methods described in Section 2.5, the $\delta^{15}N$ values given in Table 1 and assuming a $\Delta^{15}N_{collagen-diet}$ value of 4‰.

		Proportion of animal protein consumed (%) 95% Confidence interval in brackets[a]		
	Method	Vaihingen	Makriyalos	Çatalhöyük
1.	'Standard method'			
	(i) All bulk $\delta^{15}N$ values	58 (50–65%)	63 (49–76%)	63 (54–71%)
	(ii) Bulk $\delta^{15}N$ values from bone collagen isolates used in AA analyses	58 (54–61%)	63 (47–78%)	75 (38–100%)
2.	'Standard method plus plants'			
	(i) All bulk $\delta^{15}N$ values	26 (13–39%)	68 (57–79%)	55 (40–69%)
	(ii) Bulk $\delta^{15}N$ values from bone collagen isolates used in AA analyses	26 (16–37%)	69 (55–82%)	70 (27–100%)
3.	'Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values'			
	(i) $\Delta^{15}N_{Glx-Phe}{}^* = -6.9 \pm 0.2$‰	11 (8–13%)	8 (6–10%)	33 (23–42%)
	(ii) $\Delta^{15}N_{Glx-Phe}{}^* = -9.0 \pm 0.3$‰	38 (30–46%)	36 (28–43%)	61 (46–75%)
	(iii) $\Delta^{15}N_{Glx-Phe}{}^* = -8.4$‰ ± 1.6‰	30 (24–37%)	28 (21–34%)	53 (39–66%)
4.	'Bone collagen and cereal $\delta^{15}N_{Glu}$ values'	4 (0–30%)	41 (18–63%)	40 (0–82%)

[a] 95% confidence intervals for animal protein consumption were calculated using IsoError (www.epa.gov/wed/pages/models.htm; accessed 9 September 2014) for methods 1, 2 and 4. For method 3, 95% confidence intervals for animal protein consumption were calculated by taking into account the propagation of standard errors for $\delta^{15}N_{Glu[hum]}$, $\delta^{15}N_{Phe[hum]}$, $\Delta^{15}N_{Glx-Phe}{}^*$, Δ_{Glu} and Δ_{Phe}.

bulk bone collagen $\delta^{15}N$ values. This highlights the need for further studies to constrain the $\Delta^{15}N_{Consumer-Diet}$, Δ_{Glu} and Δ_{Phe} values for diets of differing protein quality and content, particularly in terrestrial mammals. When taking into account the estimated cereal grain $\delta^{15}N_{Glu}$ values, the calculated animal protein consumption is considerably lower (4%) due to the relatively high plant $\delta^{15}N$ values.

3.3.2. Makriyalos, Greek Macedonia

Animal protein consumption among humans at Makriyalos is calculated to be 63% if the 'Standard method' includes the bulk collagen $\delta^{15}N$ values of all of the individuals. However, 1 out of the 18 individuals exhibits a $\delta^{15}N$ value (under 6‰) below those of the domestic herbivores, either suggesting that this individual consumed no animal protein, or pulses and plants with low $\delta^{15}N$ values comprised a significant part of their diet. Two out of the 21 individuals whose bone collagen isotope values were determined by Triantaphyllou (2001) also exhibited very low $\delta^{15}N$ values. Conversely, one individual exhibits a $\delta^{15}N$ value ($\delta^{15}N = 9.3$‰) greater than the maximum theoretical bone collagen $\delta^{15}N$ value predicted from the consumption of pure herbivore protein (i.e. 9.1‰).

The calculated proportion of animal protein consumed is higher using the 'Standard method plus plants' (68%). This is because the cereal grain $\delta^{15}N$ values are relatively low compared to the estimated herbivore forage $\delta^{15}N$ values ($\delta^{15}N_{Herbivore} - 4$‰ $= 1.1$‰ compared to 0.4‰). There is the potential for fish consumption at Makriyalos, considering its proximity to the coast and the findings of abundant seashells. Extensive investigations of organic residues in cooking pottery from the site show no evidence, however, of the processing of aquatic commodities as judged by stable carbon isotope determinations of fatty acids and an absence of aquatic lipid biomarker proxies (Evershed et al., 2008; Cramp and Evershed, 2014; Whelton et al. unpublished). Further $\delta^{15}N$ value determinations of cereal grains from Makriyalos are necessary in order to ascertain whether the very low cereal grain $\delta^{15}N$ values measured in this study reflect those of the majority of crops.

Using the 'Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values method', animal protein consumption estimates are much lower (between 8 and 36%), regardless of the $\Delta^{15}N_{Glu-Phe}{}^*$ value used. Again, this highlights the need for better understanding of the factors contributing to amino acid and bulk collagen $\delta^{15}N$ values. The calculated animal protein consumption is also lower (41%) using the 'Bone collagen and cereal $\delta^{15}N_{Glu}$ values method' despite the fact that the cereal grain $\delta^{15}N_{Glu}$ value used in this calculation was estimated from the relatively low bulk cereal grain $\delta^{15}N$ values.

3.3.3. Çatalhöyük, Turkey

Animal protein consumption among humans at Çatalhöyük is calculated to be 63% if the 'Standard method' includes the bulk collagen $\delta^{15}N$ values of all of the individuals. Seven out of the 67 individuals exhibit $\delta^{15}N$ values (over 14‰) greater than the maximum theoretical bone collagen $\delta^{15}N$ value predicted from the consumption of pure herbivore protein. The proportion of animal protein consumption calculated using only the bulk collagen $\delta^{15}N$ values of the individuals chosen for amino acid analysis is higher than that calculated for all individuals (75%).

The calculated proportion of animal protein consumed is slightly lower using the 'Standard method plus plants' (55%). This is because the cereal grain $\delta^{15}N$ values are slightly higher than estimated herbivore forage $\delta^{15}N$ values ($\delta^{15}N_{Herbivore} - 4$‰ $= 6.0$‰ compared to 6.7‰). Cereals consumed by humans may therefore have been manured, increasing their $\delta^{15}N$ values above that of the herbivore forage.

Using the 'Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values method', animal protein consumption estimates are slightly lower (between 33 and 61%), but they are strongly influenced by the $\Delta^{15}N_{Glu-Phe}{}^*$ value used. The calculated animal protein consumption is also lower (40%) using the 'Bone collagen and cereal $\delta^{15}N_{Glu}$ values' method. The uncertainties associated with all dietary calculations are large because of the considerable variation in bulk and amino acid $\delta^{15}N$ values between individuals.

4. Discussion and conclusion

This is the first study to investigate bone collagen amino acid $\delta^{15}N$ values of humans believed to have been eating only, or predominantly, terrestrial protein, which simplifies the possible dietary inputs to the bone collagen N isotopic signature. We have used the $\delta^{15}N$ values of bone collagen, bone collagen amino acids, plant protein and plant protein amino acids in four different palaeodietary models to calculate the proportion of animal protein in human diet at three different archaeological sites. Comparison of the results of these calculations highlights limitations of these models and draws attention to the priorities for future work needed to improve their accuracy and reliability.

Bulk bone collagen $\delta^{15}N$ values average out the $\delta^{15}N$ values of their constituent amino acids, representing the net effect of dietary protein sources and metabolic cycling within the body. Calculating human animal protein consumption using bulk $\delta^{15}N$ values also relies upon the assumption that faunal bone collagen preserved on the site is representative of the $\delta^{15}N$ values of the animals

consumed. This is not the case with the 'Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values method' since human bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values provide an internal indicator of animal protein consumption, although it remains a limitation of the 'Bone collagen and cereal $\delta^{15}N_{Glu}$ values method'.

The large discrepancies between the estimates of animal protein consumption made with and without taking into account the $\delta^{15}N$ values of charred cereal grains and pulses illustrate the importance of plant $\delta^{15}N$ values in palaeodietary interpretations. The relatively high $\delta^{15}N$ values of cereal grains from Vaihingen (4.5‰) and Çatalhöyük (6.7‰) could be responsible for the relatively high human bone collagen $\delta^{15}N$ values, leading to overestimation of animal protein contribution to the diet if the 'Standard method' is used. In contrast, the relatively low $\delta^{15}N$ values of the cereal grains from Makriyalos (0.4‰) could lead to underestimation of animal protein contribution to the diet if their values are not taken into account. In order to be more certain about the plant $\delta^{15}N$ contribution to the human bone collagen $\delta^{15}N$ value, it is clear that more cereal grain $\delta^{15}N$ value determinations need to be carried out at each site and the variability in $\delta^{15}N$ values of modern plants relevant to human and animal diet needs to be ascertained.

Bone collagen $\delta^{15}N_{Glu}$ and $\delta^{15}N_{Phe}$ values of humans and the animals included in their diets have the potential to improve interpretation of human diet in the past, since they separate the influence of diet $\delta^{15}N$ ($\delta^{15}N_{Phe}$) from subsequent N metabolism ($\delta^{15}N_{Glu}$). However, this method is only of use if amino acid $\delta^{15}N$ values of plants consumed by herbivores and humans are the same. This is particularly unlikely in circumstances when humans are eating manured cereal grains and herbivores are not. This could be the case at Vaihingen and Çatalhöyük, since the determined cereal grain $\delta^{15}N$ values at these sites are higher than estimated herbivore forage $\delta^{15}N$ values ($\delta^{15}N_{Herbivore} - 4$‰).

The 'Bone collagen and cereal $\delta^{15}N_{Glu}$ values method' provides a means of combining the specificity of amino acid $\delta^{15}N$ values whilst taking into account the $\delta^{15}N$ value of cereals consumed by humans. Comparison of determined cereal grain $\delta^{15}N$ values and those estimated from herbivore $\delta^{15}N_{Phe}$ values indicates whether humans were likely to have been eating the cereals preserved on the site (Section 3.2 and Fig. 4).

Further studies are needed into: (i) the ^{15}N trophic enrichment factor between consumer and diet $\delta^{15}N_{Glu}$ (ΔGlu) and $\delta^{15}N_{Phe}$ (ΔPhe) in a terrestrial ecosystem through feeding experiments involving terrestrial mammals, and (ii) $\Delta^{15}N_{Glu-Phe}$ values of common plant foods, particularly glume wheats, pulses and plants consumed by herbivores, in order to improve the accuracy and more widespread applicability of both amino acid methods. With these provisos, analysis of amino acid $\delta^{15}N$ values offers significant potential to elucidate the factors contributing to bulk $\delta^{15}N$ values of human and non-human bone collagen and thus to achieve more reliable estimates of the contribution of animal protein to human diet.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jas.2014.11.009.

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